Instituto Juan March
de Estudios e Investigaciones

71 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Programmed Gene Rearrangement: Site-Specific Recombination

Organized by

J. C. Alonso and N. D. F. Grindley

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INTRODUCTION

J.C. Alonso and N.D.F. Grindley

Site-specific recombination, sometimes qualified by the term 'conservative site specific recombination', is a process that involves the reciprocal exchange of defined DNA segments by precise breakage and rejoining mechanisms with no loss or synthesis of DNA. A particular feature of such processes is that phosphodiester bond energy is conserved throughout the reaction. DNA breaks are made not by hydrolysis but rather by phosphoryl transfer to a side chain (a tyrosine or a serine) of the recombinase, resulting in covalent linkage of the enzyme to the terminal phosphate of the broken DNA. DNA is resealed, and the recombinase released, by reversal of the process - attack by the terminal hydroxyl of the DNA on the protein-DNA phosphodiester.

Biological roles of site-specific recombination (thus defined) include chromosomal integration and excision of bacteriophage genomes, monomerization of plasmid and bacterial chromosomes, switching of gene expression between two alternative modes, resolution of transposition intermediates and the fusion of gene cassettes into a functional gene. The vast majority of site-specific recombinases fall into two distinct groups: the Integrase family, named after the prototypical phage λ integrase, and the Resolvase family, named after the cointegrate resolving proteins encoded by the transposons, γδ and Tn*3*. Members of the integrase family include λ and many other phage integrases, the phage P1 Cre protein and the bacterial XerC and XerD proteins, the FLP protein encoded by the yeast 2μm circle, the integron-associated recombinases (responsible for the acquisition of antibiotic resistance cassettes) and the transposases of conjugative transposons. The resolvase family includes most transposon-encoded resolvases, DNA-invertases such as Hin and Gin, and a few phage integrases. These two families are unrelated in protein sequence or structure, and employ different recombinational mechanisms. In this Workshop, about half of the sessions were devoted to presentation and discussion of the latest results obtained with members of these two families (including the crystal structure of a synaptic complex).

Recombination using specific sites is, however, not the exclusive domain of the Integrase and Resolvase family of proteins. Nor are the processes used by these recombinases, such as the site-specific cleavage of DNA; the pairing of distant DNA segments to form synaptic complexes; and the formation of covalently linked protein-DNA intermediates. The other half of the Workshop was focussed on biological processes that exhibit one or more of the features found in site-specific recombination.

In mammalian cells, the phenomenon closest to the conservative definition of site-specific recombination is the rearrangement of the immunoglobulin and T-cell receptor genes that accompanies B-cell and T-cell development. Specific DNA sites are brought together in synaptic complexes by the Rag1/Rag2 recombinase, cleaved (without protein-DNA linkage), rearranged and rejoined. Although signal joints are precise, the coding joints are not and typically show addition or deletion of a few nucleotides. The process of synapsis was specifically addressed, as was the involvement and role of additional nuclear proteins. Synapsis also turns out to be an unexpected feature of the 'restriction' endonuclease, SfiI.

Workshop participants were informed about progress towards understanding three rather unusual recombination events, very different from conservative site-specific recombination, but site-directed nevertheless. One of these is the programmed deletion of

thousands of genomic segments that occurs during macronuclear development in the ciliate, *Tetrahymena thermophila*. A new *in vitro* system was described which gave results supporting a novel mechanism for this deletion. The other two involve the 'homing' of DNA encoding group I and group II introns to intron-less genes. Homing occurs by very different mechanisms; in one case, involving directed gene conversion by double strand break repair; in the other, involving the direct participation of the intron RNA as well as the intron-encoded reverse transcriptase and other functions.

Initiation of conjugative DNA transfer and site-specific recombination are topics rarely found on the same programme at a scientific meeting. However, since both processes conserve phosphodiester bond energy and ensure precise reversibility by the formation of a covalently linked protein-DNA intermediate, relaxases from several plasmids were discussed in detail.

The breadth and depth of the topics presented, the relatively small number of participants, and the intimate and congenial atmosphere of the Juan March Institute combined to produce a highly informative meeting with lively and stimulating discussions.

Session 1

Chairman: Erich Lanka

LAMBDA INTEGRASE: THE DYNAMICS AND CHEMISTRY OF STRAND EXCHANGE

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Approximately 30 site-specific recombination pathways, in both prokaryotes and eukaryotes, belong to the family, whose common features are: a) a conservative DNA cleavage mechanism that is independent of hig energy cofactors and proceeds via a covalent 3' phosphotryrosine linkage between the recombinase and DN b) staggered DNA cleavages that define a short region of homology between the recombining helices; and c conserved amino acid tetrad that is thought to comprise the cleavage-ligation active site of the recombinase There are two classes of reactions within the Int family. One is defined by the minimal elements necessary effect synapsis, strand cleavages, strand exchanges and ligations, e.g., the cre-lox system. The second class imposed upon the basic reaction an additional level of complexity that confers directionality and regulatory responsiveness, e.g., the λ Int system.

Integrative recombination between bacteriophage λ (*att*P) and *E. coli* (*att*B) DNA requires the virally-encoc Int protein, which is a typeI topoisomerase, and the host-encoded IHF protein, which is a DNA bending pro The products of this reaction, the prophage sites *att*L and *att*R, form the junctions between bacterial and vir DNA and are also the substrates for excisive recombination. Excisive recombination additionally requires t virally-encoded protein, Xis, and is stimulated by the host-encoded protein, Fis, both of which are also DN/ bending proteins.

Integrase is a bivalent DNA binding protein. The first 64 residues form a domain that recognizes the five "arm-type" sites that are located distant from the sites of strand exchange. The carboxy-terminal portion of (292 amino acids) recognizes the four low affinity "core-type" sites that define the region of strand exchang and is fully competent in cleaving and ligating DNA at these sites. This carboxy-terminal portion of Int car further subdivided into two domains with distinct, but possibly overlapping, functions. The domain consist of residues 65 to 169 is essential for efficient and specific binding to core-type DNA sites, has been shown t interact with these sites by a variety of biochemical techniques and retains its DNA binding specificity whe isolated as the product of a cloned sequence encoding this region. The carboxy-terminal domain extends fr residues 170 to 356, binds DNA very poorly and with no demonstrable specificity for core-type sites but it l the capacity to form the covalent phosphotyrosine DNA cleavage complexes that are known to be the high energy intermediates of recombination. The crystal structure of this catalytic domain (at 1.9Å resolution) reveals a protein fold that appears to be conserved among more than 70 Int family members in organisms ranging from archaea to yeast. Among the interesting features of the structure is the location of the catalyti Arg-His-Arg triad that is thought to activate the scissle phosphate for DNA cleavage and the location of the catalytic Tyr 342 on an exposed 17 amino acid loop that is flexibly tethered to the rest of the protein and ca thus be modeled for cleavage of DNA *in cis* or *in trans*.

We shall also discuss models for the exchange of DNA strands and for the realignment of DNA helices.

Structure of a Cre-loxP Site-specific Recombination Reaction Intermediate

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Cre recombinase catalyzes site-specific recombination between 34-base pair loxP sites in a process that maintains the stable inheritence of bacteriophage P1 chromosomes (1). Cre is representative of a simple, or minimal, subset of the lambda integrase family of site-specific recombinases because it requires no acessory proteins or auxiliary DNA sequences for the reaction to occur. The simplicity of the Cre-loxP system has led to its growing use in a variety of genetic engineering applications, particularly the construction of inducible gene knockouts in mice (2).

In the site-specific recombination reaction (3), two Cre molecules bind to the loxP site and two Cre-bound loxP sites associate to form a recombination synapse. One of the two recombinases bound to each substrate nicks the loxP site, forming covalent 3'-phosphotyrosine intermediates. The released 5'-hydroxyl groups in each substrate undergo intermolecular nucleophilic attack of the phosphotyrosine in the partner substrate to exchange the first pair of DNA strands. The resulting intermediate is a Cre-bound Holliday junction, which is itself a substrate for cleavage by the remaining pair of recombinases, on the second pair of substrate strands. The 5'-hydroxyl groups released in this cleavage reaction also attack the partner phosphotyrosine linkages, resulting in exchange of the second pair of DNA strands and formation of recombinant products.

We have determined the structure at 2.4 Å resolution of a covalent intermedate of the Cre-loxP site-specific recombination reaction, where one of the two Cre molecules bound to a suicide loxP substrate has cleaved the DNA to form a 3'-phosphotyrosine linkage (4). Two such Cre-loxP complexes have associated to form a site-specific recombination synapse that is trapped in the strand exchange step of the reaction. The structure of this 200 kD Cre-DNA complex reveals (i) the structure of the recombinase, (ii) the nature of the Cre-DNA interface, (iii) the stereochemical details of two different Cre active sites, one containing a 3'-phosphotyrosine linkage and one where cleavage has not occurred, (iv) protein-protein interactions involved in formation of a pseudo-fourfold symmetric synapse, (v) DNA substrates which are sharply bent into a configuration that resembles a planar Holliday junction, and (vi) possible models for allosteric control over the stepwise cleavage and strand exchange steps in the reaction.

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ASSEMBLY AND ACTION OF A SITE-SPECIFIC RECOMBINATION MACHINE

Sherratt, D.J., Arciszewska, L.K., Blakely, G.B., Colloms, S.D., Cornet, F., Cao, Y., Hallet, B., Hayes, F., Spiers, A., Alén, C., Bath, J., Grainge, I., Subramanya, H.S., Bird, L.E. and Wigley, D.B. *Dept Biochemistry, University of Oxford, Oxford OX1 3 QU, UK*

Xer site-specific recombination functions in the normal segregation of circular chromosomes and plasmids in bacteria. It uses two related recombinases, XerC and XerD, each catalysing one specific pair of strand exchanges. The requirements for and outcomes of recombination differ for recombination sites present in the bacterial chromosome and in multicopy plasmids. The presentation will focus on how the structures of the components of the Xer recombination machine relate to the overall architecture of the nucleoprotein complex that catalyses recombination, the reaction mechanism, and the biological functions of Xer recombination.

Substrate and enzyme contributions to the logic of coordinated site-specific phosphoryl transfer: the Flp model

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The Flp site specific recombination takes place within an enzyme substrate complex consisting of two DNA substrates and four recombinase monomers. The reaction is completed in two steps: the first strand exchange reaction yields a Holliday junction intermediate; the second strand exchange reaction resolves the junction into reciprocally recombinant products.

How does the system achieve the coordinated pair of phosphoryl transfers required to complete one round of recombination? The factors that contribute to the complexity as well as the flexibility of the system are (1) the shared active site of Flp, (2) the double-helical geometry as well as the plasticity of the substrates, (3) the relative spacing and phasing of the Flp monomers and the labile phosphates and (4) a mechanism for the special selectivity and temporal exclusion of active sites.

Our current model for Flp recombination is based upon results from (1) the interaction and mode of strand cleavage by Flp in linear substrates, (2) the manipulation of synthetic Holliday junctions by Flp, (3) the bimodal cleavage by Flp in Holliday substrates, (4) the selective exclusion of Flp active sites in geometrically constrained substrates and (5) the escape from active site exclusion under special circumstances.

The general features of the recombination model are consistent with the architecture of the Cre-lox recombination complex whose structure was recently obtained in the van Duyne laboratory.

DNA TOPOLOGY AND SITE-SPECIFIC RECOMBINATION *IN VIVO*. E. Lynn Zechiedrich and Nicholas R. Cozzarelli. Department of Molecular and Cell Biology, Division of Biochemistry and Molecular Biology. University of California, Berkeley, CA 94720-3204. Negatively supercoiled DNA substrate is required for the activities of many site-specific recombinases. By inhibiting topoisomerases I, II, and IV, selectively, we can now set the plasmid supercoil density in *Escherichia coli* to range from completely relaxed ($\sigma = 0$) to hyper-negatively supercoiled (σ = a gaussian distribution about -0.1). We tested the requirement for DNA supercoiling of lambda integrase and Tn3 resolvase *in vivo*. There was a remarkably sharp transition between no recombination at all and full recombination with supercoiling. No recombination occurs when the DNA is more relaxed than $\sigma = -0.05$. At this value, 50% of the plasmids are recombined and at a σ of -0.06, all of the substrate DNA is recombined. The products of integrase recombination are two intertwined circles (a catenane). The number of links in the product catenane is dependent upon the number of supercoil nodes in the substrate. We found that the more negatively supercoiled the substrate DNA was, the more catenane links resulted in the integrase products. The final step in integrase and resolvase recombination is the unlinking (decatenation) of the catenanes by topoisomerases. We found that only topo IV carries out this reaction and we were able to measure *in vivo* kinetics for decatenation. The more links present in the recombination- mediated catenanes, the faster topo IV unlinks. Topo II (gyrase), which used to be thought to decatenate, acts only to negatively supercoil the DNA. Supercoiled DNA, in turn, is then a better substrate for topo IV. ELZ is a Special Fellow of the Leukemia Society of America.

Session 2

Chairman: Michael M. Cox

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Using Cre-loxP-mediated deletion and inversion for targeted mutagenesis in mice

As the Cre-loxP recombination system had been shown to operate efficiently in mammalian cells (1), it was straightforward to make use of it in order to advance the classical gene targeting technology. Flanking the selection marker gene(s) in targeting vectors (whose introduction into the embryonic stem (ES) cell genome by homologous recombination is the basis of targeted mutagenesis in mice (2)) allows their subsequent elimination from the ES cells through transient transfection with a Cre-encoding plasmid. This provides a general method of targeted mutagenesis (gene replacement) by which any kind of mutation can be introduced into ES cells (i.e. the mouse germ line) in a clean way (3, 4). Perhaps the most important application of the Cre-loxP system in gene targeting is in generating conditional (cell type-specific and/or inducible) systems of mutagenesis (5-7). Here, a mutant mouse strain is generated in which the gene of interest or an essential part thereof is flanked ("floxed") by loxP sites in innocent positions. Crossing these mice then with cre-transgenic mice in which Cre is expressed in a cell type-specific and/or inducible manner allows conditional gene inactivation or mutation. This approach which is now beginning to be widely applied to study gene function in a variety of biological systems such as the immune and the central nervous system, allows one for the first time to genetically study the differentiated state in vivo, leaving its development intact. Inducible systems of targeted mutagenesis appear of particular importance in this respect (8). A recent addition to Cre-loxP-mediated gene targeting is based on the ability of the Cre enzyme to invert DNA sequences flanked by loxP sites in opposite orientations. The usefulness of this approach is exemplified by recent work of Kong Peng Lam in my laboratory, in which the specificity of the antigen receptor on antibody forming cells is changed at will in an inducible manner in a fraction of the population of antibody forming cells, with the aim of studying, in a novel manner, problems of receptor mediated cellular selection in vivo.

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Relaxases - DNA Strand Transferases in Bacterial Conjugation

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Relaxases of transmissible plasmids play a key role in initiation and termination of the transfer DNA replication during bacterial conjugation. The enzymes function as DNA strand transferases cleaving an unique phosphodiester bond at the *nic*-site of the transfer origin *(oriT)* [1, 2]. The cleavage reaction consists in a reversible transesterification reaction resulting in transfer of the 5' phosphoryl at *nic* to the hydroxyl group of a tyrosyl residue in the active center of the relaxase. Hence, cleavage results in the intermediate covalent attachment of the enzyme to the 5' terminus of the plasmid strand destined for transfer from a donor to a recipient cell. Relaxases cleave double-stranded DNA at *oriT* site- and strand-specific and also single-stranded DNA containing the *nic* site. The reaction requires Mg^{2+} ions. Amino acid sequence alignments of relaxases of several origins suggested the existence of at least two subclasses of these enzymes. The relaxase of the broad host range plasmid RP4 (TraI, IncPα) contains three motifs which are conserved among one subclass. Mutagenesis of invariant amino acid residues lead to the identification of domains involved in the catalytic cleavage-joining reaction [3]. The resulting phenotypes allowed us to allocate particular relaxase functions to distinct relaxase domains, e.g. substrate recognition and binding or activation of the active site tyrosine. To uncouple substrate binding and cleavage-joining we applied partially biotinylated relaxase mutant proteins that were immobilized to magnetic heads. Using this approach we could demonstrate that tight DNA substrate binding and cleavage-joining are independent processes. Enhanced toposimerase activity of some relaxase mutants was correlated with low specific substrate binding in conjunction with high cleavage-joining activity [4].

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DNA RELAXASES ENCODED BY THE STREPTOCOCCAL PLASMID pMV158

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The streptococcal plasmid pMV158 replicates through the mechanism of rolling circle (1). The plasmid encodes two DNA relaxases, RepB and MobM. RepB is involved in initiation of vegetative replication, whereas MobM is involved in conjugative mobilization. Both proteins were overproduced, purified, and shown to specifically cleave supercoiled pMV158 DNA. The target of RepB is the plasmid double strand origin (*dso*), and that of MobM is the origin of transfer, *oriT*. Through a recently developed assay (2), we have been able to determine the strand discontinuities introduced *in vivo* by Rep and Mob proteins of plasmids replicating by the rolling circle mechanism. Upon DNA cleavage, the RepB and MobM proteins leave a free 3'-OH end (3-5). However, whereas MobM remained covalently attached to the 5'-end of the nick site, covalent RepB-DNA complexes have not been detected (4, 5). RepB protein has nicking-closing (topoisomerase I-like) activities on supercoiled DNA (3, 4), but we have found only nicking activity mediated by protein MobM (5). Plasmid pMV158 offers an attractive system to study the enzymology of DNA replication initiated by relaxases, since two plasmid-encoded DNA relaxases, which share some conserved motifs, act on two different targets within the same plasmid.

Relaxase RepB. RepB cleaves the phosphodiester bond of the dinucleotide 5'-GpA-3' within the sequence 5'-TACTACG/AC-3', and it is able to perform strand transfer reactions on single stranded oligonucleotides containing the RepB target. Attempts at capturing covalent RepB-DNA intermediates were unsuccessful (4). However, employment of single stranded oligonucleotides containing a chiral phosphorothioate in the target DNA allowed us to follow the process of RepB-mediated strand-transfer reaction. This reaction occurred through an even number of steps because the chirality of the phosphorothioate at the reaction site was retained upon RepB-mediated strand transfer (6). This finding suggests the existence of a covalent intermediate between the protein and its target DNA during the strand-transfer reaction.

Based upon homologies at the *dso* and *rep* genes, several plasmid families have been defined. One of them, represented by pMV158, is constituted by a dozen replicons from Gram-positive and -negative bacteria. Alignment of the amino acid residues of their Rep proteins, allowed us to define several conserved regions (1). Two of these regions, termed R-IV and R-V, contained a conserved Tyr residue. Part of the region R-IV is conserved among Rep, Mob, and Tra (involved in conjugative DNA transfer) proteins (7). Region R-V is present only in the Rep proteins of the pMV158 plasmid family (1). In RepB, the two conserved Tyr residues are located at positions 99 and 115 within regions R-IV and R-V, respectively. Replacement of Tyr99 by Phe or Ser led to protein variants that conserved the ability to bind to the iterons but that had lost nicking-closing activity.

The MobM relaxase. The 5'- and 3'-ends of the nick introduced by MobM have been determined by DNA sequencing and by primer extension analysis (5). The nucleophilic attack exerted by MobM is at the 5'-GpT-3' dinucleotide, within the sequence: 5'-TAGTGTG/TTA-3'. The pMV158 *oriT* does not exhibit homologies with known origins of transfer of plasmids from Gram-negative bacteria (8-10). However, several plasmids from Gram-positive hosts have a region identical or very similar to the pMV158 *oriT*. Comparison of the Mob proteins of fifteen plasmids from Gram-positive bacteria showed that the highest homologies were observed within two regions, located at the N-terminal part of the proteins.

In the case of plasmid-encoded proteins that participate in RC mechanisms (replication or conjugative transfer), all of these Rep, Tra, and Mob proteins share homologies with the Rep proteins of ssDNA-coliphages and geminiviruses (7, 11). Two of the more conserved motifs have been ascribed a function, which correspond to: i) the enzymatic activity domain, and ii) a putative metal-binding domain. For the Mob proteins of the family 2 of plasmids, represented by pMV158 (11), we have identified putative domains which could correspond to the two conserved domains. The first region could be the MobM-enzymatic domain, since it contains the NYDL conserved motif (11). The second MobM-region may contain the putative MobM-metal binding domain (the so-called "HUH" motif; 11). This HUH motif is present in the IncPα family 1 of Tra/Mob proteins (8-11). The HUH motif, which is also found in Rep proteins of RCR plasmids, has been reported as a copper-binding domain in the crystal structure of the so-called "blue oxidases" (12).

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Biochemistry of F-Type Plasmid Relaxases

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Relaxases comprise a superfamily of proteins which are involved in essential initiation and termination steps in the process of plasmid conjugation. They catalyze a number of biochemical reactions in vitro:

(i) single-stranded cleavage (nicking) at a specific site in a supercoiled plasmid DNA.
(ii) formation of DNA-relaxase covalent complexes by formation of Tyr-5'P bonds.
(iii) Mg^{+2}-dependent site-specific cleavage of oligonucleotides containing the *nic* site.
(iv) DNA strand-transfer reactions between ssDNA molecules.
(v) sometimes, additional biochemical activities, such as DNA helicase or DNA primase.
(vi) interaction with other proteins involved in the *in vivo* processing of conjugative DNA.

Relaxases were divided in four protein families, according to their sequence similarities and those of the sites at which they act. P-type, F-type and Q-type are the most widespread. TraI, the P-type relaxase of plasmid RP4 has been characterized in particular detail (3).

We are analyzing the structure-function relationships of TrwC, the relaxase of plasmid R388 (4,5). This 966 amino acids protein was subjected to domain analysis, to find that the 300 N-terminal polypeptide constitutes the relaxase domain, while a 650 C-terminal polypeptide contains a DNA helicase domain. Both activities are essential for R388 conjugation. The biochemical activities of the TrwC relaxase domain were analyzed in some detail. The oligonucleotide cleavage reaction seems to be an equilibrium of the sort TrwC + 18-mer $\Leftrightarrow$ TrwC::4-mer + 14-mer, with a $K_{eq} = 3 \times 10^{-3}$. Cleavage of supercoiled DNA *in vitro* is much more efficient, and it seems to attain also a very rapid equilibrium. The oligonucleotide strand-transfer reaction is slower, and thus susceptible of kinetic analysis. The mechanism of these reactions is similar to that of gpA, the replication protein of phage ΦX174 (2), with some exciting peculiarities. As is gpA, two Tyr residues in TrwC are involved in the DNA strand transactions. However, they are not interchangeable. One Tyr residue is specifically used for the initial reaction on SC DNA, while the second Tyr is only used for the second strand-transfer reaction. As a result, relaxase activation results in a single replication event, since the product of the second strand-transfer reaction cannot be directly used for a second round of replication (1).

TrwC displays a unique and differential property of F-type relaxases: it is able to cleave SC DNA without the need of accessory proteins. This fact does not imply that other proteins are not involved in *oriT* processing. The relaxase activity of TrwC is enhanced *in vitro* by the accessory protein TrwA. TrwA plays a dual role in conjugation, since it works also as a repressor of the MOBw operon. Structurally, TrwA appears to belong to the Arc repressor family. Besides TrwA, protein IHF also binds to a specific site in R388 *oriT*. In doing so, it acts as a negative modulating factor: R388 relaxosomes are not constitutively assembled in the presence of IHF, as they do in its absence (G. Moncalián and F. de la Cruz, unpublished observations). We suggest that both TrwA and IHF play important regulatory roles in triggering TrwC action *in vivo*.

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Characterization of the recombination site of the integrase IntI1, from bacterial integrons.

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Bacterial integrons are genetic elements present in transposons, plasmids and chromosomes of many bacteria where they play a role in the assembly and reshuffling of antibiotic resistance genes (ARG). The integrons are characterized by the presence of a 5' conserved region encoding a recombinase that belongs to the lambda integrase family, a 3'conserved region containing a gene for resistance to sulfamides and a central variable region containing several ARG's. The integrase and the ARG's are adjacent to each other and they are expressed from divergent promoters.

Primary sites of recombination for this integrase are known as 59 bp elements and consist in sequences of around 60 bp length, and with some extent of sequence symmetry.

We have characterized secondary recombination sites for IntI1 integrase. The minimal requirement for recombination was a pentanucleotide with only two conserved residues. Next in complexity was a "double site" that consisted in two pentanucleotides in opposite orientation separated by a 3-8 bp spacer. Finally, 59 bp elements could be seen as two "double sites" separated by a palindromic GC rich region. Recombination frequency increases with site complexity from pentanucleotides to 59 bp elements (primary sites).

The use of secondary sites could explain how new genes could become associated into integrons and suggest a role for integrons as a general mechanism for the genesis of bacterial operons. In addition, they allow the use of the integrase as a biotechnology tool for the integration of genes into the chromosome.

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Session 3

Chairman: S. Dusko Erhlich

THE RAG PROTEINS AND OTHER FACTORS IN V(D)J RECOMBINATION. Martin Gellert, Dale A. Ramsden, Tanya T. Paull, Kevin Hiom, Meni Melek, and Dik C. van Gent*. Laboratory of Molecular Biology, NIDDK, National Institutes of Health, Bethesda, Maryland 20892-0540, U.S.A., and *Dept. of Cell Biology and Genetics, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

V(D)J recombination is initiated by the RAG1 and RAG2 proteins acting together to make DNA double-strand breaks at a pair of recombination signal sequences (RSSs). The break generates a blunt end on each RSS and a hairpin end on the adjacent DNA (this would be antigen receptor coding sequence in the natural situation). The purified proteins first bind the RSS in a "stable cleavage complex" that is highly resistant to competitor DNA. This complex requires both RAG proteins and both halves (heptamer and nonamer) of the RSS. Binding and cleavage can be greatly stimulated by the ubiquitous HMG1 or HMG2 proteins, which are known to bind DNA non-specifically and introduce a sharp bend.

The reactions of the RAG proteins display chemical similarities to transposition. A stereochemical test showed that hairpins are made by a one-step transesterification (like the DNA strand transfers of phage Mu transposase and HIV integrase), without a covalent protein-DNA intermediate, and more recent results have identified a "disintegration-like" reaction that extends the analogy.

We have also worked on the later steps of V(D)J recombination that rejoin the broken ends. This part of the process is known to be relatively non-specific and to share many factors with DNA double-strand break repair. We have recently been able to reconstitute complete V(D)J recombination in a cell-free system. Incubation of a DNA substrate with the RAG proteins, followed by a second incubation with a HeLa cell fraction, leads to the formation of both coding joints and signal joints. The continued presence of the RAG proteins after cleavage is absolutely required to make coding joints, but inhibits the joining of RSSs. The RAG proteins evidently influence the ability of other factors to act on coding ends and signal ends.

Coding joints from the cell-free reaction often contain self-complementary tracts that arise from the asymmetric opening of hairpin ends; such "P nucleotide" insertions are commonly found in junctions from in vivo recombination. The cell-free reaction displays a strong preference for joining at sites of short DNA homologies, but the addition of human DNA ligase I leads to a more diverse set of junctions, similar to those found in vivo. It is possible that different ligases are used for homology-dependent and independent joining.

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Investigating the role of Ku86 in V(D)J recombination.

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The Ku86 protein plays critical but poorly defined roles in V(D)J recombination and repair of DNA double-strand breaks induced by ionizing radiation. Along with Ku70, Ku86 forms a heterodimeric DNA end binding complex termed Ku that is capable of recruiting and activating the catalytic subunit of the DNA-dependent protein kinase (DNA-PK). Cells or mice bearing mutations that inactivate Ku86 are severely defective for formation of both standard products of V(D)J recombination: signal joints and coding joints. The rare junctions that have been recovered from these cells exhibit abnormal deletions, leading to the suggestion that Ku might be responsible for protecting broken DNA recombination intermediates from degradation. However, we have shown that V(D)J recombination intermediates are abundant and full length in Ku86-deficient cells, indicating that Ku86 is not required for protection of coding or signal ends (2,3). Unexpectedly, we found that formation of nonstandard V(D)J recombination products, termed hybrid joints, occurs with normal efficiency in Ku86-deficient mice and cell lines (1,2). Furthermore, most of the junctions result from joining an intact signal end to a full-length coding end. These data show that Ku is not required for some types of V(D)J joining events. We propose that hybrid junctions can be formed directly by the RAG proteins by a mechanism analogous to disintegration reactions performed by retroviral integrases (2).

Nucleotide sequences of 99 junctions from Ku86-deficient mice revealed that over 90% of the coding joints, but not signal or hybrid joints, exhibit short sequence homologies, indicating that base pairing interactions may be important for joining coding ends in the absence of Ku86. Furthermore, the addition of extra nucleotides (N nucleotides) mediated by terminal transferase (TdT) is absent in Ku86-deficient mice (1). These data suggest that Ku86 plays an unexpected role in the addition of N nucleotides by TdT.

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Role of the TCR α Enhancer in Regulating Recombination and Expression of the TCR α/δ Locus

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The TCR α/δ locus is a complex locus with gene segments that encode for the TCR δ chain contained within the boundaries of those that encode for the TCR α chain. As a consequence, V to J rearrangement of the TCR α locus results in excision of the TCR δ locus, a process that has been proposed to be important in T cell lineage determination. Several *cis*-acting elements have been defined in the TCR α locus that are involved in transcriptional regulation of the locus. There is a single transcriptional enhancer just downstream of the constant region gene. In addition, there are two transcriptional silencer elements between the constant region gene and the enhancer, and a locus control region downstream of the enhancer. There is a transcriptional promoter (TEA) upstream of the most 5' J segment that drives germline transcription of the J α region and has been proposed to be important in opening the locus during development. There is a single defined enhancer within the TCR δ locus.

We have used gene targeting strategies to target and delete the TCR α enhancer and have generated mice with the targeted deletion on one or both alleles. We have also used the cre-*lox* system to delete the drug resistance gene from the loci to insure that the effects we observe are due to the lack of the enhancer and not due to the insertion of a drug resistance gene. Development of α/β T cells proceeds normally in mice with the TCR α enhancer deleted on a single allele. However, rearrangement of the targeted TCR α allele occurs in only a small fraction of mature T cells. Furthermore, mice with the TCR α enhancer deleted on both alleles show a block in T cell development at the CD4/CD8 double positive thymocyte stage. Neither germline nor mature transcripts from the TCR α locus are observed in these mice. TCR δ rearrangement appears grossly normal in these mice.

These results demonstrate that the TCR α enhancer is critical for mediating accessibility of the TCR α locus to the V(D)J recombinase and that it functions in a *cis*-acting manner. In addition, the TCR α enhancer also appears to be important in regulating germline transcription of the TCR α locus. Accessibility of the TCR δ locus does not appear to require the integrity of the TCR α enhancer. However, transcriptional regulation of this locus may depend on the integrity of this element, a possibility that we are currently investigating.

Is *Sfi*I a Recombinase?

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It has long been thought that restriction enzymes may have functions in bacterial cells outside of their acknowledged roles in the restriction of foreign DNA entering the cell. Recent experiments on the *Sfi*I endonuclease have shown that this enzyme has more in common with recombinational enzymes than with other type II restriction enzymes (1-3). The type II enzymes are generally dimeric proteins that recognize palidromic DNA sequences, typically 4-6 bp long, and interact with these in symmetrical fashion so that each active cleaves one strand of DNA (4). *Sfi*I deviates from this pattern in several respects. First, its recognition sequence, GGCCnnnn↓nGGCC (5), is unusually long for a type II enzyme and instead looks more like the recombination sites for enzymes such as Cre, Xer or FLP. Second, *Sfi*I exists in solution as a tetramer of identical subunits rather than the conventional dimer. Third, before any DNA cleavage reactions can occur, *Sfi*I has to bind to two copies of its recognition site. Both sites are then cleaved in a concerted process in which four phosphodiester bonds are cleaved in consecutive reactions before the enzyme dissociates from the DNA.

The two *Sfi*I sites can be located on the same DNA molecule, in which case the enzyme loops out the intervening DNA, or they can be on different molecules, with the enzyme bound simultaneously to both. However, looping interactions across sites on the same DNA molecule are greatly favoured over bridging interactions between separate molecules. Consequently, the primary reaction of the *Sfi*I endonuclease is the excision of the DNA between two *Sfi*I sites but, at its physiological temperature, the enzyme remains attached to the cleaved ends for ~30 min before releasing them.

The relationship between restriction and recombination-like activities of *Sfi*I is currently being explored by determining the stereochemistry for phosphodiester hydrolysis by this enzyme. This will reveal whether the reaction proceeds with inversion of configuration at the phosphoryl centre, as found for both *Eco*RI and *Eco*RV, or with the retention of configuration seen with lambda integrase. Further experiments are being carried out to determine if the *Sfi*I endonuclease can act *in vivo* as a restriction enzyme, by examining whether DNA with unmethylated *Sfi*I sites can be transformed into cells containing the *Sfi*I restriction-modification system. Finally, the *Sfi*I system is being utilised as a test system for the analysis of long-range interactions between distant DNA sites.

We thank New England Biolabs for providing the *Sfi*I system, and the BBSRC and the Wellcome Trust for financial support.

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Nucleosome mediated inhibition of V(D)J recombination

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V(D)J recombination is the site specific recombination reaction which assembles functional immunoglobulin and T cell receptor genes from a pool of gene segments during lymphocyte development in vertebrates. The reaction is initiated by the introduction of a nick on one strand, immeditely 5' of the recombination signal sequence (RSS). This nick is then converted into a double-strand break (DSB) at the border between a gene segment and its flanking RSS. Binding and site-specific cleavage at RSS is performed by the products of the recombination-activating genes, RAG1 and RAG2. Although identical RSS are found at all rearranging loci, cutting by RAG proteins is tightly regulated at several levels: cell type, cell stage, locus, and allelic specificity are all observed. Very little is known about how this regulation is achieved. The appearance of germline transcripts at rearranging loci and the stimulation of recombination by enhancer sequences in transgenic substrates suggest that modification of chromatin structure is probably an important level of control by regulating accessibility of the RSS to RAG proteins. To test the accessibility model directly, we assembled RSS-containing mononucleosomes in vitro and tested their ability to be cleaved by purified RAG proteins. Compared with cutting on a free DNA substrate, RAG-mediated cleavage on a nucleosome substrate is severely inhibited. Both the nicking and second strand cleavage steps are blocked. Competitor titrations suggest that this is due to inefficient binding of RAGs to the nucleosome substrate. These results suggest a potential regulatory step of V(D)J recombination at the level of RAG-mediated cleavage on mononucleosomes.

Session 4

Chairman: Arthur Landy

Site-specific Recombination by γδ Resolvase: Holding the Pieces Together

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The site-specific recombination enzyme, γδ resolvase, performs recombination within a nucleoprotein complex, the synaptosome, consisting of two copies of the 114 bp *res* site and at least six resolvase dimers (1). Each *res* site contains three resolvase dimer binding sites. Strand exchange occurs within one of these (site I); the other two (sites II and III) are crucial for *res* site synapsis and proper organization of the synaptosome.

X-ray crystallographic studies of γδ resolvase identified an interaction (called the 2,3' interaction) between resolvase dimers (2). The amino acid residues responsible for this interaction are Arg2, Arg32, Lys54 and Glu56. These residues are clustered together, forming a single compact surface (which we shall call the 2/32/56 surface) on each resolvase subunit. This surface from one protomer engages the identical surface of a second protomer through a set of reciprocal and complementary interactions. An important biological role for this interface is indicated by the behavior of resolvase mutants (3). Amino acid substitutions at each of the four positions resulted in mutants that bound to *res* but failed to exhibit the cooperativity characteristic of the binding of wildtype resolvase. In addition the mutant proteins were unable to promote synapsis or recombination. A study with the 2,3'-defective mutant, R2A, found that mixtures of R2A and E128K (a mutant unable to bind to site III) were recombinationally inactive, whereas R2A with the catalytically defective mutant, S10L (which lacks the Ser10 nucleophile essential for DNA breakage and joining) exhibited substantial recombination activity (4). These and other results led to the proposal that the 2,3' interaction is essential at binding sites II and/or III (where it plays a critical role in synapsis) but may not be used by the resolvase subunits performing strand exchange at site I.

Which resolvase subunits within the synaptosome make use of the 2,3' interaction? The 2,3' interaction between two resolvase dimers involves just one protomer of each dimer; the 2/32/56 interface of the second protomer is exposed and, in the synaptosome, could remain free or could interact with a third resolvase dimer. It seems likely, then, that only a subset of the 12 resolvase protomers in the complex needs an active 2/32/56 interface. We have now identified which half sites within *res* must be occupied by a 2,3'-competent subunit. Our approach makes use of covalently linked heterodimers of resolvase, with one subunit containing one of the 2,3'-defective mutations (R2A, R32S or E56K). These heterodimers are specifically oriented at appropriate dimer binding sites by a combination of the altered-specificity mutation, R172L, in the DNA-binding domain of one subunit, and a G2T base substitution in one half of the binding site. Both recombination and DNA binding assays have been used for the analysis.

Our results indicate that 2,3'-proficient resolvase subunits are required at the left half of site II, the right and possibly also the left halves of site III, and the right half of site I. We propose that the resolvase protomer bound to site II-L interacts with one at site III and that this interaction is critical for *res* site synapsis (1, 5). The protomer bound to site I-R is likely to interact with the other protomer at site III; this interaction may be responsible

for appropriately positioning the two copies of site I for pairing and strand exchange, for ensuring the correct topology of the complete synaptosome or for activating the site I-bound resolvase for catalysis.

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Hybrid site-specific recombination systems

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The Tn*3* recombination site *res* has three binding sites for dimers of resolvase. Strand exchange takes place exclusively at the centre of site I. The accessory binding sites, II and III, are required for efficient resolution. Various experiments have defined roles for sites II and III in synapsis. With their bound resolvase, they are the main agents in bringing the two recombining *res* sites together. Also, it is proposed that requirement for antiparallel pairing and intertwining of the two sets of accessory sites in the synaptic complex results in the selectivity and specific product topology of recombination (Refs. 1-4).

In order to analyse further the functions of the accessory sites, we have constructed hybrid recombination sites with binding sites II and III from *res* and a 'crossover site' from a different system. Previous experiments where the crossover site was Tn*21 res* binding site I, or the phage Mu *gix* site for Gin recombination, will be described briefly in the talk. More recently, we have made hybrid *'les'* sites containing the phage P1 Cre recombination site *loxP*. First, *loxP* was placed in the approximate position of *res* site I relative to sites II and III, in either orientation. Recombination test substrates were made containing two of these hybrid *les* sites, with the pair of *res* sites II/III in direct repeat, and the *loxP* sites either in direct or inverted repeat. Cre-mediated recombination was inhibited by the presence of resolvase. When the spacing between *loxP* and site II was increased to about 100 bp, Cre recombination in the presence of resolvase gave three-noded knot inversion products and four-noded catenane resolution products, whereas in the absence of resolvase the products were mainly unknot and free circles respectively. When the *loxP*-site II spacing was increased further, to 190 bp or 300 bp, the product topology was still constrained, but more knot or catenane nodes were observed. When the *les* sites were inverted with respect to each other, no gross change in product topology was observed when resolvase was present.

Similar constrained topology of Cre recombination was observed when *loxP* sites were placed adjacent to binding site I of full *res* sites.

The product topologies in these hybrid systems are consistent with a resolvase/*res* synaptic complex in which intertwining of sites II and III is sufficient to introduce the three interdomainal supercoils required to account for the topologies of the standard resolution reaction. *les* recombination products are as predicted for antiparallel alignment of *loxP* sites by Cre followed by strand exchange without introduction of any further topological nodes. The hybrid system products are strikingly analogous to those of Xer-mediated recombination at *cer* and *psi* sites (Ref. 5).

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Site-specific recombination by the β recombinase from the streptococcal plasmid pSM19035.

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The β recombinase encoded by the Gram-positive bacterial plasmid pSM19035 is unable to mediate DNA recombination *in vitro* unless a chromatin-associated protein of bacterial (e.g. bacterial Hbsu, HU), algae (HlpA), plant or vertebrate (HMG1) origin is provided. pSM19035 has extraordinarily long inverted repeats that comprise about 80% of the molecule. The β coding region, the β target site (*six* site) and the minimal replicon are within the inverted repeated segments. The *six* site is a 90 bp DNA segment containing two binding sites (subsites I and II) for β protein dimers. The β recombinase binds sequentially to both sites, having a different affinity and interaction with each one.

The β recombinase in the presence of simple buffer, 80 mM NaCl, and highly purified Hbsu protein, is able to catalyze DNA inversion when the two *six* sites are in an inverse orientation, and DNA resolution, when the two *six* sites are directly oriented. The point of crossing over has been localized to the centre of subsite I, while subsite II seems to play an architectural role by aligning the crossing over sites at the synaptic complex. The chromatin-associated protein facilitates the joining of distant *six* sites and promotes the aligment of a properly oriented recombination complex.

A number of site-directed mutants at residues conserved between the β protein and other recombinases of the same family have been constructed and the wild-type and mutant products purified. The analysis of the recombination and DNA binding ability of each mutant protein showed that the mutations affect the catalytic activity and, in two cases, the dimerization of the protein. The results suggest that the β protein probably mediates recombination by a catalytic mechanism similar to that proposed for the resolvase/invertase family. Since the β recombinase differs from DNA resolvases and DNA invertases in its lack of bias towards one of these two activities, the results presented support the hypothesis that its unique properties might lie in the architecture or assembly of the recombination complex. In addition, two β protein mutants that can no longer form dimers in solution have provided new insights into the way the protein binds to DNA.

The β recombinase catalyzes both DNA inversion and DNA resolution on supercoiled DNA substrates with inversely oriented target sites, and only DNA inversion when the substrate is relaxed or linear

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The β recombinase belongs to the Tn3/γδ family of recombinases, although it has several distinctive characteristics. In the presence of a chromatin-associated protein such as Hbsu, the β recombinase is known to catalyze resolution on supercoiled substrates containing directly oriented target (*six*) sites, and inversion on supercoiled substrates having two inversely oriented *six* sites. We have studied the recombination reaction on DNA substrates carrying two inversely oriented *six* sites. We have found that, under low ionic strength conditions, both inversion and resolution occur when the DNA substrate is supercoiled, while only inversion products are generated when the substrate is relaxed. Interestingly, although resolution activity was strictly dependent upon DNA supercoiling, inversion products were efficiently generated even on linear DNA substrates. This unusual property is not found in the resolvases and invertases of the Tn3/γδ family, to which the β protein belongs, and gives new insights into the recombination mechanisms of this important family of recombinases. The results support the notion that the topological filter of the recombination reaction catalyzed by the β recombinase is less strict than that of other recombinases of the family, and that the pathway leading to DNA resolution depends more on the supercoiling of the DNA substrate than on the relative orientation of the recombination sites.

The role of the pAMβ1 resolvase in plasmid replication

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Two types of replication have been observed for plasmids from Gram-positive bacteria, one called rolling circle replication (RCR), the other theta replication (TR). They tend to be used by small and large plasmids, respectively, presumably because RCR is more error-prone than TR. The most thoroughly studied TR plasmids belong to the pAMβ1 family. Replication region of pAMβ1 can be divided into three sub-regions, corresponding to copy control, primary replication functions and secondary replication functions. The copy control subregion contains two elements, a repressor gene and a counter-transcript driven transcriptional attenuation system. They independently regulate transcription from a promoter, which has two functions. One is to provide the message for the synthesis of the essential Rep protein, and the other is to provide the primer for initiation of plasmid replication. The subregion that carries primary replication functions (« basic replicon ») comprises the *rep* gene and the replication origin. Leading strand synthesis is initiated 27 bp downstream of the end of the *rep* gene, within the replication origin. Three different secondary replication functions are found downstream of the replication origin. The first is a single-strand initiation site (*ssiA*), located on the lagging strand template, ~150 bp downstream from the leading strand initiation site. *ssiA* priming activity requires proteins involved in primosome assembly. The second function corresponds to a site-specific resolution system, comprising a *res* site and a resolvase of the Tn*3* family. The *res* site is localized ~20 bp downstream of *ssiA*. The third function is a type I topoisomerase, which appears to relax specifically plasmids that replicate in the pAMβ1 mode.

Replication of pAMβ1 appears to involve the following steps: (i) The transcription initiated at the rep promoter reaches the replication origin. Its processing, somehow involving the Rep protein, provides a primer for DNA synthesis. (iii) DNA polymerase I (Pol I) initiates the synthesis. It replicates the template strand while displacing simultaneously the complementary strand and thus generates a D-loop. (iv) The synthesis is arrested between *ssiA* and *res* by the action of topoisomerase, which modulates plasmid superhelicity, or at *res* by the resolvase, which forms a road-block for Pol I. (v) A primosome assembles at *ssiA*, which is on the displaced strand of the D-loop, while DNA polymerase III (Pol III) loads at the end of the newly synthesised strands. (vi) A helicase displaces the resolvase and thus allows Pol III to complete the round of replication.

The central feature of this model is a programmed switch between the initiating and elongating polymerase. It is tempting to speculate that in different systems, where the initiation and elongation replication machineries differ, a programmed switch may exist, based on proteins with high affinity for DNA, such as resolvase, to arrest the progression of the initiating polymerase.

Site-specific recombination by actinophage øC31 integrase.

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øC31 is a temperate bacteriophage that infects over half of the known strains of *Streptomyces* and has been used widely to develop vectors for the genetic manipulation of this commercially important genus. øC31 integrates into the host chromosome via a site-specific recombination reaction. Previous work (1) has shown that the øC31 integrase gene, *int*, is located between the early and late genes and immediately downstream of the *attP* site. This work (1) also strongly suggested that øC31 integrase is not a member of the λ integrase group of recombinases which catalyse strand exchange via a phospho-tyrosine intermediate. *int* encodes a 68 kDa protein which has primary sequence similarity at its N-terminus to the resolvase/invertase group of recombinases (2) and is therefore reminiscent of a small group of other phage enzymes, notably the lactococcal phage TP901-1 (3) and actinophage R4 (4). This group also have similarities to the cellular-encoded resolvases, CisA and XisF involved in *B. subtilis* sporulation and heterocyst formation in *Anabaena*, respectively. We have been studying the properties of the øC31 integrase with a view to making comparisons with those of resolvase/invertases and the λ integrase type enzymes.

Using compatible plasmids in *E. coli* we expressed øC31 integrase and analysed recombination between the cloned *attB* site from *S. ambofaciens* and the *attP* site. The observation that the reaction occurs in *E. coli* indicated that no other phage or *Streptomyces* encoded proteins were required. The reaction occurred when the *att* sites were located *in trans* or in a tandem orientation *in cis* (in which case a deletion occurred). A functional *attB* site was 30 bp in length and the *attP* site can be less than 80 bp. No recombination was observed between *attL* and *attR* sites in this system. The integrase was purified from *E. coli* and used in binding assays; integrase bound to all the *att* sites with approximately equal affinities. Finally integrase could catalyse the recombination reaction *in vitro* between supercoiled substrates. Our findings demonstrate that øC31 integration shows properties reminiscent of both the resolvase/invertase and the λ integration systems. It is hoped that further studies of this enzyme and those similar to it will help to gain a deeper understanding of the mechanisms of site-specific recombination.

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Session 5

Chairman: David J. Sherratt

Activation of strand exchange by resolvase

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The resolvases from Tn3 and γδ, along with the DNA invertases Gin and Hin, are among the best-studied members of a big family of site-specific recombinases. These enzymes carry out strand exchange by a mechanism fundamentally different from recombinases of the phage lambda integrase family. Instead of making a Holliday Junction, resolvase cuts both DNA strands in each recombination site, making a recombination intermediate in which four half-sites are covalently attached to four resolvase subunits. At this stage the four half-site DNA's are no longer covalently joined to each other in any way. Resolvase nevertheless prevents their escape, ensuring that each half-site is ligated to its correct recombination partner, ie. a "left" half-site is always joined to a "right" half-site. The relative motions of the half-sites in the interval between cleavage of the substrate and religation of the recombinant are tightly constrained, giving a fixed change in the *local* DNA linkage (*local* $\Delta Lg = +2$ for the forward reaction of resolvase, compared to $\Delta Lg = 0$ for lambda integrase etc.).

We have previously suggested that strand exchange by resolvase is coupled to a rotational exchange of the four protomers within an active tetramer. This idea is in accord with the topological data, but has been ridiculed on structural grounds. There are three main problems:

1. destruction of the existing dimer interface during recombination would be energetically costly.
2. no relevant dimer-dimer interface has yet been identified.
3. there is no identifiable means for maintaining the connectivity of the four protomers and half-sites during rotation.

It has thus been argued that the stuctural integrity of the resolvase dimers is likely to be maintained throughout the catalytic cycle. We will present experimental data that address the first and second points, and we will describe a new structure-informed strand exchange model that deals with the third problem.

Certain mutations affecting the resolvase dimer interface display a curious "activated" phenotype. The altered proteins (eg E124Q γδ resolvase) have a relaxed requirement for substrate supercoiling, exhibit reduced topological selectivity, and seem to stabilize the covalent intermediate. These mutant proteins also participate in unusual

"cleavage-ligation" reactions in which half-sites are joined to "incorrect" partners, as might be expected if half-sites are able to escape from the original synaptic intermediate, and rejoin to new partners. Consistent with this interpretation, we have now demontrated the catalytic activity of purified "half-site" complexes, in which resolvase is covalently attached to half of a recombination site. In the cleavage-ligation assay, the half-site complexes are more recombinogenic than intact linear res sites. These results imply that a resolvase dimer complexed to an intact crossover site can be broken into two unconnected half-site-monomer complexes that retain recombination activity. A more highly choreographed version of the same process is thus a good candidate for the mechanism of the normal strand exchange reaction.

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Activation of Hin-catalyzed site-specific DNA inversion by Fis

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The alternate expression of flagellin genes in *Salmonella* is controlled by a site-specific DNA inversion of a 1 kb segment of the chromosome (1,2). The Hin recombinase, encoded within the invertible segment, is responsible for cleaving and exchanging DNA strands. Hin binds to the ends of the invertible segment, *hixL* and *hixR*, and is able to assemble these two recombination sites into a catalytically incompetent structure. Catalytic activation of this complex requires the incorporation of a recombinational enhancer segment together with two bound Fis protein dimers into an invertasome structure (3).

Analysis of Fis-chemical nuclease chimeras together with gel electrophoresis experiments have shown that the degree of DNA bending varies at different Fis-DNA complexes (4). These experiments have enabled us map the path of DNA within the recombinational enhancer. This information, combined with a model of Hin based on the structure of resolvase (5) plus the DNA binding domain of Hin (6) and topological analysis (7,8), has enabled us to generate a molecular model for the Fis-activated invertasome.

The region on Fis that is responsible for Hin activation had been only partially resolved by X-ray crystallography (9,10). A new crystal structure of a Fis mutant now reveals the entire activation region, which includes an N-terminal flexible β-hairpin arm from each subunit that protrude over 20 Å from the protein core (11). Crosslinking assays employing cysteine substitutions throughout the β-arms are consistent with this structure in solution and directly demonstrate the mobility of the arms. Mutation and chemical modification studies combined with the crystal structure have identified the important structural and functional amino acids. Three amino acids near the tip of the arms are particularly important and probably contact Hin.

Active Fis dimers must be present at both binding sites within the enhancer in order to promote Hin inversion. In order to test whether both subunits are required within the dimer, heterodimers containing one active and one inactive subunit were generated in vitro. His-tagging of the mutant form combined with nickel affinity chromatography ensured that no active homodimers were present and disulfide linkage within the protein core ensured that subunit mixing after purification did not occur. Recombination assays using the heterodimers demonstrated that only one of the Fis dimer arms is required to activate Hin inversion. The activity of the heterodimer is consistent with efficient activation by Fis mutants in which the N-terminal arms within a dimer are covalently linked by disulfide bridges. Experiments employing mixes of Fis-independent and Fis-dependent subunits of Hin are in progress to test whether one or both subunits within a Hin dimer are required to assemble a catalytically active Hin complex.

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SITE-SPECIFIC RECOMBINATION OF MOBILE INTRONS Marlene Belfort[1] and Alan Lambowitz[2]. [1] Molecular Genetics Program Wadsworth Center, New York State Department of Health, and School of Public Health, State University of New York at Albany, PO Box 22002, Albany, New York 12201-2002, [2] Depts. of Molecular Genetics and Biochemistry, The Ohio State University, 484 West 12th Street, Columbus, OH 43210.

Group I and group II introns are self-splicing at the RNA level and mobile at the DNA level. However, they differ in RNA structure and splicing pathway, as well as in mobility mechanism. Group I introns home to cognate intronless sites by a DNA-based double-strand-break repair (DSBR) mechanism initiated by an intron-encoded endonuclease. In contrast, group II introns home by an RNA-based pathway that involves maturase, reverse transcriptase and endonuclease activities encoded by the intron, in a process termed retrohoming.

Studies on group I homing of the phage T4 *td* intron have shown that the process is tightly coupled to recombination-dependent DNA replication (RDR). In addition to functions known to be required for RDR (e.g. T4 recombinases UvsX/Y, the T4 replisome, and DNA ligase), 5'-3' exonucleolytic activities of T4gp46/47 and RNaseH and the 3'-5' activity of T4 DexA have been implicated in processing the ends of the recipient DNA in group I intron homing. Both classic DSBR and synthesis dependent strand annealing pathways are implicated as mechanisms in group I intron homing.

To dissect pathways and functional requirements of group II intron homing, the *Lactococcus lactis* LtrA intron has been expressed in *E. coli*, in a collaboration with the laboratory of Dr. Gary Dunny (University of Minnesota). We have shown that the LtrA intron splices in *E. coli*, expresses maturase, reverse transcriptase and endonuclease activities, and that while the protein cleaves the one DNA strand of the recipient, the intron RNA cleaves the other, precisely at the site of intron insertion. Furthermore, genetic experiments demonstrate that mobility occurs by a retrohoming pathway via an RNA intermediate. The system is now amenable to mechanistic studies in *E. coli*.

Programmed Genomic Deletion In *Tetrahymena thermophila* In Vitro, Sergei Saveliev and Michael M. Cox, Department of Biochemistry University of Wisconsin, Madison, WI 53706

Excision of thousands of specific genomic sequences (Internal Eliminated Sequences, IES) occurs during macronuclear development in *Tetrahymena thermophila*. In earlier studies of the reaction intermediates appearing in vivo during IES excision in *Tetrahymena*, we proposed a transposition-like mechanism for excision. According to the mechanism, a single cleavage at one IES boundary initiates the IES excision. The 3' hydroxyl group on the MAC-retained side of the cleavage then acts as nucleophile in attacking a phosphodiester bond at the opposite end of the IES. This one-step phosphoryl transesterification reaction creates the final chromosomal junction on one strand.

Now we have developed an in vitro assay for the proposed transesterification step. We have found that this step of IES excision occurs in nuclear extracts of *Tetrahymena* if one boundary of the IES is precleaved before the incubation. The reaction is heat-inactivated and divalent cation-dependent. The reaction exhibits a strong dependence on the presence of a 3' terminal adenosine residue, for use as the 3'-OH nucleophile in transesterification. Target sites occur within a 15 bp region at one end of the IES. The excision activity is found in conjugating cells undergoing IES excision. However, it is absent in vegetative cells in which genomic rearrangements are complete and in starved cells in which programmed DNA rearrangements have not yet commenced. These properties of IES excision in vitro generally conform to those expected for transesterification associated with IES excision in vivo.

THE β RECOMBINASE FROM THE STREPTOCOCCAL PLASMID pSM19035 CATALYSES SITE-SPECIFIC RECOMBINATION IN EUKARYOTIC MICROENVIRONMENT

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Site-specific recombination is a well known mechanism used by prokaryotic and eukaryotic organisms for the dynamic modification of their genome. Basic site-specific recombination mechanism is simple because mainly it needs first target sequences and second the recombinase enzyme that will recognize that sequences and will promote recombination. Therefore, it turns out that it will be possible to introduce this in higher organisms to induce targeted changes in their genome.

Different authors have taken till now advantage of two site-specific recombination mechanisms: the Cre-loxP and the Flp/FRT systems. Both are quite similar in their way of action; their target sequences comprise 34 base pairs and both recombinases (Cre and Flp) are able to catalyze recombination either in the same molecule (intramolecular recombination) or in different ones (intermolecular recombination).The last capability has proven useful for targeted integrations in the genome of mice and plants. However, this last attribute implies the risk of undesired rearrangements if the same system is used for two different recombination events in the same animal or plant due to the existence of two recombination sites per modification.

In this context we have focused on a new recombination system already characterized for the prokaryotic microenvironment. The β recombinase from the streptococcal plasmid pSM19035 belongs to a new family of site-specific recombinases, and is able to catalyze both deletions and inversions on DNA sequences. This recombination system needs the existence of two 93 base pair recognition sites termed *six* and a host factor to catalyze the reaction. Nevertheless, although the recombination machinery is more complex, this recombinase catalyzes only intramolecular recombination, therefore reducing the risk of uncontrolled recombinations and allowing the possibility of engineering two or more recombination events with the only condition of being in different chromosomes or at least far enough from each other. Furthermore this recombinase could be potentially used in combination with the already existing systems simplifying the design of multiple targeting experiments.

We have cloned the β recombinase gene in an eukaryotic expression vector and transfected this vector into the simian COS-1 cell line. The pattern of expression reveals a nuclear localization of the protein on cells and it can also be detected by Western blot analysis. To assess whether this expression leads to the production of an active protein we made transient cotransfection experiments of the expression plasmid with different constructions harboring a reporter gene fragment flanked by two *six* sites. The recombined species could be monitored by PCR with specific primers. In the cotransfection experiments the recombined band could be seen only when the expression plasmid and the target construction were cotransfected. This result not only reveals that the β recombinase is active in eukaryotic cells but also shows that the mammalian microenvironment provides the host cofactor(s) needed for the recombination event. Finally, to know if the β recombinase could be active on target sequences integrated in the chromosome structure, we developed stable clones of the reporter construction by transfecting the mouse NIH 3T3 cell line. These stable clones harbored different copies of the recombination reporter structure. When these clones were transiently transfected with the expression plasmid, the PCR of the genomic DNA revealed the presence of recombined structures. The intensity of the diagnostic band as shown by Southern Blot seemed to correlate with the number of copies of the target construction.

Further experiments remain to be done regarding the efficiency of the system as well as the maintenance of the intramolecular restriction in eukaryotic cells. Anyhow, this new system could be used in combination with the already existing ones for the targeted modification of the eukaryotic genome.

POSTERS

The mechanism of Xer site-specific recombination.

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Xer site-specific recombination functions in stable inheritance of the *Escherichia coli* chromosome and natural multicopy plasmids, by converting multimers, that can arise by homologous recombination, to monomers. Xer recombination requires two related recombinases, XerC and XerD, each of which mediates exchanges of one specific pair of DNA strands. In order to understand the respective roles of XerC and XerD in recombination, their substrate requirements were analysed using synthetic Holliday junction-containing substrates 'tethered' so that they can adopt only one of two possible right-handed antiparallel stacked conformations. Both recombinases preferentially catalyse exchange when their substrate strands are 'crossed' consistent with the 'strand-swapping' model for λ integrase family recombinases. By using chemical probes of Holliday junction structure we have determined that recombinase binding induces unstacking of the bases in the centre of the recombination site indicating that the junction branch point is located there. The implications of these results will be discussed.

We have determined the crystal structure of XerD. The arrangement of active site residues supports a cis cleavage mechanism. The structure based model of XerD interaction with DNA of its binding site accomodates extensive bichemical and genetic data. We have been extending these studies even further to relate the structure of XerD to its function in binding, catalysis and interactions with its partner recombinase XerC.

Characterization of the site-specific recombination system from the *Lactobacillus bulgaricus* temperate bacteriophage mv4 : functional flexibility of the integrase and characteristics of its target sites.

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Temperate phage mv4 is representative of a widespread genetic group of phages of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *lactis*. mv4 genome is a double stranded DNA of 36 kb, circularly permuted. The late genes encoding the main capsid proteins of 34 and 18 kd, as well as the genes involved in cell lysis have been characterized previously. Phage mv4 DNA integrates in the chromosome of *Lactobacillus delbrueckii* subsp. *bulgaricus* strain by recombination between a 17 bp core sequence common to phage (*attP*) and bacterial (*attB*) attachment sites. This mechanism is catalyzed by the phage encoded integrase, following the Campbell model. Analysis of the *attB* site revealed that mv4 integrates into a tRNA-serine gene, a general phenomenon among various bacterial species.

A non replicative vector containing the *attP-int* region has been constructed. This integrative vector (pMC1) has a wide host range. It is able to integrate into the chromosome of different organisms such as *Lactobacillus plantarum* (LP80), *Lactobacillus casei* (TGS1.4 and A22), *Lactococcus lactis* (MG1363), *Streptococcus pneumoniae* (R800) and *Enterococcus faecalis* (JH2.2).

Sequence analysis of the pMC1 chromosomal insertion site in the different host bacteria revealed that the entire sequence of the tRNA gene is not required to get pMC1 integration and showed the flexibility of the integrase for the bacterial integration site. Furthermore, an *attB* consensus sequence has been determined. The *attB* and *attP* sites have been further characterized (minimal size and nucleotide requirements) using a recombination test developped in *Escherichia coli* since in this bacterium mv4 integrase is able to mediate site-specific recombination between two plasmids carrying respectively the *attP* site and *attB* site. The minimal lengths required for functional *attB* and *attP* sites are 16 and 201 nucleotides respectively. As in the case of Lambda recombination system, this difference of size could illustrate a difference of complexity of the two sites. However, compensation events have been observed between the two sites: *attB* sites of a longer size than the minimal one were able to compensate the absence of DNA fragments on the *attP* site suggesting that the *attB* site might be more complexe than in the Lambda system. Purification of the mv4 integrase has been undertaken in order to analyse its interaction with its target sites and an *in vitro* recombination test has been developed.

Characterization of the minimal crossing over site, and of the dimerization and catalytic domains, of the β site-specific recombinase.

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The β recombinase from the broad-host-range Gram-positive plasmid pSM19035 shows 28 to 34% identity with DNA resolvases and DNA invertases of the resolvase/invertase family. It catalyzes intramolecular site-specific recombination between two directly or inversely oriented *six* sites in the presence of a chromatin-associated protein of Bacteria or Eucarya origin. The region necessary and sufficient for β mediated recombination has been localized to a 90 bp DNA fragment within the defined *six* site. It contains two adjacent binding sites for the β recombinase (sites I and II). The point of crossing over has been localized to the centre of site I.

We have constructed a number of site-directed mutants at residues conserved between the β protein and other recombinases of the same family. The analysis of the recombination and DNA binding ability of each mutant protein shows that the mutations affect the catalytic activity and, in two cases, the dimerization of the protein. The results suggest that the β protein probably mediates recombination by a catalytic mechanism similar to that proposed for the resolvase/invertase family. Since the β recombinase differs from DNA resolvases and DNA invertases in its lack of bias towards one of these two reactions, the results presented support the hypothesis that its unique properties might lie in the architecture or assembly of the recombination complex. In addition, two β protein mutants that can no longer form dimers in solution have provided new insights into the way the protein binds to DNA.

Programmed Gene Rearrangement: Site-Specific Recombination
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Use of site-specific recombination to circumvent position effect in transgenic mice

Most transgenes are susceptible to chromosomal position effect. In many research projects this necessitates the evaluation of a number of different transgenic lines derived with the same DNA construct. This may increase the labour and expense by as much as 5- to 10- fold. The cost implications are particularly serious in the case of large animal (sheep, pigs, cows) which have very high unit costs.

In this work we propose to circumvent this problem in ES cell-derived transgenic mice by using the **Cre/*Lox*P site-specific recombination system.**
The idea is that if we establish that a particular chromosomal site permits high-level expression, then different transgenes introduced at that site by site-specific homologous may be expected to show equivalent expression. The use of two different *lox* sites will allow us to lock the inserted DNA into the site by excision of one of the *lox* sites.

The strategy we are using involves 2 steps of Cre-mediated recombination (deletion then insertion). In a first step a transgene has been electroporated into the HPRT⁻ E14TG2a ES cell clone. This fragment carries two different *lox* sites (*lox*P, *lox*2), which have been showed not to be able to recombine together, the HPRT and HSVtk2 selectable markers and the MUP gene (1) whose expression will be evaluated *in vivo*. The transfected clones have been selected in presence of HAT and the integrity of the construct has been checked by Southern blotting. Six independent clones have been injected into blastocyst and transgenic mice have been produced. MUP gene expression in the different mouse lines will be evaluated very soon.
The next step consists in deleting the MUP and HSVtk2 genes in the selected ES cell clones and then inserting the WAP gene (2) by site-specific recombination. The insertion of the WAP gene can occur either *via* the *lox*P or the *lox*2 site and this sould result in a configuration where the WAP gene is locked between two different *lox* sites. The latter ES cell clones will be selected for the loss of the HPRT gene. Transgenic mice will be produced and WAP gene expression will be compared to those of the MUP gene in the corresponding mouse line.

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Specific photocrosslinking of PI-*Sce*I-DNA complexes: Identification of phosphate backbone contacts

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The PI-*Sce*I nuclease from *S. cerevisiae* is a protein-intron encoded highly sequence-specific endonuclease which requires Mg^{2+}-ions as a cofactor for DNA cleavage and yields a staggered break with 3´OH overhangs of four bases. It is a homing endonuclease which can mediate transfer of its own coding sequence into an allele that lacks it. Hypothetically this process follows the DSBR pathway of recombination.

Analysis of the minimal sequence which is absolutely necessary for DNA cleavage indicated a dependence on the structure of the substrate. Efficient cleavage is observed with a DNA containing 30 bp of the natural recognition sequence using supercoiled plasmids, 40-50 bp using prelinearized plasmids and >50 bp using synthetic double-strand oligodesoxynucleotides.

In order to identify amino acid residues responsible for binding and catalysis of PI-*Sce*I a sequence-specific photoaffinity DNA substrate was synthesized. Azidophenacyl bromide was chemically coupled to uniquely positioned phosphorothioate residues in the DNA phosphate backbone. The derivatized DNA forms specific complexes with PI-*Sce*I and, following irradiation with 365 nm certain modified nucleotide residues of the DNA causespecific crosslinks to amino acid residues of the PI-*Sce*I within a distance of 0.11 nm. Isolation of the covalent complexes, followed by tryptic digestion and Edman-degradation of the blotted crosslinked peptides which is in progress should allow to detect amino acids involved in DNA-binding.

In order to identify all potential crosslink positions of phosphate backbone contacts in one assay we developped a reverse-footprinting method in which different from conventional footprint techniques the footprint of the DNA on the protein is measured. This method will be very useful for characterizing enzymes which cover an extended recognition sequence on DNA.

CRE RECOMBINASE EXPRESSED IN TOMATO INDUCES A PHENOTYPE AND EFFICIENT SITE-SPECIFIC RECOMBINATION

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To enable the molecular dissection of the tomato genome (0.7 x 10^9 bp), we started to introduce and map specific recombination sites (*lox*; 34bp) into the genome. These recombination sites can function as landmark positions between which rearrangements can be induced resulting in chromosomal deletions/duplications, inversions or translocations.
To efficiently retrieve plants with linked *lox* sites, we exploit the characteristics of the *Ac*/*Ds* transposable element system. A T-DNA containing two *lox*-sites of which one is located within the *Ds*-element is introduced into the tomato genome. Subsequent activation of the *Ds*-element with *Ac*-transposase will result in a high percentage transposition events over relative small distances.
To induce specific rearrangements (inversions/deletions) between the physically linked *lox*-sites in these plants, they were crossed with a Cre recombinase expression tomato plant. Interestingly, Cre expression driven by the CaMV35S promoter alone already induces a distinct phenotype in tomato plants. Details on the characteristics of this phenotype will be discussed.
The presence of an inverted repeat *lox* substrate locus in the same plant affected the phenotype in a different way compared to a substrate locus with the *lox*-sites in direct repeat.
Generally, the recombination reaction induced by Cre in tomato cells between *lox*-sites over distances up to 290 Kbp apart occurs very efficiently, with frequencies up to 100%.

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The role of a chromatin-associated protein in β-mediated site-specific recombination is to facilitate the joining of distant recombination sites.

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The β site-specific recombinase is unable to mediate *in vitro* DNA recombination between two directly oriented recombination sites unless a bacterial chromatin-associated protein (*Bacillus subtilis* Hbsu or *Escherichia coli* HU) is provided. We have shown that the chromatin associated proteins from alga plastid (Chryptomonas ϕ HlpA), plant nuclei (maize HMGa, HMGc1, HMGc2 and HMGd) and mammalian (rat HMG1) origin can subtitute for the bacterial Hbsu protein in β-mediated recombination. These proteins, however, differ in their efficiency to enhance the site-specific recombination reaction. The HMG1-like proteins share neither sequence nor structural homology with the bacterial (Hbsu) or the plastid (HlpA) proteins. The plastid HlpA protein resembles the bacterial protein. All these chromatin-associated proteins share the property of binding to DNA in a relatively non-specific fashion, bending it, and having a marked preference for altered DNA structures as four-way junctions. Hbsu, HU, HlpA or HMG1-like proteins probably could bind specifically at the synaptic region (crossing-over region), since at limiting protein-DNA molar ratios they could not be competed by an excess of a DNA lacking the crossing over site.

By electron microscopy, we show that the role of Hbsu is to help in the joining of the β–recombination sites to form a stable synaptic complex. All these data show that the role of the chromatin associated proteins in β-mediated site-specific recombination is to modulate the DNA structure (bender). The bender could act as an archictectural element that facilitate the joining of distant recombination sites to form the nucleoprotein complexes at the crossing over to help β–recombinase to mediate site-specific recombination, rather than by serving as a bridge between β–recombinase dimers through a protein-protein interaction (adaptor).

THE PENICILLIN GENE CLUSTER IS AMPLIFIED IN TANDEM REPEATS LINKED BY CONSERVED SEQUENCES

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The penicillin biosynthetic genes (*pcbAB*, *pcbC* and *penDE*) of *Penicillium chrysogenum* AS-P-78 were located in a 106.5 kb DNA region that is amplified in tandem repeats (five or six copies) linked by conserved TTTACA sequences. The wild type strains *P. chrysogenum* NRRL 1951 and *P. notatum* ATCC 9478 contain a single copy of the 106.5 kb region. This region was bordered by the same TTTACA hexanucleotide found between the repeats in the strain AS-P-78. A penicillin overproducer strain, *P. chrysogenum* E1, contains a large number of copies in tandem of a 57.9 kb DNA fragment that mostly overlaps the right half of the amplified region in the strain AS-P-78. The tandem repeats in strain E1 are linked by the sequence TGTAAA, wich is the reverse complementary of that appearing in strains AS-P-78 and NRRL 1951. The occurrence of one or the other pattern of amplification seems to depend on the orientation of a 3.4 kb fragment (shift fragment: SF) located at the right border of the amplified region. The penicillin non-producer mutant *npe*10 showed a deletion of a 57.9 kb fragment that corresponds exactly to the amplified region in strain E1. The conserved hexanucleotide sequence was reconstituted at the deletion site in strain *npe*10. The amplification has occurred within a single chromosome (chromosome I), no other copies were located in any of the remaining chromosomes in the strains studied. The tandem reiteration and deletion appear to arise by mutation-induced site-specific recombination at the conserved hexanucleotide sequences.

Actions of site-specific relaxases at oriT (pMV158) and mrs (RP4)

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The origin of transfer (oriT) of the rolling circle plasmid pMV158 is the target for the plasmid-encoded relaxase, the MobM protein, which is able to nick supercoiled plasmid DNA. Purified MobM protein was shown to bind to oriT, consisting of two sets of inverted repeats flanking the nic-region. MobM binding activities were investigated by gel retardation assays as well as DNaseI footprinting on various oriT fragments, i) the entire oriT, ii) the right part of oriT including the nic-site and IR2-R and iii) the left part of the nic-site including all the inverted repeat structures except for IR2-R. The region protected from DNaseI digestion comprises the inverted repeat elements, IR1-R, IR2-L and IR-2-R including the dinucleotide at position 3591/3592 representing the nic-site. The molar ratio of DNA : protein bound in the three MobM - oriT complexes detected in the gel retardation assays was determined.

The MobM protein concentration in S. pneumoniae cells harbouring pMV158 was determined to approximately 200 molecules/cell by the quantitative ECL immuno detection system applying anti-MobM-antiserum. Western Blotting with anti-MobM-antiserum on different cellular fractions of S. pneumoniae cells harbouring pMV158 gave first indications for membrane association of MobM.

The application of the recently developed Runoff DNA Synthesis Assay (Zechner et al., 1997) in detecting recombination intermediates at the mrs-site of the broad-host-range plasmid RP4 via induction of the site-specific resolvase protein, ParA, in Gram-negative bacteria, is under investigation.

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SITE-SPECIFIC INTEGRATION IN THE *M.smegmatis* CHROMOSOME MEDIATED BY pSAM2 INTEGRASE

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An improved integrative cassette from plasmid pSAM2 has been constructed containing plasmid *int* and *att* P genes but excluding the *xis* gene which should result in increased stability by suppression of the excision reaction. This cassette was included in both suicide and thermosensitive plasmids and used for integration in *Mycobacterium smegmatis*. Suicide plasmids containing this cassette integrated at a single site (*attB* 1) in the *M.smegmatis* chromosome. The sequence of the *attB* 1 site has been determined and was identified as a putative tRNA ^{pro} gene. Thermosensitive plasmids containing the cassette integrated both at the same *attB* 1 site, and at other different sites, often giving rise to simultaneous integration at two sites. A second integration site (*attB* 2) has been sequenced, which was located in the region coding for rRNA 16S of one of the two *rrn* operons of *M.smegmatis*.

List of Invited Speakers

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PROGRAMMED GENE REARRANGEMENT: SITE-SPECIFIC RECOMBINATION

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