Instituto Juan March de Estudios e Investigaciones

122 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Mechanisms of DNA-Bound Proteins in Prokaryotes

Organized by

R. Schleif, M. Coll and G. del Solar

A. K. Aggarwal
J. C. Alonso
A. Antson
D. Bastia
S. Busby
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M. M. Cox
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Introduction

R. Schleif, G. del Solar and M. Coll

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The replication, conservation, and utilization of the information stored on DNA molecules is performed by proteins, many of which bind to DNA as they carry out their functions. The technologies of both X-ray diffraction and NMR have dramatically improved, and at present, the structure of many DNA-binding proteins, alone and/or complexed with their DNA targets, have been solved. In most cases, however, examination of the structure alone is insufficient to reveal the most important information, that is the protein's mechanism of action. As a consequence, it has become necessary to combine the knowledge gained from structural analysis with genetic, biochemical, and biophysical studies to learn the fundamentals of the mechanisms of action of DNA-bound proteins. New avenues of research in prokaryotic systems are closest to bringing biological understanding to the atomic level. In prokaryotes, not only can biochemical and biophysical experiments readily be done, but genetics allows powerful selections to be performed for rare variants. In combination with knowledge of structure of the molecules involved, the molecular biology of prokaryotic systems is currently well poised to join structure with function and to begin bio-atomic engineering.

The Workshop on "Mechanisms of DNA-bound proteins in prokaryotes" was an important effort to bring together the areas of structural and mechanistic studies on DNA-bound proteins. The main outcome of the Workshop was a thorough discussion on the present knowledge of this field. The final aim was directed towards a comprehensive understanding of the mechanism of action of these proteins, to which a general discussion and final summing-up talk were devoted. Experts from around the world met with their Spanish counterparts for several days of intense interchange under nearly ideal conditions. The meeting topic recognizes the fact that the pursuit of fundamental understandings and basic operating principles in a variety of diverse biological systems is converging on questions of how proteins function. While we all believe that structure determines function, increasingly, as the 14,000 plus structures have been deposited in the protein data bank, it has become clear that for the majority of the structures, the structure does not advertise function, or more properly, activity. In reality, the relationship between protein structure and activity turns out to be very complicated and subtle, similar to the obscurity of an undocumented computer code. Because much of the excitement in biological sciences over the past 30 years has centered around nucleic acids, the focus of the meeting was the mechanisms employed by DNA-bound proteins. The meeting included topics in the areas of general and site-specific recombination, replication, transcription, gene regulation, and structure determination. Thus,

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we learned of mechanistic analyses on DNA helicases and how they may work. DNA replication in chromosomes or extrachromosomal elements were addressed, as well as the role of proteins involved in recombination acting as molecular motors. Site-specific proteins-mediated recombination is an exciting field in which the role of the so-called "auxiliary" proteins seem to play a regulatory role. Bacteriophages are an excellent example not only for replication studies but also for structure-based studies of polymerases, on which unexpected complexity was shown, although unifying principles affecting DNA and RNA polymerases have been observed. Further, bacteriophages were an excellent example on resolution of structures in which toroidal rings (constituted by protein subunits) are generated and on how these structures may explain the mechanisms by which the phage DNA is packaged. An important and new concept has been developed here and it relates with the flexibility in protein structure and how such a flexibility may allow activity on substrates of diverse structure. Concerning the mechanisms that govern transcription, an important finding was the observation of the remarkable flexibility and adaptability of the interactions between RNA polymerase and the proteins that regulate RNA polymerase activity. Furthermore, proteins that control transcription initiation play a crucial role of which examples of surprising complexity were reported.

A number of general principles were made ever more apparent at the meeting. One is that Nature is complicated. That is, even for processes that we might expect to be simple, evolutionary pressures for optimization have added multiple refinements that complicate the activity. Examples of this "gratuitous" complexity were found in virtually every area covered. Notable is the variety of ways or mechanisms found in the regulation of different genes. Another principle made apparent was that it is hard to answer mechanistic questions. In a few cases (like toroidal clamps that hold proteins near DNA), the structure of a protein explains its activity, but in most cases, difficult and ingenious experiments must be used to learn even conceptually simple facts about mechanism. Much work was required to demonstrate that a helicase functions as a dimer or to find the residues of RNA polymerase that interact with transcription regulators. A recurring concept in the action of proteins was the role of rigidity or flexibility in providing or preventing energy differences or energy barriers in a process important to a protein's function. In addition to endonuclease VII, rigidity was seen to be important to the processivity of T7 DNA polymerase, the mechanism of AraC action, the mechanism of lambda phage in protein action, and the behavior of phage 434 repressor protein. In some cases, the biologically relevant energy differences are so small that a

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significant change in the expression rate of a protein can in principle be achieved by a slight change in the strength of an RNA polymerase-regulator protein. Hence, we may never be able to deduce biological properties without explicit measurement.

A number of tools are now being employed in mechanistic studies. While often difficult to perform, fluorescence studies provided a significant amount of the data presented. Genetics has shifted from a blunt tool often used for the elimination of a protein´s activity to a highly refined and structure-based tool, and a number of mechanistic studies utilized the alteration of a specific amino acid whose choice was determined from the tertiary structure of the protein. Although much of our desire to investigate nature arises from simple curiosity, additional motivation undoubtedly results from our desires to apply our knowledge and understanding to the solution of specific problems. That is, we would like some of what we do to be useful in an engineering sense. Both from the viewpoint of curiosity and from the perspective of biological engineering, it was also notable just how little we actually know and how much more there is to learn. Absent from the meeting because of our current lack of knowledge were titles such as "Predicting DNA Binding Affinities from the Known Structure of a Protein-DNA Complex", or more ambitiously "Determining Ligand Identity and Binding Affinity from a Known Protein Structure", textbooks to the contrary, we do not seem close to "The Mechanism of General Recombination" or "Mechanisms of Site-specific Recombination". We have yet to see work that would justify the title "Mechanism of Allolactose Modulation of Lac Repressor Activity", and clearly much is needed before we are likely to see the general title "Structure-based Calculation of ...".

In summary, the meeting showed that while we know a lot, and work from many different biological areas is heading in the same direction, that of determining the mechanism of a protein´s action and of engineering relatively minor modifications to this activity, much work and creativity and the combination of multiple disciplines will be involved in future advances.

Robert Schleif, Gloria del Solar and Miquel Coll

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Session 1: DNA replication
Chair: Michael M. Cox

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DNA unwinding by the *E. coli* SF-1 helicases, Rep and UvrD

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E. coli Rep and UvrD are 3' to 5' DNA helicases in the SF-1 helicase superfamily [1]. Rep is involved in DNA replication and UvrD participates in both DNA repair and replication. Crystal structures of the monomeric forms of these proteins [2], as well as the PcrA monomer from *B. stearothermophilus* [3], indicate that all three proteins are structurally homologous. Although the Rep protein is a stable monomer in the absence of DNA, biochemical studies of its DNA binding, DNA unwinding and ATPase activities have led to the proposal that the active form of the helicase is dimeric [4-7]. Similarly, single turnover DNA unwinding studies indicate that an oligomeric form of UvrD is required for optimal helicase activity *in vitro* [8]. However, crystal structures of the PcrA protein bound to a ss-ds-DNA junction show the protein bound as a monomer [9]. Based on this, it has been proposed that a PcrA monomer is the functional form of the helicase [9].

In order to determine whether a particular oligomeric form of a protein has helicase activity it is necessary to examine DNA unwinding activities of the proteins under conditions that promote formation of either monomeric-DNA complexes or oligomeric-DNA complexes. To this end we have used pre-steady state single turnover DNA unwinding experiments (stopped-flow and quenched-flow) to examine DNA unwinding. Such single turnover DNA unwinding experiments, as opposed to multiple turnover experiments, enable one to examine the helicase activity of protein that is bound to the DNA in a particular oligomeric state in the absence of re-binding and re-initiation of unwinding by any free (unbound) protein[8]. The results of these studies demonstrate that monomers of Rep and UvrD display no detectable helicase activity *in vitro* and that oligomeric forms of these proteins are required to promote helicase activity *in vitro*.

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Relating structure to mechanism in DNA helicases

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Helicases are enzymes that unwind nucleic acid duplexes. They are involved in processes such as replication, repair and recombination of DNA, as well as transcription and translation. Consequently, cells encode many isozymes, for example at least 12 DNA helicases have been identified in *Escherichia coli*. Mechanistically, there are two classes of helicases: those that require a 3' flanking strand of single-stranded DNA and those that require a 5' flanking strand (known as 3'-5' helicases and 5'-3' helicases, respectively). There are 5 superfamilies of helicase based on sequence homology. Most 3'-5' helicases are members of superfamilies I and II, which have similar sets of seven conserved motifs. In order to begin to understand how helicases work, we have solved the crystal structure of PcrA, a 3'-5' helicase from *Bacillus stearothermophilus*, alone and in a complex with ADP. The enzyme comprises two large domains with a large cleft between them. The ATP-binding site is situated at the base of this cleft and is lined with residues from the conserved motifs. The structure suggests how these residues might contribute to ATP hydrolysis. However, the biochemical mechanism for strand separation was not clear from these structures. In order to try to understand more about this process, we have determined crystal structures of complexes of PcrA with a 3'-tailed DNA substrate. Two different complexes were obtained, one with a non-hydrolysable ATP analogue (thus mimicking a "substrate" complex) and the other mimicking a "product" complex. The structures favoured an "inchworm" rather than "active-rolling"mechanism. The mechanism that we proposed for the enzyme had two major implications. First, that the step size for the enzyme would be just one base pair separated for each ATP molecule that was hydrolysed, well below the theoretical estimates of up to a dozen base pairs. Secondly, it also suggested that the enzyme actively destabilises the duplex DNA ahead of the fork in order to facilitate strand separation. The enzyme couples these two processes to produce helicase activity.

In order to test our proposal, we have investigated each of these processes biochemically. First, we developed a novel pre-steady state assay system which has allowed us to measure ATP hydrolysis during DNA translocation by PcrA helicase. The results show that one ATP molecule is hydrolysed for each base that is translocated with a translocation speed of around 50 bases per second. Secondly, we combined site-specific mutagenesis and DNA footprinting techniques to show how duplex destabilisation is promoted by binding of ATP to the helicase:DNA complex. Finally, these experiments lead us to conclude that the so-called "helicase" motifs are instead characteristic of nucleic acid *translocases*, with helicases being a subfamily of this much larger group of enzymes.

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Interaction between RepA and DnaA initiators and initiation of DNA replication as revealed by yeast forward and reverse dihybrid techniques

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The replication of the plasmid pSC101 and several other plasmids requires both a plasmid-encoded initiator and the ubiquitous, host-encoded initiator DnaA. We wanted to unravel the relative roles played by these two different initiators in plasmid replication. Our earlier work had shown that initiation of replication of pSC101 required the plasmid-encoded RepA protein, the DNA bending protein IHF and DnaA (1,2). Our earlier work had suggested that IHF bends DNA to bring into direct contact DnaA and RepA proteins that were bound to physically noncontiguous regions of the ori. We had also shown that RepA directly interacted with the helicase DnaB and that this direct physical interaction was essential for recruiting the helicase to the plasmid ori (2).

We have used yeast forward 2-hybrid method to show direct physical interaction between RepA and DnaA and have used reverse 2-hybrid to isolate a noninteracting mutant of RepA that shows reduced interaction with DnaA. Biochemical and immunological methods have confirmed the physical interaction between wt RepA and DnaA and the lack of this interaction in the mutant form of RepA obtained by the reverse 2-hybrid approach. We have shown that RepA interacts both with the domains I and IV of DnaA and that the mutant disrupts the interaction of RepA mainly with the domain I of DnaA. We show that the interaction between DnaA and RepA is absolutely essential for helicase loading. DnaA appears to be necessary in the preloading step that results in melting of the AT-rich region of the ori. We show that the noninteracting mutant of RepA fails to unwind the ori and thus fails to provide the melted region needed to load DnaB helicase.

The domains I and IV of DnaA are the only domains needed to support pSC101 replication (3). We show that when domains I and IV are separately fused to leucine zippers and coexpressed in a DnaA-deleted host, the two domains noncovalently associate to support pSC101 replication. Thus, the last observation provides us with a strategy to design a replication activation-based bacterial 2-hybrid system.

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The interaction of bacteriophage T7 replication proteins with DNA

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Bacteriophage T7 efficiently replicates its DNA using a minimal number of proteins, making it an attractive system for studying the interaction of replication proteins with DNA. In recent years we have collaborated with the laboratory of Dr. Thomas Ellenberger to determine the crystal structure of the two major components of the T7 replication fork: the T7 DNA polymerase (1) and the T7 helicase (2). The structure of T7 DNA polymerase was solved complexed with its processivity factor, thioredoxin, a primer-template, and a nucleoside triphosphate in the polymerase active site.

In order to further understand the interactions of T7 DNA polymerase with DNA and with its processivity factor, helicase, primase, and single-stranded DNA binding protein, we are carrying out site-directed mutagenesis of the T7 DNA polymerase based on unique features in its structure not found in the structures of other related, Pol I-type DNA polymerases. For example, T7 DNA polymerase contains a unique, four residue loop in its thumb that contacts the template DNA four bases from the 3' end of the primer. This small loop is located at the site where the 5' end of a tetranucleotide RNA primer must be bound for use by the polymerase. Deletion of this loop has no noticeable effect on DNA synthesis catalyzed by T7 DNA polymerase but it dramatically reduces its ability to extend primers synthesized by the gene 4 protein. We believe that this loop is an important contact for either the primase, the RNA primer, or both.

The structure of T7 DNA polymerase suggests a critical role of the C-terminal residue, His704, which forms a hydrogen bond with an oxygen atom on the penultimate phosphate diester of the primer strand. To probe the contribution of this electrostatic interaction, we have examined the physiological and biochemical properties of a mutant T7 DNA polymerase in which this histidine has been changed to alanine. Phage expressing this mutant are nonviable, even when the wild-type polymerase allele is coexpressed. No DNA synthesis can be detected *in vivo* by ^{3}H-thymidine uptake assays. Biochemical analysis of the purified, genetically altered enzyme shows that polymerase activity on primed M13 DNA is only 12% of that of the wild-type enzyme. The rate of strand displacement DNA synthesis by the mutant polymerase when coupled to the T7 helicase is likewise reduced several fold, although the processivity of the complex is unimpaired. The decrease in polymerase activity is concomitant with an increase in hydrolytic activity, as judged by the turnover of nucleoside triphosphate into the corresponding monophosphate (percentage turnover = 65%) during DNA synthesis. These data suggest that this terminal amino acid residue of T7 DNA polymerase may play a critical role in the controlled shuttling of DNA between the polymerase and exonuclease sites.

We have been exploiting the high efficiency of the T7 replication proteins to develop an efficient system for the nonspecific replication of DNA. This system is capable of carrying

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out a 10^{13}-fold amplification of input DNA in a 30 minute reaction, resulting in the production of 50 µg of DNA starting from a single plasmid or chromosomal DNA molecule. This is useful for the immortalization of precious genomic DNA samples.

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Insights in recognition of ϕ29 origin of replication

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and Margarita Salas

The linear double-stranded genome of phage phi29 contains a terminal protein (TP) covalently linked at each 5' DNA end, called parental TP. Initiation of phi29 DNA replication starts with the recognition of the origins of replication, constituted by the parental TP-containing DNA ends, by a heterodimer containing phi29 DNA polymerase and primer TP. It has been argued that origin recognition involves protein-protein interactions between parental and primer TP. Analysis of the TP sequence revealed that the region between amino acids 84 and 118 has a high probability to form an amphipatic a-helix that could be involved in the interaction between parental and primer TP. Therefore, this TP region may be important for origin recognition. To test this hypothesis we introduced various mutations in the predicted amphipatic a-helix and analyzed the functionality of the corresponding purified TP mutants. The results obtained show that the identified putative amphipatic a-helix of TP is an important determinant involved in origin recognition.

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Session 2: DNA recombination and repair
Chair: Margarita Salas

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RecA protein as a motor protein

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The RecA protein of Escherichia coli is a DNA-dependent ATPase, structurally related to several helicases and the F1 ATPase. *In vitro*, RecA protein promotes a variety of DNA strand exchange reactions that are central to homologous genetic recombination processes in bacteria. Recent work has demonstrated that the primary function of bacterial recombination systems is the repair of stalled replication forks (1, 2).

The fundamental processes of DNA pairing and strand exchange require bound ATP, but not ATP hydrolysis. Considerable DNA strand exchange is observed with ATP analogues that are not hydrolyzed, or with RecA mutants that bind but do not hydrolyze ATP. RecA, bound to a single strand of DNA, will take up a homologous duplex and align it with the single strand. The DNA strand exchange occurs within the filament, facilitated only by binding energy.

ATP hydrolysis enhances the DNA strand exchange process in several ways. When ATP is hydrolyzed, RecA protein can promote longer strand exchange reactions much more efficiently. DNA strand exchange is rendered unidirectional. RecA can also promote strand exchange through heterologous DNA insertions of up to about 200 bp, and promote strand exchange reactions involving 4 DNA strands. These latter two processes exhibit an absolute requirement for ATP hydrolysis.

There are two models for how ATP hydrolysis is coupled to the latter stages of DNA strand exchange. One involves the dissociation of RecA filaments (3-5). The other involves a postulated motor function of RecA in which ATP hydrolysis is used to facilitate the rotation of one DNA about the other (6, 7). Several predictions of both models have been tested (6, 7) and the results will be presented. In general, the results argue strongly for the existence of a RecA motor function.

A potential function for a RecA motor is seen in the RecA-mediated regression of stalled replication forks. We have demonstrated that RecA protein will promote the regression of fork models in vitro. This reaction is completely dependent on ATP hydrolysis.

A part of the RecA structure required for ATP hydrolysis-dependent DNA strand exchange has also been identified in the C-terminal domain (S. Lusetti and M. Cox, unpublished data). We have constructed a series of C-terminal deletions of the RecA protein. These deletions do not affect DNA binding, or the fundament DNA pairing process. ATP hydrolysis and the exchange of RecA monomers into and out of the filament is also unaffected. However, loss of even a few amino acid residues from the C-terminus eliminates the capacity to promote DNA strand exchange through heterologous insertions, and to promote exchanges involving 4 strands. The eukaryotic RecA homologues like Rad51 lack a

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comparable C-terminal domain, and also lack the capacity to promote DNA strand exchange involving barriers or 4 DNA strands.

An overview of this problem and its current status will be presented.

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Higher order complexes in site-specific recombination

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The large λ Int family of site-specific recombinases from archaebacteria, eubacteria and yeast catalyze recombination between DNA sequences with little or no sequence homology to each other. Many of these recombinases, like the integrase protein (Int) of *E. coli* phage λ, are responsible for the integration and excision of viral chromosomes into and out of their respective hosts. They comprise a large subgroup of recombinases with the ability to simultaneously bridge two different and well-separated DNA sequences called arm- and core-type, respectively. This heterobivalent DNA binding is a key architectural element in the formation of higher-order complexes predicted to be approximately 500 kDa in size. Binding sites for one or more accessory DNA bending proteins (IHF, Xis and Fis in the case of λ) always separate the arm- and core-type Int binding sites.

We shall discuss experiments pointing to two rather different views of how the λ integrase functions. The architectural view highlights the role of the DNA bending proteins in bringing the flanking arm- and core-type Int binding sites into close proximity so that Int, bound through its amino-terminal domain to a high affinity arm-type site, is "delivered" to the lower affinity core-type site, where the carboxy-terminal domain binds, cleaves and religates DNA strands. A different view reveals that, independent of any delivery or bridging function, the small amino-terminal domain greatly influences the DNA binding and cleavage functions of the large carboxy-terminal domain in a context-sensitive manner. DNA as an allosteric effect of 434 repressor function.

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DNA-binding proteins that promote survival under extreme stress

Abraham Minsky and Eyal Shimoni

The enhanced stress resistance exhibited by starved bacteria represents a central facet of virulence since nutrient-depletion is regularly encountered by pathogens in their natural *in-vivo* and *ex-vivo* environments. We find that the regular stress responses which are mediated by enzymatically-catalyzed chemical transactions and promote endurance during logarithmic growth phase, can no longer be effectively induced during starvation. We show that survival of bacteria in nutrient-depleted habitats is promoted by a novel strategy: finely-tuned and fully reversible intracellular phase transitions. These non-enzymatic transactions, detected and studied in bacteria as well as in defined in-vitro systems, result in DNA sequestration and generic protection within tightly packed and highly ordered assemblies. The protein responsible for such transactions is Dps, expressed in large amounts in starved bacteria, and capable of forming highly stable crystalline structures with DNA. Since this physical mode of defense is uniquely independent of enzymatic activity or de-novo protein synthesis and consequently does not require energy consumption, it promotes virulence by enabling long-term bacterial endurance and enhancing antibiotic resistance in adverse habitats.

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Novel roles of protein DNA clamps in replication and repair

Francisco López de Saro

The beta subunit of DNA polymerase III is implicated in replication, recombination and repair. During chromosomal replication, the beta subunit forms a clamp around DNA that anchors the polymerase to the template thus assuring the high processivity of the polymerase reaction. Recent studies suggest a more diverse function for protein clamps on DNA. For example, it has been suggested that beta clamps left over by the replication machinery could also serve as 'DNA markers' for proteins involved in the repair of errors created by the DNA polymerase. Further, the *E. coli* beta clamp is known to function with DNA polymerases II and V, indicating that beta also interacts with more than just the chromosomal replicase, DNA polymerase III. We have found several new protein-protein interactions with the beta clamp. MutS, a protein involved in post-replicative mismatch repair, binds the beta subunit and it does so in the same face of the clamp as the polymerase. We also show that DNA polymerases I and DNA ligase interact directly with the beta clamp, highlighting a wide role of protein clamps in DNA synthesis and repair.

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Structural selectivity of T4 endonuclease VII

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T4 endonuclease VII (Endo VII) is a junction-resolving enzyme with a broad substrate specificity recognizing a variety of branched DNA structures. In addition to Holliday junctions and cruciform DNA, Endo VII will cleave Y-junctions, heteroduplex loops, single-strand overhangs, curved DNA, but also abasic sites and single-base mismatches, in contrast to other resolvases like e.g. yeast CCE1 or E.coli RuvC, which are specific for 4-way junctions. Endo VII is active as a dimer and nicks both strands in a divalent cation-catalysed reaction 2-6 base pairs 3' of the branch point in independent, however temporally closely correlated reactions.

We have determined the crystal structure of Endo VII, which revealed a novel fold with an unusual domain-swapped dimer architecture, not seen in any of the known junction resolvases (Raaijmakers et al., 1999). Based on the structures of the Ca^{2+}-bound wild-type and the inactive N62D mutant, which allowed us to identify the active site, we have proposed a model for the complex with a four-way DNA junction. This model assumes binding of the positively charged face of the Endo VII dimer to the antiparallel stacked X structure of the junction. The model is in line with the hypothesis, that Endo VII accepts as substrates branched DNA with either intrinsically inclined helical segments, or DNA which can easily adopt properly inclined configurations due to the presence of a structural perturbation in the double helix. While the overall features of this model are in agreement with available experimental data, a detailed picture of the interactions has to await the determination of the crystal structure of a substrate complex, particularly in view of possible conformational changes upon binding.

The recent analysis of a second wild-type crystal form demonstrated a surprisingly high conformational flexibility of the Endo VII dimer (Raaijmakers et al, 2001). A comparison of the two wild-type and the N62D mutant structures indicates, that the opening of the DNA binding cleft, the dimerization interface and the orientation of the C-terminal domains are sensitive to the crystal packing environment. In particularly, the rearrangements within the extended hydrophobic interface were highly unexpected. This conformational flexibility is most likely of functional significance for the broad substrate specificty of the enzyme.

Bound sulfate ions in the new wild-type structure and their positions relative to the active site metal ions and catalytically essential residues suggest a possible catalytic mechanism. A comparison with the active site geometries of other magnesium-dependent nucleases, among them the homing endonuclease I-PpoI and *Serratia* endonuclease, shows common features suggesting related catalytic mechanisms.

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Session 3: Transcription I
Chair: Stanley Tabor

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Mechanisms of phage ø29 regulatory proteins p4 and p6 for transcriptional activation and repression

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Bacillus subtilis phage ø29 has a linear, double-stranded DNA 19,285 bp long. Transcription of early genes takes place from several promoters (among them A2b, A2c and C2) and is carried out by the host RNA polymerase (RNAP) with the major σ^A subunit. Trancription of late genes occurs from a single promoter (A3) and requires, in addition to the σ^A RNAP, the product of the early viral gene 4 (p4).

Protein p4 is a transcriptional activator that promotes binding of the RNAP to the late A3 promoter by interaction of amino acid residue Arg120 in p4 with the C-terminal domain (CTD) of the RNAPα subunit. By site-directed mutagenesis at the α-CTD we have shown that a mutation of residue Glu297 into Ala destabilizes the open complexes formed at the A3 promoter.

In addition to activating the late promoter, protein p4 represses the early A2b and A2c promoters. In the first case, protein hindrance and the bending of the DNA upon p4 binding results in displacement of the RNAP from the A2b to the A3 promoter. In the A2c promoter, repression by p4 also occurs by interaction of Arg120 with the α-CTD of RNAP, although in this case the interaction between the two proteins impairs the escape of the RNAP from the A2c promoter.

The ø29-encoded protein p6 is a histone-like protein, very abundant in infected cells, that in addition to its role in the initiation of phage DNA replication, is involved in regulating the viral switch between early and late transcription. On the one hand, p6 represses the early C2 promoter by destabilizing the close complex formation. On the other, there is a functional interaction of p6 with the regulatory protein p4 through the formation of a nucleoprotein complex in which the three proteins, p4, p6 and RNAP, are present. This results in activation of the A3 promoter by enhancing binding of p4 to its recognition site and in repression of the A2c promoter through the impairment of open complex formation. The p4 positive control mutant R120Q, that prevents interaction with the RNAP α subunit, and thus repression of the A2c promoter, is able to repress the latter in the presence of protein p6.

The switch from early to late transcription in the ø29-related phage GA-1 will be also discussed.

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Examination of the transition to a stable elongation complex using the single subunit T7 RNA polymerase

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As is the situation with all other RNA polymerases (RNAPs) T7 RNAP forms an unstable initiation complex (IC) that synthesizes and releases transcripts 2-8 nt in length before disengaging from the promoter and isomerizing to a stable elongation complex (EC). While considerable information is available concerning the structure of the IC, little is known about the EC. We have explored the trajectory of the RNA in the EC through the use of RNA:DNA and RNA:protein crosslinking techniques. We found that the RNA remains in an RNA:DNA hybrid up to 8 nt upstream from the active site and does not emerge to the surface of the enzyme until after 12 nt have been synthesized. Strikingly, as the transcript is displaced from the template it becomes associated with a portion of the specificity loop of the RNAP that makes contacts with the upstream region of promoter during initiation. It seems likely that association of the nascent RNA with the specificity loop facilitates disengagement from the promoter, and is an important part of the process that leads to a stable EC (*1*).

In addition to the synthesis and release of abortive products, T7 RNAP also synthesizes poly (G) products that arise by slippage of the transcript on the 3 Cs in the template (T) strand from +1 to +3 (*2*). This process is in competition with abortive initiation, and is observed even under optimal conditions of transcription (*3*). During both of these processes, T7 RNAP apparently maintains contacts with the upstream region of the promoter while drawing the downstream template DNA into the active site (*4,5*). It has been proposed that this is accomplished by "scrunching" of the intervening portion of the template strand of the DNA into a hydrophobic pocket, and that filling of the pocket triggers the isomerization event (*6*). We found that at promoters in which the integrity of the T strand is interrupted between the binding region and the initiation region, poly(G) synthesis is nearly completely suppressed. We present a model in which this is attributed to a different exit pathway for the interrupted template strand that does not require scrunching, resulting in a lowered thermodynamic barrier for translocation.

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How bacterial transcription factors talk to RNA polymerase

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At a large number of bacterial promoters, transcription initiation is activated by direct interactions between transcription factors and holo RNA polymerase. Many transcription activators contact the C-terminal domain of the RNA polymerase α subunit, whilst others contact Region 4 of the RNA polymerase σ^{70} subunit. Recent progress concerning the identification of the contact sites for different transcription activators in both the RNA polymerase α and σ subunits will be described. At some activator-dependent promoters, the binding of a single activator is sufficient for transcription initiation, whilst at others, the binding of several activators is required. The organisation of different classes of activator-dependent promoters will be reviewed briefly.

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Review

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Copy number control and segregation of plasmid pSM19035 shares a common regulatory pathway

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The 71 amino acids long ω protein encoded by the *Streptococcus pyogenes* non-conjugative pSM19035 co-ordinates expression of genes responsible for copy number control (*cop*S) and stable maintenance (δω and ωεζ) (1). The promoters of the *cop*S (*P*cop1 and *P*cop2), δ (*P*δ) and ω (*P*ω) genes have been mapped. The DNA binding site of ω consists of several copies of 7-bp repeats (5'-WATCACW-3') (2). The co-operative binding of ω protein to the repeats on the *P*cop1, *P*cop2, *P*δ and *P*ω promoters represses transcription initiation by a mechanism that does not exclude σ^{A}RNAP from the promoters. Using gel retardation assays, surface plasmon resonance and DNase I footprinting, we show that the ω protein requires two 7-bp repeats as a minimal binding unit. For co-operative and stable binding, however, a cluster of three 7-bp repeats composed of three direct repeats or two direct plus one inverted repeat is needed. The higher binding affinities are observed with four 7-bp repeats or with full-length cognate site. Our data suggest that the binding of ω protein to DNA appears to be rotationally flexible. A functional ω binding site is formed by extending a minimal recognition sequence with two discrete heptamers. Crystals of ω protein have hexagonal space group $P6_1$. Two monomers related by a non-crystallographic two-fold axis form a homodimer that occupies the asymmetric unit. Each polypeptide chain is folded into two α-helices and one β-strand forming an antiparallel β-ribbon in the homodimer. The N-terminal regions (1-23 and 1-22 in subunits I and II, respectively) are not defined in the electron density due to proteolysis during crystallisation and partial disorder. ω protein belongs to the structural superfamily of MetJ/Arc repressors featuring a ribbon-helix-helix DNA binding motif with the β-ribbon located in the major groove of the DNA substrate. According to a modelled ω protein-DNA complex, residues Arg31 on the β-ribbon can recognise the guanine base in operator DNA in the major groove. These results indicate that ω protein regulates plasmid maintenance by controlling the copy number in one hand, and by interacting with parA-like δ protein, required for partitioning, and with the εζ, complex, involved in programmed cell death in another hand.

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Interactions between the transcriptional repressor CopG and its target DNA

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Control of replication in some plasmids is exerted by the combined action of an antisense RNA (whose concentration is proportional to the plasmid copy number) and of a transcriptional repressor protein (3). Such is the case of the rolling circle-replicating plasmid pMV158, which is the prototype of a family composed by more than 20 replicons. Plasmid pMV158 encodes the transcriptional repressor protein CopG, which is involved in copy number control (2). The genes encoding CopG and the initiator of replication RepB proteins are co-transcribed from a single promoter, P_{cr}. CopG binds to a DNA region that spans about 50 bp and that includes the P_{cr} promoter. The centre of the CopG binding region contains a 13-bp pseudo-symmetric sequence (SE), which is not sufficient for high affinity binding of the protein to its target DNA (4). The purified CopG protein is a homodimer (composed of 5.1 kDa subunits) with a Stokes radius of 16 Å. The protein remains as a dimer in solution, even at high protein concentrations. Deletions in gene *copG* affecting regions other than the C-terminal end of the protein, strongly reduced the intracellular stability of the otherwise long-lived wild type CopG (1). These findings indicated that the protein has a compact structure, being constituted by a single folding domain. The crystal structure of CopG has shown that the protein displays a homodimeric ribbon-helix-helix arrangement. Solution of the crystal structure of CopG in complex with either a 19-bp or a 22-bp double stranded oligonucleotide (containing the SE) showed that two CopG dimers bind to two successive major grooves of the DNA, introducing a bend of about 60°, which is generated by a marked CopG-mediated compression of both DNA major and minor grooves facing the protein (ref. 5 and Fig. 1). Electrophoretic mobility shift assays (EMSA) together with hydroxyl radical footprinting analyses showed that several molecules of the protein bind in a highly cooperative way to the same face of the DNA helix, resulting in formation of, at least, four different CopG-DNA complexes. Point mutations at the SE, even in some of the bases contacted by CopG, resulted in neither significant alterations in the EMSA patterns nor in the affinity of CopG for the operator DNA containing these mutant SE sequences. In addition, the SE element alone, when cloned into a foreign DNA context, was not sufficient to provide specific and high affinity CopG binding.

Analysis of the mechanism of action of CopG showed that the binding of the protein to its target DNA inhibited transcription by hindering the binding of the RNA polymerase (RNAP) to the promoter P_{cr}. This conclusion derives from competition experiments between CopG and RNAP, which also showed that the RNAP previously bound to the P_{cr} promoter was rapidly displaced by the binding of CopG. This occurred in spite of the high stability of the RNAP-DNA complexes and the short half-life of the CopG-specific complexes. The implications of the interplay of CopG and RNAP on the P_{cr} promoter DNA region will be discussed.

Two CopG mutants having a single amino acid substitution (CopG7, A30E; CopG8, G25E) have been purified and characterised. DNase I footprinting experiments showed that both mutant proteins bind to the central DNA region containing the SE. However, the

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footprints generated by the CopG variants did not extend through the entire region occupied by the wild type CopG. The CopG mutants showed a decreased affinity for the target DNA and an impaired inhibitory capability on the transcription from the P_{cr} promoter. EMSA assays revealed the formation of a single high-mobility complex upon binding to the target DNA. The amino acid substitutions were located on the protein dimer-dimer interface of the functional tetramer bound to the SE, as seen in the co-crystals of the wild type CopG. This suggests that the CopG mutants could be affected in their cooperative binding to DNA.

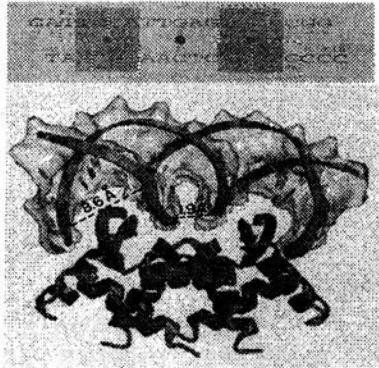

Figure 1. Upper part: Sequence of the 19-bp double stranded wild type DNA used for co-crystallization with CopG. The centre of symmetry is depicted by a dot. In addition, the DNA contains two internal symmetrical sequences (shadowed, with their centre indicated by a small dot) which are located at the left and the right of the centre of symmetry. The bases contacted by CopG are shown (broken lines). Lower part: The structure of two CopG dimers complexed with a 19-bp double stranded DNA containing the IR. Note the compression of the major and minor grooves of the DNA introduced by CopG (taken from ref. 5).

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Session 4: Transcription II
Chair: Steve Busby

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Characterization of the *Pseudomonas putida* Pm promoter

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and Juan L. Ramos

The Pm promoter, which depends the on TOL plasmid XylS regulator activated by benzoate effectors, drives the transcription of the meta-cleavage pathway for the metabolism of alkylbenzoates. This promoter is unique in the sense that in vivo transcription is mediated by RNA-polymerase with different sigma factors. In vivo footprinting analysis show that XylS interacts with nucleotides in the -40 to -70 region. In vivo and in vitro methylation of Pm show extensive methylation of T at position -42 in the bottom strand, suggesting a key distortion point that could favor XylS/RNA polymerase interactions. Methylation of T-42 was highest in cells bearing XylS and in the presence of an effector. Gs in the -47 to -61 region appeared to be more protected in cells harbouring XylS in the presence of the effector than in its absence. Almost 100 mutants in the Pm region between -41 and -78 were generated; transcriptional analysis of these mutants defined the XylS target as two direct repeats whose sequence is TGCAN6GGNCA. These motifs cover the -70 to -56 and -49 to -35 regions. Single point mutations revealed that nucleotides located at -49 to -46 and at -59, -60, -62 and -70 are the most critical for appropriate XylS/Pm interactions. XylS has been analyzed with regard to its potential interactions regulators with the C-terminal domain of the a subunit of RNA polymerase (a-CTD). For these studies we expressed a derivative of a deprived of the entire C-terminal domain (a-D235) and found that expression from Pm with XylS decreased significantly. To discern whether a-CTD activation depended on interactions with DNA and/or XylS proteins we tested a large collection of alanine substitutions within a-CTD. Most substitutions that had an effect on XylS-dependent transcription were located in or adjacent to helix 1 and 4, which are known to be involved in a-CTD interactions with DNA. Two alanine substitutions in helix 3 (residues 287 and 291) identified a putative region of a-CTD/XylS regulator interactions.

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Mechanisms of Ligand-Mediated Regulation of DNA Binding Affinity

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Two general methods have been developed for investigating the mechanisms by which the binding of ligands to DNA binding proteins modulate the proteins' DNA binding affinities. When applied to AraC protein, these methods show that the binding of arabinose to AraC changes the protein's DNA binding properties by changing the relative positioning of its DNA binding domains rather than changing the intrinsic DNA binding ability of a DNA binding domain. The methods also show that essentially no signal is sent from the dimerization domains to the DNA binding domains in the presence of arabinose. Both of these results are in accord with the light switch mechanism of AraC action.

Direct biophysical support for the light switch mechanism has now been provided by plasmon resonance experiments showing that peptides corresponding to the arm of AraC bind to arm-deleted dimerization domain in an arabinose dependent way. Further, sequence alterations in the arm that are expected on the basis of their regulatory properties to bind either more tightly or less tightly to the dimerization have the predicted effects on peptide binding.

Modeling and molecular dynamics calculations have been applied to wild type and mutant dimerization domains in an effort to understand the mechanical basis of several mutations.

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cAMP-CRP and the regulation of translation

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The *E. coli* cAMP receptor protein (CRP) is a global transcriptional regulator that can serve as an activator, repressor, co-activator and co-repressor. CRP accomplishes this diversity of functions by the differential use of its DNA-binding and –bending capacities and its ability to make contacts to other proteins. Evidence that CRP interacts with other proteins is available from its action as an activator (RNA polymerase) and its action as a co-repressor in the CytR regulon (1-4). Moreover, there are a number of observations suggesting that cAMP-CRP (directly or indirectly) regulates gene expression at the translational level. Work on the *gal* operon, for example, has revealed that the cAMP-CRP complex influences synthesis of the products of this operon differentially (5).

The first gene within the *gal* operon encodes GalE, the second encodes GalT and the third encodes GalK. The operon processes two promoters and two corresponding transcriptional start sites (S1 and S2). The cAMP-CRP complex positively controls transcription from S1. In wild-type cAMP and CRP proficient cells equimolar amounts of GalETK polypeptides are made, showing complete coordination in their expression. However, in wild-type cells deficient in cAMP and CRP (e.g. glucose grown) the *gal* operon exhibits "natural polarity": i.e. the molar amount of GalE is about 4 fold higher than that of GalK. This "polarity" proved to be independent of the promoter and start site used for transcription. Since the "polarity" is more severe in the absence of cAMP and/or CRP and can be suppressed in *cya* strains by the addition of cAMP to the growth medium, the cAMP-CRP complex somehow plays a role in altering the relative expression of the *gal* cistrons.

CytR was identified as a negative regulator of operons involved in nucleoside and deoxynucleoside uptake and degradation and has subsequently been shown to play a broader role in gene expression (1,6). Among the newly discovered genes controlled by the concerted action of CytR/CRP is the *spf* gene which encodes a small unstable RNA composed of 109 unmodified nucleotides (Spot 42 RNA). The role of Spot 42 RNA was unclear, even though the RNA has been studied extensively (7). However, several observations suggested to us that Spot 42 RNA might be a riboregulator involved in differential expression of the *gal* cistrons. First, Spot 42 RNA contains an U-turn-like loop structure. U-turn loop motifs are present in antisense RNAs and tRNAs and facilitate rapid bi-molecular RNA-RNA interaction (8-10). Second, *spf* is negatively regulated by CytR/CRP (i.e. there are intriguing parallels between "polarity" in the *gal* operon and *spf* expression). Third, three stretches of Spot 42 RNA are complementary to the region surrounding the *galK* Shine-Dalgarno sequence.

Comparison of Gal E-, T-, K-polypeptides in various *spf*$^+$/*spf* strains revealed that the "polarity" in the *gal* operon was eliminated in *spf* cells. Subsequent biochemical studies showed that a duplex between Spot 42 RNA and the 5'-region of the *galK* message can be formed and that the Spot 42 RNA interaction specifically inhibited binding of ribosome to the *galK* Shine-Dalgarno sequence. The regulatory RNA interacts with its target through three regions: the 5'-end region, the loop of the first hairpin structure and a region located just

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downstream of this hairpin. Moreover, Spot 42 RNA interacts with a general post-transcriptional regulator, the Hfq protein, and the half-life of the RNA is dramatically reduced in *hfq*⁻ strains. Thus, it appears that Hfq protects Spot 42 RNA from nuclease attack.

Prospect - Our results have produced answers to two unsolved issues: the physiological role of Spot 42 RNA and how polarity can be modulated by cAMP and CRP. Important objectives for further work include determination of other targets for Spot 42 RNA and determination of Hfq's role in turnover and/or in the activity of the Spot 42 RNA.

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In vivo and *in vitro* UV laser footprinting of the *Pseudomonas putida* Pu promoter

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The *Pu* promoter is among the bacterial promoters transcribed by the RNA polymerase associated to the alternative sigma factor σ^{54}. This promoter regulates transcription of the *upper* operon of *P. putida* mt-2 TOL plasmid pWW0, which encodes the first enzymes for toluene and *m/p*-xylene degradation. The *Pu* promoter is regulated through a mechanism that involves: [a] Binding of the XylR regulatory protein to upstream activating sequences (UAS) and [b] Looping of the DNA region between the UAS and the RNA polymerase-binding site, an event assisted by the integration host factor (IHF). DNA looping seems to facilitate the interaction between upstream-bound activator protein and the RNA polymerase.

An early observation in the study of the *Pu* promoter was that its activity is inhibited when cells grow exponentially in rich medium, a phenomenon that was called *exponential silencing*. A *P. putida* strain containing the *Pu* promoter integrated in monocopy in the chromosome along with the *xylR* gene faithfully reproduces this expression profile, thus providing a simpler experimental system. While overexpression of XylR or IHF does not affect such a metabolic inhibition effect, production of the σ^{54} alleviates the silencing of the promoter in exponential growth. On the other hand, strains lacking any of these proteins exhibit no *Pu* expression. Thus, although the influence of the bacterial metabolic state on *Pu* has been well established, neither the protein through which the physiological signal is acting nor the exact mechanism is known.

To monitor the occupation of the different protein binding sites in the promoter at different growth phases would help to determine which of them is responsible for the silencing during exponential growth. UV laser footprinting has been shown to be a promising way to determine protein-DNA binding either *in vitro* or *in vivo*. The technique is based on the fact that 260 nm laser light applied to DNA generates the dimerisation of adjacent tymidine residues in the molecule. The process takes place in nanoseconds and is affected by the physicochemical environment (e.g. the presence of DNA-bound proteins). Since polimerisation is arrested when DNA polymerase encounters a tymidine dimer, linear amplification of UV laser treated DNA using a radioactively labelled oligonucleotide, followed by denaturing polyacrylamide gel electrophoresis results in a footprinting pattern of the amplified region. The technique has been successfully applied to whole bacterial cells for *in vivo* footprinting.

In this study, we have examined the influence of the IHF protein in the exponential silencing by monitoring *in vivo* its binding to *Pu* promoter throughout the growth curve. Our results show differences in the occupation of the IHF site depending on the growth phase. Interestingly, the footprinting pattern indicates that this DNA-bending protein is bound to *Pu* promoter only in stationary phase. This is the first time that *in vivo* UV laser footprinting has been applied to the study of a sequence in monocopy, integrated in a bacterial genome.

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Characterization of the repressor of the parD operon: the Kis-Kid complex

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Kis and Kid are an antidote-toxin protein pair coded by the *parD* system of plasmid R1 (Bravo et al., Mol. Gen. Genet. 210, 101-110, 1987). The two proteins interact strongly and regulate coordinatly the *parD* operon (Ruiz-Echevarría et al., Mol. Microbiol. **5**, 2685-2693, 1991; J. Mol. Biol. **247**, 568-577, 1995). The antidote of the system alone has a poor regulatory activity.

CD analysis suggests that the Kis antidote is poorly structured and that upon interaction with the Kid toxin it gains structure and stability. Analysis of the Kis-Kid complex by analytical ultracentrifugation reveals that this complex tends to be an octamer with an oval shape. An analysis of the interactions of this complex with the *parD* promoter-operator will be presented.

Genetic analysis indicates that in the Kid toxin, formation of the regulatory complex with Kis and inactivation of its cell target involves different amino-acid residues. However both functions require the carboxyl-end of the toxin. The genetic analysis also shows that the amino and carboxyl-ends of the Kis antidote play essential, but different roles in the formation of an active *parD* repressor.

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DNA as an allosteric effect of 434 repressor function

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Although 434 repressor binds to its specific DNA sites only as a dimer, formation of the dimers in solution occurs at concentrations three orders of magnitude higher than those needed to bind the 434 operator DNA. To begin to resolve these apparently paradoxical observations, we have used steady-state fluorescence and fluorescence anisotropy measurements to study the structure 434 repressor in the absence and presence of various DNA binding sites. The results of these investigations suggest that both specific and non-specific DNA induce conformational changes in repressor that leads to formation of repressor dimers. Our data also show that the repressor conformational changes induced by specific and nonspecific DNA occur at DNA concentrations much lower than those needed to bind repressor. This finding suggests that the alternative conformations of repressor persist even if the protein is not in direct contact with DNA. This suggestion implies that even brief collisions between 434 repressor and DNA induce these conformational changes. Although we have not yet determined the lifetime of the DNA-induced conformations, time resolved DNA anisotropy measurements show that in the case of the nonspecific DNA, the protein-DNA contact that affects repressor structure has a lifetime of ≤ 3 nsec. Hence our data indicate that DNA acts in a "catalytic" fashion to induce a steady state amount of an alternative repressor conformation.

In effort to discern the functions of the dimeric repressor forms induced by nonspecific DNA, specific DNA or increased repressor concentrations, we measured the relative affinities of these protein species for a specific binding site. Our data show that the repressor dimer species formed in the presence of specific or non-specific DNA has an enhanced affinity for specific DNA, and that the dimer species formed at higher repressor concentration has a lower affinity for specific DNA. We find that the dimeric species induced by high repressor concentrations is not formed in vivo. We considered these observations in an attempt to discern a possible pathway for the assembly of repressor-DNA complexes. We suggest that the repressor conformer induced by non-specific-DNA is the form of the repressor that is optimized for searching for DNA binding sites along non-specific DNA. Upon finding a binding site, the repressor protein an additional conformational change, that allows it to "lock-on" to its specific site. We will present additional evidence supporting the existence of this pathway and that elucidates the functional role of each repressor conformer.

Typical of many transcriptional regulatory proteins, the bacteriophage 434 repressor also binds cooperatively to two sites on DNA. Cooperative binding is essential for establishment and maintenance of phage lysogeny. In the phage, two dimers, one bound at each of the adjacent O_R1 and O_R2 operator sites, form a tetramer that mediates the cooperative binding of the repressor. The mechanism by which the tetramer forms on the two adjacent sites is unknown. Bacteriophage 434 repressor does not form tetramers in the absence of DNA and the disparate intrinsic affinities of repressor for O_R1 and O_R2 suggest that tetramer formation precedes multisite binding of repressor. We propose that repressor DNA binding

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may stimulate repressor to form tetramers. Consistent with this idea, a complex containing 3-4 repressor molecules readily assembles on a single operator site. Mutations that block cooperative tetramer binding to the adjacent O_R1 and O_R2 sites, also block formation of the complexes assembled on a single O_R1 site. This finding shows that the complexes that form on a single site assemble using the same interface as does the tetramer assembled on adjacent operator sites. Adding additional O_R1 to dissociates these complexes into dimeric repressor-O_R1 complexes. In contrast, adding O_R2 to these complexes results in the formation of a repressor tetramer containing an O_R2 and an O_R1 site. Together these findings suggest that an O_R1-bound repressor may cooperatively help repressor bind to O_R2 by the recruiting an additional repressor molecule from solution that subsequently occupies O_R2. Further support for this idea derives from the observation that purified carboxyl terminal domain of 434 repressor, which lacks the DNA binding domain is capable of forming a hetero-oligomeric complex with O_R1 DNA bound intact repressor dimers.

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Session 5: Structure and function
Chair: Deepak Bastia

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B.subtilis TRAP and bacteriophage SPP1 portal protein: the role of circular organisation in interaction with nucleic acids

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Multisubunit circular assembly provides a mechanism by which proteins can acquire new biological properties. The results of the X-ray structural analysis will be presented for two circular protein assemblies. The first is the *trp* RNA-binding attenuation protein (TRAP) which interacts with the single-stranded RNA during transcription. The second is bacteriophage SPP1 portal protein (gp6) which plays a key role in viral DNA translocation.

TRAP regulates transcription of *L*-tryptophan biosynthetic genes (*trpEDCFBA*) in *B.subtilis* by sensing the intracellular levels of the amino acid [1,2]. By binding to its target RNA TRAP prevents the formation of an "antiterminator" stem-loop structure allowing formation of an alternative "terminator" hairpin that leads to transcription termination. In the TRAP/RNA complex, the 11-subunit circular assembly of TRAP[3] is matched by eleven GAG or UAG triplets of RNA separated by two or three non-conserved nucleotides[4,5]. In this case, the periodic repetition of TRAP subunits within the circular assembly favors binding of the repeating single stranded RNA sequence which forms a belt around the protein molecule.

Bacteriophage SPP1 portal protein (gp6) controls movements of DNA into and out of the viral capsid[6]. As an isolated protein, gp6 is a circular assembly of 13 subunits[7,8]. In the mature capsid, however, this protein is present as a 12-subunit circular assembly, suggesting a structural change during viral assembly. The X-ray structure of the 13-subunit oligomer of gp6 is now being determined at 3.4 Å resolution. In this case, the circular organization generates the central channel which is ideally suited for conducting DNA translocation.

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The extraordinary specificity of restriction endonucleases: How is it achieved?

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Restriction endonucleases are paradigms for the study of protein-DNA recognition. Their specificity is extraordinary. A single variation in the DNA sequence results in over a million-fold loss in activity. To explore the basis of this extreme specificity, we have determined the structures of restriction endonuclease BamHI at different stages of its catalytic pathway(1,2). These structures provide a complete picture of BamHI specificity, that is derived partly from binding and partly from catalysis.

Using the knowledge gained form these structures, attempts have been made to alter the specificity of BamHI, but these have proved unsuccessful(3). To understand why the BamHI scaffold cannot be modified to recognize a closely related DNA site, we have determined the structure of the closely related endonuclease BglII. We find that the DNA is contorted differently in the BglII-DNA complex, leading to different protein-DNA contacts(4).

Together, the BamHI and BglII structures reinforce a sense that protein-DNA recognition in restriction endonucleases must be considered in the context of the whole protein and not just the few residues that interact directly with DNA.

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DNA translocating machines

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DNA transfer between cells, across membranes, is a central biological issue of unknown molecular mechanism. Bacterial conjugation, which implies such trans-membrane passage, is the major route for horizontal gene transfer in prokaryotes. It is a means for rapid acquisition of genetic information resulting in organisms with new ecological or pathogenic characteristics, a way for the evolution of bacterial genomes. Acquisition of antibiotic resistance by pathogens is also mediated by the conjugative transfer of mobile genetic elements. Furthermore, trans-kingdom gene transfer from bacteria to plants or fungi is a special case of conjugation. Even bacterial sporulation involves a similar DNA transfer mechanism from the mother cell to the forming spore. An integral membrane DNA-binding protein, called TrwB in the *E. coli* R388 conjugative system, is essential in the conjugation process. This large multimeric protein of 507 residues per monomer is responsible for recruiting the relaxosome DNA-protein complex and coupling it to the transmembrane transport apparatus. We have solved the three-dimensional structure of a soluble variant of TrwB[1], lacking the first 70 amino acids. The molecule consists of two domains: a nucleotide-binding domain of α/β topology, reminiscent of RecA and DNA ring helicases, and an all-alpha domain. Six equivalent protein monomers associate to form an almost spherical quaternary structure strikingly similar to F_1-ATPase. A central 20 Å-wide channel traverses the hexamer.

ϕ29 is a complex double-stranded DNA bacteriophage that infects *Bacillus subtilis* cells. The viral particles are formed by a prolate icosahedral capsid, or head, and a tail. Between these two structures there is a connecting region called portal vertex or connector. This large oligomeric protein, composed of 12 identical protomers of 420 kDa each[2], translocates the DNA into the prohead during packaging and plays an important role in the first steps of head assembly. The connector is also involved in the process of DNA transfer into the host cell. We have crystallized[3] the connector particle and solved and refined its three-dimensional structure to 2.1 Å. Each monomer is composed by a central domain that includes a three-helix bundle, a distal α/β domain and a proximal all-β domain. The protomers assemble into a 12-mer funnel super-structure with a central channel, 40 Å wide at its half distal part. The channel opens up at the proximal end, internal to the viral capsid. The surface of the channel is mainly of electronegative nature but includes two lysine rings. The structure of the connector suggests a simple DNA translocation mechanism, where the connector rotates respect to the capsid while moving longitudinally the DNA.

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Structural studies of prokaryotic RNA polymerases

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The 3.3 Å-resolution X-ray crystal structure of *Thermus aquaticus* core RNA polymerase (1) provides a basis for further structural and functional studies. The path of the transcript RNA and template DNA through the RNA polymerase structure was tracked using RNA-protein and DNA-protein crosslinks, resulting in a model of the ternary elongation complex (2). A co-crystal structure of core RNA polymerase complexed with rifampicin reveals the structural mechanism of the antibiotics' inhibitio n mechanism (3). Work currently in progress includes analysis of conformational flexibility in the RNA polymerase molecule by comparison of different crystal forms and high-resolution X-ray structures of σ factor domains comprising most of *Thermus aquaticus* σ^A. Finally, the structure of an open promoter complex of *Thermus aquaticus*holoenzyme with promoter DNA, obtained by fitting the high-resolution structures of core RNA polymerase, σ factor domains, and DNA into a 6.5 Å-resolution X-ray map, will be described.

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Prokaryotic histones and nucleosomes

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Prokaryotic histones and nucleosomes, true homologs of their eukaryotic counterparts, are present throughout the Euryarchaea branch of the Domain Archaea, and they provide a unique perspective on the structure and function of archaeal and eukaryal chromatin. Both types of histones share a common three-dimensional structure, the histone fold, but the archaeal histones represent the minimal, unembellished fold, which will be shown in high resolution crystal structures (1). In view of the relative simplicity of the archaeal system and the clear ancestral homology between the two types of histones and nucleosomes, archaeal histones provide an ideal experimental system for structure-function studies, as a single recombinant archaeal histone can form homotetramers that organize ~80 bp of DNA into an archaeal nucleosome. The structures of both the histone fold and the archaeal nucleosome have been probed in vitro by generating archaeal histone variants by site-directed mutagenesis and applying a variety of biochemical techniques to study the archaeal nucleosomes they assemble. Based on these analyses, functions have been assigned to most of the 69 residues in the representative archaeal histone rHMfB. The buried hydrophobic residues in a histone dimer comprise the monomer-monomer interface and complementary hydrophobic side chain packing determines the range of potential dimerization partners for a histone monomer (1). A subset of these hydrophobic residues accounts for the extreme thermal stability of histones from hyperthermophiles (2). Positively charged surface residues dictate a path for DNA across the surface of the archaeal histones, and these residues interact cooperatively with DNA in nucleosome formation (3). DNA-contacting residues are properly oriented by both a surface electrostatic network and a novel proline tetrad structure (1). Surface hydrophobic patches on histone dimers are buried upon tetramer formation in the nucleosome, and movement of the two dimers about this hydrophobic interface permits the formation of nucleosomes with either positive or negative DNA topologies. By SELEX selection, the sequences of DNA molecules that are most readily bound and wrapped by rHMfB into archaeal nucleosomes in vitro have been identified, and these define DNA structures that position archaeal nucleosome assembly (4). By all measures, the protein and DNA components of the archaeal and eukaryal nucleosomes are conserved and it seems reasonable to conclude that this structure originated in a prokaryote for the purpose that it now serves in contemporary Euryarchaea -- to package and maintain the chromosome in a configuration that prevents aggregation and preserves its flexibility.

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Abstract: Protein-protein Interactions in the Formation of an IS911 Pre-cleavage Paired End Complex
Robert J Alazard, Christophe Normand, Laurence Haren and Michael Chandler
Two proteins encoded by the bacterial insertion sequence IS*911* are required for efficient intermolecular transposition: the transposase, OrfAB and a regulatory factor, OrfA. This protein shares the majority of its amino acid sequence with the N terminal part of OrfAB, which includes a putative helix-turn-helix and three of four heptads of a leucine zipper (LZ) motif. OrfA strongly stimulates OrfAB-mediated intermolecular transposition both *in vivo* and *in vitro*.
We present evidence that this is accomplished by direct interactions between the two proteins via the LZ. We showed that OrfA not only undergoes oligomerisation but is capable of engaging with OrfAB to form heteromultimers and that the LZ is necessary for both types of interactions.
The synaptic complex formed between a derivative of OrfAB deleted for the C terminal catalytic domain and different IS*911* left and right end fragments was investigated. Gel shift experiments demonstrated that this protein was capable of bridging two IS911 ends to generate a species whose composition was that expected for a synaptic complex. Addition of OrfA dramatically altered this complex suggesting a role for this protein in modulating transposition. A model describing the involvment of OrfA on synaptic complex formation will be discussed. The effect of staggered deletions in the ends of IS*911* on synaptic complex formation and footprinting patterns of these complexes will be presented.

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The thumb subdomain of T7RNAP is a processive clamp and a putative element for Class II termination/pausing recognition

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Bacteriophages are the archetype of "genomic economy". It's not surprising that the RNA polymerase of bacteriophage T7 (T7RNAP) is able to carry out all the transcriptional steps with out any accessory proteins, regulate the phage life cycle by its allosteric interaction with T7 lysozyme, and have alternative roles in DNA packing and DNA replication. Thus, T7RNAP is an example of "protein economy". In this work we present evidence for two different functions of the helix N of the thumb subdomain. Residues R391 to R394 are involved in the classical role of the thumb subdomain as a processive clamp, exerting interactions with the RNA:DNA hybrid when it reaches 6 or more nucleotides. The thumb's contribution to the complex stability depends of the equilibrium between the formation of extended or short RNA:DNA hybrids during transcription. The fate of this equilibrium is determined by the topology of the template. In double stranded templates, reannealing of the non template strand favors the formation of short RNA:DNA hybrids, whereas in supercoiled or partial single stranded templates long RNA:DNA hybrids are favored. Residues Y385 and D388 are involved in the recognition of Class II termination/pausing sequences. The Class II termination/pausing sequence is only effective in linearized templates and its transcribed recognition sequence is identical to an "RNA bulge" motif presented in the HIV-1 TAR element, suggesting that the reannealing of the non template strand determines if the transcribed RNA folds into a motif that is in part recognized by the thumb subdomain.

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Specific and overimposed transcriptional regulation of the 3-hydroxyphenylpropionic acid degradation pathway from *Escherichia coli*

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To study the transcriptional regulation of the inducible mhp genes responsible for the catabolism of 3-hydroxyphenylpropionic acid (3HPP) in Escherichia coli, genetic (lacZ translational fusions) and biochemical (gel retardation assays, primer extension analyses) approaches were used. A pathway-specific regulatory system mediated by the mhpR gene product was observed. The MhpR protein (277 aa, 31.2 kDa) belongs to the IclR family of transcriptional regulators and possess the putative DNA-binding helix-turn-helix motif at its N-terminus end. The MhpR protein has been shown to be a transcriptional activator that binds to the promoter (Pa) running the expression of the mhp catabolic genes. The operator region of the Pa promoter is a 15-bp palindromic sequence motif located 50-bp upstream of the transcription start site. Although binding of MhpR to Pa is no dependent on the presence of 3HPP in vitro, transcriptional activation requires such inducer molecule. Structural analogues of 3HPP such as 2-hydroxyphenylpropionic acid, 3-hydroxycinnamic acid, and the corresponding 2,3-dihydroxy derivatives of phenylpropionic and cinnamic acids are also inducers. However, phenylpropionic derivatives without hydroxy groups in the aromatic ring or with hydroxy groups in para position, as well as 3-hydroxy derivatives with a shorter or larger side chain than that of 3HPP were not inducers. The specific regulation of mhp expression is subordinated to a more general control mechanism that is dependent on the physiological state of the cell and mediated by the interaction between the MhpR protein and some global transcriptional regulators such as the CRP protein. The molecular links between these two regulatory levels have been also analysed.

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Two very similar transcriptional regulators possess two totally different functions in the regulation of bacteriocin production

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Bacteriocin production in *Lactobacillus plantarum* C11 involves two very similar response regulators (PlnC and PlnD), in addition to the peptide pheromone plantaricin A and a histidine protein kinase (PlnB). In this study, the functionality of these four regulatory components, whose genes are located on the same operon (plnABCD), was investigated. By using the b-D-glucuronidase gene (gusA) fused with the plnA promoter as a reporter system, we demonstrated that the cloned plnABCD operon codes for an auto-regulatory unit, capable of activating its own promoter through the action of the cognate response regulators. Mutational analyses confirmed that both the inducer gene and the kinase gene are required for specific activation of the regulators. Further studies with a heterologous expression host revealed that PlnC, but not PlnD, plays a major role in gene activation. The role of the regulators in the global regulation of bacteriocin production was also accessed. By studying them in the bacteriocin producer C11-strain, it was shown that the two regulators possess opposite regulatory functions: PlnC activates while PlnD represses bacteriocin production. To our knowledge this is the first report dealing with a regulator protein that can play a key role in down-regulating bacteriocin production, a process which is still poorly understood in bacteriocin research.

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Plasmid pMV158 encodes two proteins with nicking-closing activities: RepB, the initiator of replication, and MobM, involved in mobilisation

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The streptococcal promiscuous plasmid pMV158 replicates by the rolling circle mechanism, and its is able to be mobilised among several bacteria. These two functions rely on two plasmid-encoded proteins (Fig. 1), namely RepB (the initiator of replication) and MobM (the initiator of transfer). Both proteins have strand- and site-specific nicking-closing (Topoisomerase I-like) activity and cleave supercoiled [or single-stranded (ss)] plasmid DNA on two physically distant targets: the double-strand origin (*dso*), and the origin of transfer (*oriT*). RepB and MobM are *trans*-acting proteins, and optimal requirements for its activity have been tested. Both proteins require Mg^{2+} or Mn^{2+}, and do not need energy cofactors. Optimal temperatures are 60°C (RepB), or 30°C (MobM). Whereas purified RepB protein is a hexamer in solution, MobM behaves as a monomer.

```
                          T
                       C     A
                      A        C
                      T      G        nic
                       C    A
              C          GC
           T     C       GC
             CG          GC
             TA          GC
             TA          GC
             TA          GC
             TA          GC   T
             TA          TA                     bind
             TA          AT
             AT
TGTTGTGGGGG CTAAAAT AGTGCCG--64bp--TCTCGGCGACTTTTCGGCGACTTTTCGGCGACTTT

                                      DR1          DR2          DR3
```

Figure 1. Nucleotide sequence of the *dso* region of pMV158. The RepB protein binds to the three 11-bp direct repeats and introduces cleaves the phosphodiester bond between nucleotides G and A.

Initiation of replication is mediated by protein RepB (210 residues), which binds to a set of three directly repeated sequences (the *bind* region), and cleaves DNA at the sequence 5'-TACTACG↓AC-3' (the *nic* region; being ↓ the phosphodiester bond cleaved). Cleavage is performed by transesterification mediated by the Tyr99 residue. The *bind* region is located 84 nucleotides downstream of the nick site, although close secondary binding sites have been found. This indicates that, upon binding to its primary recognition region, RepB is somehow transferred to a region close to the nick site. Whether this "transfer" is mediated by DNA looping or by a RepB-mediated DNA bending is currently under investigation.

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All plasmids of the pMV158 family have a conserved *nic* region located on a terminal or internal loop within a secondary structure. Sequence differences are found at the *bind* region.

This is consistent with the notion that replicon specificity is provided by the *bind* region, whereas cleavage is replicon-independent. *In vitro*, RepB is able to cleave supercoiled or single-stranded DNA of plasmids of the same family. Accordingly, we have found the existence of putative domains in the Rep proteins of the pMV158 family involved in cleavage (the more conserved N-terminal moiety), oligomerization (Leu-zipper), and DNA-binding domain (the more divergent C-terminal moiety).

MobM (494 residues) binds to the *oriT* region, which contains two inverted repeats (IR1 and IR2), cleaving its target DNA at the sequence 5'-AGTGTG↓TTA-3' within IR2, thus initiating the conjugative transfer. DNase I footprinting experiments showed that the region protected by MobM includes the *oriT* and the *mobM* promoter, suggesting that the protein auto-regulates its own synthesis. The pMV158-*oriT* is fully conserved among several plasmids of Gram-positive bacteria. In addition, a homologous mobilisation cassette is also present in Tn4555 from the Gram-negative bacteria *Bacteroides*. MobM exhibits various putative domains. The N-terminal region contain motifs that are found in proteins involved in DNA cleavage in conjugation (relaxases) and in initiators of rolling circle replication (Rep proteins). This N-terminal domain includes the active Tyr residue, and the so-called "HUH motif" (U being a hydrophobic residue) probably involved in cation coordination. In addition, molecular modelling indicates that MobM exhibits two αhelical-rich regions located within the C-terminal moiety which include a putative Leu-zipper domain and a C-terminal α-helical region. Two plasmid with mutations at these regions have been constructed. *Streptococcus pneumoniae* cells harbouring pMV158 or its derivatives were fractionated. Cell fractions were analysed by immunological detection with specific polyclonal anti-MobM antibodies, allowing us to show that MobM is associated to the cell membrane.

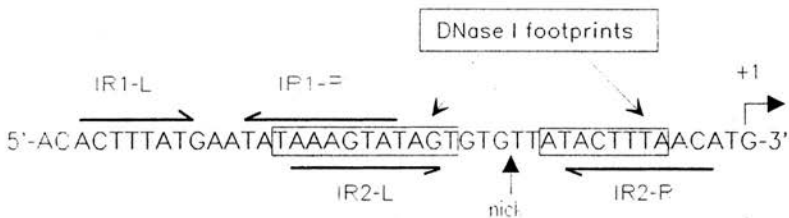

Figure 2. Structure of the *oriT* of pMV158. Initiation of transcription (+1), inverted repeats (IR), regions protected by MobM from Dnase I cleavage (boxed), and nick site (vertical arrow) are shown.

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Transcriptional regulation of the hpa cluster of *Escherichia coli*: Molecular characterization of the HpaR repressor

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HpaR protein is a repressor belonging to MarR family, which represses the expression of the meta operon of the hpa cluster of the *Escherichia coli* for the degradation of the 4-hydroxyphenylacetic acid (4-HPA) in this bacteria (Prieto et al., 1996. This operon is controlled positively by two global regulators, i. e., catabolite repression protein (CRP) and integration host factor (IHF). The hpaR gene is located adjacently to the meta operon in the chromosome and is transcribed divergently to it. Pg and Pr promoters drive the expression of meta operon and hpaR gene respectively. We have demonstrated by gel retardation assays using purified HpaR that this repressor binds to both, Pg and Pr promoters, which suggests that HpaR is controlling likewise its own expression. Furthermore, the HpaR operators (OPR1 and OPR2) in Pg and Pr promoters, respectively, have been identified by DNAse I footprinting assay. Whereas OPR1 overlaps the transcriptional start site of Pg, OPR2 is located at 37 pb downstream of the transcriptional start site of Pr. This finding suggests that different mechanisms control the repression mediated by HpaR in Pg and Pr promoters. In vivo and in vitro transcription assays demonstrated that 4-HPA is the effector of the HpaR protein.

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A new hyperrecombination mutation identifies a novel yeast gene, THP1, connecting transcription with mitotic recombination

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To investigate the genetic control of the incidence of recombination and genetic instability and, in particular, its connection with transcription, we have undertaken a dearch for hyperrecombination mutants among a large number of strains deleted in genes of unknown function.. We have identified a new gene, THP1(YOL072w), whose deletion mutation strongly stimulates recombination between repeats.In addition, thp1* impairs transcription, a defect that is particulary strong at the level of elongation through particular DNA sequences such lacZ. The hyperrrecombination phenotipe of thp1* cells is fully dependent on trasncription elongation of the repeat construct. When transcription is impeded either by shutting off the promoter or by using a premature transcription terminator, hyperrecombination between repeats is abolished, providing new evidence that transcription-elongation impairment may be a source of recombinogenic substrates in mitosis. We show that Thp1p and two other proteins previously shown to control transcription-associated recombination, Hpr1p and Tho2p, act in the same "pathway" connecting transcription elongation with the incidence of mitotic recombination. Our current interest is to know whether Thp1p interacts with either DNA or RNA to understand its putative role connecting transcription and recombination.

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Estimating protein-induced DNA-bending *in vivo*

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Transcription from many *Escherichia coli* promoters can be activated by the cAMP-CRP complex. One possible mechanism of activation involves contacts between the DNA upstream of the CRP binding site and the "back" of RNA polymerase, most likely involving the alpha-subunits. We wanted to verify *in vivo* the importance of theses contacts for transcriptional activation and determine de conformation of the DNA upstream of the core promoter.

We used a modified *malT* promoter with a CRP binding-site centered at –65.5 from the transcription start site, inducing an unfavorable conformation of the DNA. We annihilate the repressive DNA conformation and restore a DNA geometry favorable for transcriptional activation by randomly inserting a random number of additional CRP binding sites upstream of the first site. Transcriptional activation factors from these constructions are correlated with the predicted geometry of the DNA. We thereby constrain the possible parameters describing CRP-induced DNA-bending *in vivo*. CRP bends DNA *in vivo* by 90° with a 20° out of plane bend as observed in the crystal structure. In order to further confirm our model we designed a strongly activated artificial promoter by precisely positioning five CRP-binding sites upstream of the core promoter.

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Interplay of three global regulators in oxygen control of *cydAB* expression in *Escherichia coli*

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The *Escherichia coli* cydAB operon, encoding cytochrome d oxidase, is microaerobically activated by ArcA and repressed by Fnr in anaerobiosis (1, 2). In this work we have used gene fusion, in vitro gel retardation and footprinting and primer extension techniques to investigate the role of the nucleoid protein H-NS in the transcriptional regulation of cydAB. Aerobically, an hns mutant showed increased expression of a cydA-lacZ fusion, due to enhanced transcription from four of the cydAB promoters. In anaerobiosis, an hns mutation did not significantly affect the cydAB expression levels unless arcA was also inactivated. As with several other H-NS regulated promoters, the cydAB control region showed altered mobility on a polyacrylamide gel, suggesting that it may contain bent DNA. The purified H-NS protein bound specifically at the cydAB promoter element. H-NS protected extended regions from DNAse I digestion, that span the ArcA and Fnr binding sites relevant to anaerobic cydAB regulation. Our results indicate that H-NS is a repressor of the cydAB operon. H-NS repression is due to direct interaction with the cydAB promoter region, probably through recognition of a bend in the DNA strand. The targets for cydAB regulation are the P1, P2, P3 and P4 promoters. H-NS control is indirectly modulated through the action of ArcA, which antagonizes H-NS repression at low oxygen tensions (3).

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Expression of the stress response sigma factor, σ^H, is temporally and spatially regulated by the developmental transcription factor BldD in the multicellular prokaryote Streptomyces coelicolor A3(2)

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Morphological changes leading to aerial mycelium formation and sporulation in the mycelial bacterium *Streptomyces coelicolor* rely on establishing distinct patterns of gene expression in separate regions of the colony. σ^H was previously identified as one of nine homologous sigma factors associated with stress responses or morphogenesis in *S.coelicolor*. Transcription studies showed that *sigH* and the upstream gene, *prsH* encoding a putative anti-sigma factor, form an operon transcribed from two developmentally regulated promoters *sigHp1* and *sigHp2*. Moreover, transcription from *sigHp2* was induced by heat-shock or ethanol. Localization of *sigHp2* activity using a transcriptional fusion to *egfp* showed that *sigHp2* transcription is spatially restricted to sporulating aerial hyphae in wild-type *S.coelicolor*.

Analysis of mutants unable to form aerial hyphae showed that *sigHp2* transcription is dramatically upregulated in a *bldD* mutant and that the *sigHp2-egfp* fusion was expressed ectopically in the substrate mycelium in the *bldD* background. We have shown that the tissue specific regulation of *sigHp2* is mediated directly by the developmental transcription factor BldD that belongs to the HTH-3 family of DNA binding proteins. BldD binds to the promoter *sigHp2* and protects sequences extending from –34 to +10 in a DNaseI footprinting experiment. The *sigHp2* promoter is repressed by BldD in vegetative hyphae, but this repression is released in sporulating aerial hyphae. The possible mechanism of regulating BldD activity, including proteolysis, covalent modification or inactivation via protein-protein interaction, will be discussed.

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A DNA-protein complex in bacterial conjugation: Interactions at the relaxosome of plasmid R388

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During bacterial conjugation, a DNA-protein complex is formed, named the relaxosome, which includes several proteins bound to the plasmid origin of transfer (oriT). In plasmid R388, proteins TrwA and TrwC were shown to bind two specific oriT sequences. TrwC is the protein that introduces the specific nick at the transferred strand; TrwA assists in the nicking reaction. A third protein, TrwB, is thought to form part of this complex. TrwB belongs to a family of conjugative proteins that have been proposed to play the role of coupling the relaxosome to the DNA transport apparatus during conjugation. It is believed that it carries out its role by interacting with other proteins that specifically bind oriT and by bridging the relaxosome to the transport complex by means of its amino-terminal transmembrane domains. Then, it could motor the DNA through the transmembrane pore by tracking along the displaced DNA strand. In support of this role, a soluble domain of TrwB (TrwBDN70) binds DNA unspecifically and cooperatively, and also binds NTPs. In addition, we present evidence that TrwB interacts with proteins TrwA and TrwC, that form the R388 relaxosome. TrwBDN70 was fused to GST and immobilized in a glutathione-sepharose column, where it retained both TrwA and TrwC proteins. The interaction with TrwA was significantly stronger. A weaker interaction between TrwA and TrwB could be detected in vivo by using the bacterial two-hybrid assay. On the basis of the recently published 3-D structure of TrwBDN70, these data are integrated into a model for TrwB´s coupling role in conjugation.

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Monitoring signal integration in catabolic promoters of *Pseudomonas putida*

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The m-xylene responsive Pu promoter of the upper operon of the TOL plasmid pWW0 of *P. putida* mt-2 is regulated by the XylR protein, that belongs to the family of prokaryotic enhancer-binding activators that act in concert with s54. This occurs through a mechanism that involves the binding of the regulator to upstream activating sequences (UAS) and the looping-out of the complex into close proximity to the s54-containing form or RNA polymerase bound to the -12/-24 region of the promoter, an event assisted by the integration host factor (IHF). Pu activity is inhibited when cells grow exponentially in rich medium. This effect seemed not to require the activity of the whole complement of TOL genes, since it can be faithfully reproduced with only the regulatory elements that control transcriptional activity of Pu. *P. putida* cells devoid of the TOL plasmid but added with chromosomal insertions of the xylR gene and a Pu-lacZ fusion were unable to accumulate ß-galactosidase when growing exponentially in LB, regardless of the presence or absence of m-xylene. However, as soon as the cells leave exponential growth and enter stationary phase, the same Pu-lacZ fusion becomes extremely responsive to the aromatic inducer. Since Pu is functional in vitro by just mixing purified and preactivated XylR with s54-containing RNAP and IHF, it is clear that additional elements adjust transcription to the physiological state that governs the cells when they become induced. At least in part, these effects can be traced to modulation of the activity of the sigma factor iself, because its overproduction relieves the inhibition. That some physiological signals are entered through the sigma factor is suggested as well by the observation that ftsH mutants of E. coli (encoding a membrane-bound protease involved in the turnover of s32) fail to activate Pu. Overproduction of s54 defeats the inhibition caused by the loss of FtsH protein. Furthermore, increased levels of FtsH not only restore promoter activity but it also relieves the physiologi

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Interactions of the H-NS family and the Hha family of bacterial proteins

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The bacterial nucleoid-associated protein H-NS is one of the best characterized central regulators of gene expression. It is implicated in the response to changes in osmolarity and temperature. H-NS affects the expression of a large number of genes in different bacterial species of Enterobacteriaceae and Vibrionaceae. The Hha protein of *Escherichia coli* and the YmoA protein of *Yersinia enterocolítica* belong to a family of proteins implicated in the regulation of gene expression, mainly of virulence operons. Members of both these families are present in large antibiotic resistance conjugative plasmids too. A possible way how the members of the Hha family of proteins could exert their regulatory role has been discovered recently when we found that Hha can interact with H-NS (Nieto J.M., et al. 2000. Mol. Gen Genet.263, 349-358).

Interaction of the Hha and H-NS proteins: Nickel-NTA affinity purification is a convenient method to detect protein-protein interactions. We constructed a recombinant Hha protein containing a 6 Histidine tag at the N-terminal end. We expressed this recombinant protein under a T7 polymerase system. The cleared lysate was bound to Nickel-NTA Agarose and washed with increasing concentrations of imidazol, which progressively liberated the bound protein. The His-Hha protein copurified with a protein, later found to be H-NS by N-terminal Edman analysis. We observed that DNA copurified as well. Degradation of this DNA allowed us to see that it was not necessary for the interaction of both proteins.

Hha and H-NS form a complex with DNA: Purified Hha and H-NS proteins and DNA (a fragment of the hemolysin operon) were used for gel-retardation studies. We found that the concentration of Hha needed to show a retardation was much higher that that of H-NS. When both proteins were put together with the DNA, a much lower amount of Hha showed an effect. The complex formed by both proteins and the DNA behaved like the H-NS-DNA complex in front of a competitor DNA (polydI-dC). This result suggests that in the complex Hha is bound to H-NS, wich is bound to DNA.

The interaction is a property of both the H-NS and the Hha families: By using the Nickel Affinity methodology, we have found that YmoA of *Y. Enterocolitica*, binds H-NS of *E. coli*. Additionally we have identified the previously unkonwn H-NS protein *Y. enterocolítica* and cloned its gene.

Present investigations: We are trying to obtain an hns mutant of *Y. enterocolitica*. We obtained aminoacid substitutions of Hha and we are studying how these affect the interaction with H-NS.

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Domain architecture of a bacterial transcriptional factor containing the eukaryotic "AT-hook" DNA-binding domain

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The transcriptional regulatory protein CarD participates in light-induced carotenogenesis and starvation-induced fruiting body formation in the bacterium *Myxococcus xanthus*. It is also the only known example of a prokaryotic protein with the "AT-hook" DNA-binding domain and an adjacent highly acidic region characteristic of the eukaryotic high mobility group I(Y), HMG-I(Y), proteins. The latter are non-histone nuclear proteins that modulate various DNA-binding activities by functioning as architectural factors to remodel DNA and chromatin structure. We have examined the structural and functional domain organization in protein CarD. Structural domains in purified CarD were probed by: (i) limited proteolysis coupled with N-terminal sequencing, mass spectrometry, and Western blotting using anti-CarD monoclonal and polyclonal antibodies of defined epitope specificities; (ii) the cloning, expression and purification of specific CarD fragments. We find that the N-terminus of CarD constitutes a relatively stable domain, as does the combined segment formed by the acidic and the adjacent AT-hook regions, where the stabilization appears to be largely mediated by favorable electrostatic interactions. CarD fragments containing these regions could be purified in amounts sufficient for spectroscopic examination. HMG-I(Y) proteins bind specifically to DNA in the minor groove of AT-rich sequences four to eight base pairs in length that occur in at least two appropriately spaced tracts. We show that this characteristic is shared by both CarD as well as its fragment with only the acidic and AT-hook regions. The conformation and DNA-binding affinities of HMG-I(Y) proteins have been shown to be affected by phosphorylation within the acidic region by casein kinase II, and within the AT-hook region by Cdc2 kinase in a cell-cycle and cell-differentiation dependent manner. We find that the acidic region of CarD like its counterpart in HMG-I(Y) can serve as a substrate for casein kinase II. We will present details of our ongoing examination of the DNA-binding, phosphorylation and conformational properties of CarD and their parallels with the mammalian HMG-I(Y) proteins.

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An unconventional glucose effect represses the catabolism of 4-hydroxyphenylacetic acid in *Escherichia coli* W

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The peculiar regulation of the Pg promoter which controls the expression of the meta operon of the 4-hydroxyphenylacetic acid (4-HPA) catabolic pathway of *Escherichia coli* W, has been examined through in vivo and in intro experiments. By using Pg-lacZ fusions we have demonstrated that Pg behaves as a stationary phase promoter when cells are grown on glucose as the sole carbon and energy source. This unusual and strict catabolite repression control is mediated by the cAMP receptor protein (CRP). This unconventional behavior does not require the presence of the specific HpaR repressor or the 4-HPA permease (HpaX), excluding the implication of a typical inducer exclusion mechanism. However, the acetic acid secreted at stationary phase by the cells growing in glucose acts as an overflow metabolite, providing the energy to produce cAMP and to rapidly adapt the cells to the utilization of a new less-preferred carbon source such as the aromatic compounds. Although Pg is not a sigma s dependent promoter, it is activated by the global regulator IHF (integration host factor) at the stationary phase of growth. Gel retardation assays have demonstrated that both CRP and the integration host factor (IHF) bind to the Pg upstream region.

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Regulation of the *Pseudomonas oleovorans* GPo1 alkane degradation pathway: identification of factors involved in catabolic repression

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Bacteria are endowed with systems that can sense environmental and/or physiological signals, integrate them, and transmit an output response that frequently leads to the stimulation or repression of transcription. One such regulatory system is catabolic repression (CR), a mechanim used by bacteria to select which substrate will be preferentially used when confronted to a mixture of carbon sources. The molecular mechanisms mediating CR in *Pseudomonas* sp. are not well understood, despite their great environmental, biotechnological and clinical importance. To analyze this problem we have selected as model the pathway for the degradation of alkanes encoded in *Pseudomonas oleovorans* GPo1. Expression of these genes is regulated by two mechanisms. One is specific of the pathway, and responds to the presence or absence of alkanes by means of the transcriptional regulatory protein AlkS. The second system responds to the presence of a number of growth substrates that cells prefer prior to alkanes. We have recently proposed a new AlkS-mediated regulatory scheme based on a positive autoamplification system. AlkS would bind immediately upstream of the –35 region of the two promoters of the pathway, triggering an autoamplification response that boosts its own levels when alkanes are present. The CR response in overimposed to the AlkS-specific regulation, possibly interfering with it. We have identified a number of factors that participate in the CR effect exerted by amino acids. The most important one is Crc, a protein known to participate in an as yet unclear way in the regulation of some *Pseudomonas* promoters. Interestingly, Crc does not participate in the catabolic repression effect triggered by organic acids. Current advances in the role of Crc, AlkS, and other factors, on the regulation of this pathway, will be presented.

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Instituto Juan March (Madrid)

The ϕ29 membrane protein p16.7, involved in phage DNA replication, interacts with single-stranded DNA

Alejandro Serna-Rico, Margarita Salas and Wilfried J.J. Meijer

Recent advances have provided strong evidence that eukaryotic DNA replication takes place at specific sites in the cell (for review see Cook, 1999). The general idea is that the DNA polymerase(s), together with probably all other replication proteins, form so-called replication factories that are maintained at relatively static positions in the cell through attachment to underlying structures. A similar situation has recently been demonstrated for the bacterium *Bacillus subtilis* (Lemon and Grossman, 1998). Over the last decades compelling evidence has been obtained that replication of prokaryotic DNA, including that of bacterial chromosomes, resident plasmids and infecting phages, occurs at the cell membrane. Hardly anything is known, however, about the factors involved in attachment of the replication machinery to the membrane. Detailed knowledge of the *in vitro* DNA replication mechanism of the linear genome of the lytic *Bacillus subtilis* bacteriophage phi29 forms a sound basis to study this fundamental process. An early-expressed operon, located at the right side of the phi29 genome, contains an ORF, ORF16.7, whose deduced protein sequence is conserved in related phages. We showed that ORF16.7 encodes an integral, dimeric membrane protein, p16.7, which is early and abundantly expressed after infection (Meijer et al., 2000b). Using immunofluorescence techniques we demonstrated that protein p16.7 is required for efficient redistribution of replicating phage DNA from its initial to additional replication sites that surround the bacterial nucleoid (Meijer et al., 2000a). These latter results explain why *in vivo* phage DNA replication was strongly delayed in the absence of protein p16.7, but did not shed light on the mechanism of action of p16.7. Interestingly, analyses of a purified soluble variant, p16.7A, in which the N-terminal membrane anchor had been replaced by a histidine-tag, showed that it binds with high affinity to ssDNA and with lower affinity to dsDNA. The replication intermediates of phi29 contain long stretches of ssDNA (Gutiérrez et al., 1991). Thus, p16.7 appears to recruit specifically replicating phage DNA to the membrane through its affinity for ssDNA. To the best of our knowledge, this is the first integral membrane protein described that has ssDNA binding affinity.

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The molecular machines of DNA repair: SFM analysis of their architecture

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DNA repair processes, as well as many other DNA transactions, require the concerted action of many proteins. Extensive biochemical and genetic analyses have resulted in the identification of the protein components required for specific DNA repair pathways. We are now at a stage where we wish to know how these proteins act together as molecular machines to repair DNA. Knowledge on the three dimensional arrangement of the proteins and DNA in functional complexes is a necessary first step toward understanding the dynamic mechanisms of DNA repair. The SFM provides a useful tool to characterise the structure of DNA-protein complexes in the nm resolution range. We have initiated experiments to determine the architectural organisation and dynamic rearrangements that occur in the molecular machines responsible for removing damaged bases from DNA by nucleotide excision repair (NER) and repair of double strand DNA breaks by homologous recombination (HR). In the NER pathway we have observed the presumptive DNA damage recognition proteins XPA and XPC bound to DNA with a single damaged site. Experiments to observe increasingly complex elements in the NER pathway are underway. In the area of HR we have focused on one human protein, hRad54. The Rad54 protein can change DNA topology in an ATP dependent manner but does not appear to either stretch or wrap DNA. Large multimeric protein complexes are formed on DNA in the presence of ATP. DNA topology could be altered by these large Rad54 complexes dividing the assay plasmid into two domains and moving along the DNA twisting it both ahead and behind the point of movement.

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