Instituto Juan March
de Estudios e Investigaciones

102 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Helicases as Molecular Motors in Nucleic Acid Strand Separation

Organized by

E. Lanka and J. M. Carazo

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Introduction

E. Lanka and J. M. Carazo

Double helical nucleic acid is known for its stability, and yet for most critical transactions during the cell cycle such as replication, transcription, recombination and repair, and DNA transfer during bacterial conjugation, the two complementary strands of the duplex must be separated, at least transiently. Strand separation is achieved by helicases.

Helicases are motor proteins which couple nucleotide (NTP) binding and hydrolysis to nucleic acid unwinding. Almost 25 years ago enzymes of this class were originally named and described as chemo-mechanical agents for the control of DNA structure by Hoffmann-Berling (Heidelberg, Germany). Since then helicases have been the subject of extensive biochemical investigations which have shed light into the complex interplay between NTP and/or nucleic acid binding, and nucleic acid unwinding.

The attraction to study the function of helicases of the cell life cycle increased considerably by the discovery of helicase-related mammalian diseases, like Xeroderma Pigmentosum, Werner's syndrome, and Bloom's syndrome. However the progress in understanding mechanistic principles of strand separation is still guided by research on prokaryotic enzymes. A large variety of helicases is known, twelve in *E. coli* and about 140 are expected for yeast. However, not all of them are essential for cell viability. In this context we like to mention the importance of the replicative helicase DnaB of *E. coli*. The DnaB protein, the function of which was discovered in 1986 by McMacken, has been the subject of intense study over the last two decades and still is one of the most important model systems. However, the work on the replicative helicase of *E. coli* has been greatly inspired by work on corresponding proteins of the replication systems of the lytic bacteriophages T4 and T7.

The organization of this first workshop entirely dedicated to helicases was determined by questions concerning the structure-function relationship of nucleic acid unwinding machines. The multidisciplinary approach to the field by the combination of ideas of geneticists, biochemists, biophysicists and structural biologists was supposed to integrate the comprehensive knowledge with very recent developments on helicases into models on the mechanism of unwinding. Since structural data provide a new discussion level for explanations in enzymology, the workshop became timely appropriate when the first crystal

structures of a monomeric and dimeric helicase (PcrA of *B. stearothermophilus* and Rep of *E.coli*) were published.

The discussion of structural data of monomeric, dimeric and hexameric helicases comprised the first part of workshops leading into the evaluation of mechanistic principles and culminating in the discussion of helicases in the interplay with other components in replication, recombination and repair, transcription, and bacterial conjugation. The latter of the three sections was thought to provide insight also into the large variety of helicases in general.

Highlights of the discussions were certainly the novel information on crystal structures of hexameric replicative helicases extending earlier data obtained by electron microscopy. These data provide insight into the dynamics of the helicase/substrate interaction in the form of "snap shots". As a consequence, the need to expand on these approaches became obvious. In particular, it is critical to obtain structures of complexes between helicases and nucleic acids, substrates and inhibitors. Another important issue that still needs additional attention are biophysical studies of the molecular dynamics of helicases.

Thus, based on the speed of progress seen in the field at the occassion of this first helicase workshop, we look forward with great expectations, in particular to a new helicase meeting which will probably be held sometime in the near future.

Erich Lanka & José Maria Carazo

Session 1: Approaches to solve helicase structures

Chair: Erich Lanka

Rotary mechanism of ATP synthesis

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The ATP synthase from mitochondria bacteria and chloroplasts is a complex multi-subunit enzyme that works by a rotary mechanism. The lecture will describe details of the structural transitions through which catalytic sites in the F_1 sector pass in both the ground state and in the transition state [1, 2], and how these changes are impelled by the rotation of a central α-helical structure in γ-subunit.

The structure of the α-helical region of the γ-subunit changes during the catalytic cycle suggesting that it has some degree of elasticity. The exposed central stalk linking the F_1- domain to the rotation generator in F_o contains the remainder of the γ-subunit and also the δ- and ε-subunits. It resembles a riding boot, with the foot in contact with a ring of 10 c-subunits in the F_o domain [3].

The interface between the foot and the c-ring is extensive, suggesting that γ, δ, ε and the c-ring rotate as an ensemble. The mechanism of rotation requires the participation of both subunit a and the c subunits, and as the structure of subunit a remains, unknown the precise mechanism of coupling of the proton motive force to rotation remains a mystery.

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The domains of *E. coli* DnaB: structures, insights and prospects

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Native DnaB, the major replicative helicase of *E. coli*, is a hexamer of 52-kDa subunits. It is loaded onto ss-DNA at replication origins via specific mechanisms that involve its interaction with its loading partner DnaC. The simultaneous overproduction of DnaB and DnaC in *E. coli* has enabled isolation of DnaB, DnaC and the DnaB.DnaC complex in large quantities.

Although crystals of DnaB and DnaB.DnaC have been obtained, none has yet diffracted X-rays sufficiently to enable structure determination. Lower-resolution structural information on the hexameric complexes has been obtained by negative-stain and cryoelectron microscopy [1-3]. Under appropriate conditions, the DnaB hexamer exists in solution in conformational states that display either threefold (C3) or sixfold (C6) symmetry [2,3], and it is tempting to speculate that it is the interconversion between these states, driven by ATP hydrolysis, that drives the helicase as a molecular motor in the 5' to 3' direction along ss-DNA.

Results of limited proteolysis [4] and sequence comparisons suggest that DnaB is comprised of at least two structural domains, separated by a flexible "hinge" region. Recently published data suggest that the larger hexameric C-terminal domain may be further divided into two smaller (sub-) domains, one of which carries the ATPase active site while the other binds ss-DNA [5].

The smaller N-terminal domain is required for helicase activity. Its structure in solution has been determined by NMR methods [6], and in the crystalline state by X-ray crystallography [7]. NMR experiments with fragments comprising residues 1-161 and 1-142 of DnaB were first used to demonstrate that residues 1-23 at the N-terminus and 137-161 in the putative hinge region were unstructured [8]. Thus, residues 24 to 136 comprise the structured core of the N-terminal domain in the C3 to C6 conformational transition. A model for this transition will be presented and extended to a proposal for a mechanism for "rotation" of the helicase hexamer during its translocation on a DNA template.

NMR studies could demonstrate no interaction of DnaB(24-136) with ss-DNA, ATP or the DnaC protein. However, there is clear evidence of interaction of DnaC with residues between V131 and A144 in helix α6 and the unstructured "hinge" region of DnaB(1-161). This implicates this region as one of the points of contact of DnaC with the DnaB hexamer [9].

By exploiting this information, it has now been possible for the first time to achieve simultaneous high-level overproduction of DnaC and soluble hexameric C-terminal domains of DnaB [10]. Structural studies with these fragments are in progress.

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Electron microscopic studies of helicases in replication and recombination

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It is now clear that the core of all helicases is same as the nucleotide-binding core of both the F1-ATPase and the RecA protein[1]. The F1-ATPase exists as a hetero-hexamer with C3 symmetry[2]. The only active form of the RecA protein has been assumed to be a helical filament. But RecA[3] and certain hexameric helicases, such as DnaB[4] and papilloma E1[5], exist as homohexamers in both C6 and C3 symmetries. We have now shown that the active form for a eukaryotic homolog of RecA, Dmc1, which is active in meiosis, appears to be an octameric ring[6]. Since the Rad51 protein, from both yeast and humans, also forms a ring as well as a filament, we suggest that RecA and Rad51 exist in two active forms, rings and filaments, while Dmc1 exists only as a ring. The *E. coli* rho transcription termination protein is a hexameric helicase, and is believed to function by separating an RNA-DNA hybrid. Unlike hexameric DNA helicases where a single strand of DNA passes through the central channel, it has been proposed that the RNA wraps around the outside of the ring. The symmetry of rho has been controversial, and we can now show that it has C6 symmetry[7]. We have generated a three-dimensional reconstruction of rho, and localized a tRNA molecule bound to the primary RNA-binding site to the outside of the ring. An atomic structure of the N-terminal domain of rho fits into our reconstruction so that the residues involved in RNA-binding are on the outside of the ring. Thus, rho shares a common structural core with the F1-ATPase and other hexameric helicases, with a divergence in function due to rho's N-terminal domain which has no homology to other helicases. This suggests that the functional divergence of all "helicases" may be great, in part due to the diverse domains that have been added to the conserved RecA-like core.

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Crystallographic studies of DNA helicases and single-strand DNA binding proteins

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Escherichia coli SSB is a homotetrameric protein which plays essential roles in DNA replication and repair by destabilizing (unwinding) the DNA helix and binding preferentially and with high affinity to single stranded (ss) DNA. The crystal structure of the DNA-binding domain of SSB (SSBc) in a complex with $dC[pC]_{34}$ was determined at 2.8 Å resolution. A model of SSBc bound to single continuous ssDNAs of 30 nucleotides was built and refined. This binary complex structure reveals the structural basis for essential elements of SSB function which will be discussed.

Session 2: Structure of hexameric replicative helicases

Chair: José M. Carazo

An analysis of DnaB and its complexes by electron microscopy

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Structure/function analysis are basic in the understanding of how biological macromolecules play their role in the cell. In this work we have focused our attention into a key replicative helicase, such as DnaB of *E.coli*, studying the structure of this enzyme as well as some of its complexes with other proteins by means of electron microscopy and image processing.

Most structural analysis tend to present a static picture of a macromolecule, whose implication in its dynamics are not obvious. In an effort to cast light into how DnaB may actually work, we have identified *in vitro* conditions in which DnaB adopt different conformations. This study has also been performed with the DnaB.DnaC complex. Along this line we have found that the previously reported polymorphism between three-folded and six-folded architectures of DnaB can be controlled by changes in the pH of the buffer. In this way, these two views coexist at pH values below 7.6, while at higher pH values only the three-folded conformation is present. Furthermore, this polymorphism is not found when the complex DnaB.DnaC is studied, irrespective of the pH of the buffer. This latter fact could be linked to the known lack of activity of DnaB when complexed with DnaC, indicating that a mechanism of structural symmetry alternance triggered in some way by electrostatic charges could be at the heart of the dynamics of DnaB.

Low-resolution three-dimensional reconstructions of both DnaB and DnaB.DnaC have already been reported. As a step forward in this direction we are currently mapping defined structural regions on DnaB by means of monospecific antibodies purified by a matrix spot peptide synthesis approach. Additionally, a study of the interacting surfaces between DnaB monomers, DnaB and DnaC, as well as DnaB and one its inhibitors, the Kid protein, is currently underway.

Finally a new, higher resolution reconstruction of the complex DnaB.DnaC has been performed, which will be further discussed in this meeting by Barcena et al.

Negative staining and 3D Cryo-EM structural studies of the large T antigen at the SV40 origin of replication

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The large T antigen (T-Ag) of Simian Virus 40 (SV40) is a multifunctional regulatory protein, responsible for both the control of viral infection and the required alterations of cellular processes. T-Ag is the only viral protein required for viral DNA replication. The functional complex at the SV40 origin of replication (*ori*) is a double hexameric oligomer, whose formation is dependant on ATP (or ADP) binding but not on ATP hydrolysis. The T-Ag double hexamers cover the complete core origin. We have prepared such nucleoprotein complexes using a DNA probe encompassing the *ori* region flanked by some 20 base pairs at the 5`end. By using electron microscopy and negative staining, we visualised T-Ag double hexamers bound to the SV40 origin. Image processing of side views of these nucleoprotein complexes revealed bilobed particles of 24 nm in length and 8 to 12 nm in width, which indicates that the two T-Ag hexamers are oriented "head-to-head". We also described a previously undetected structural domain of the T-Ag hexamer that we identified, by monoclonal antibody decoration, as the functional domain responsible for DNA binding. In addition, the host range domain of the T-Ag protein has also been mapped, at the very edge of the propeller-shaped T-Ag hexamers. Taking into account all the biochemical data known on the T-Ag–DNA interactions at the replication origin, we present a model in which the DNA passes through the inner channel of both hexamers.

These complexes were also imaged in their frozen-hydrated state. A collection of cryo-EM side views of T-Ag double hexamer was selected for further studies. We first obtained an average 2D-projection image of the whole of the particle. The former did not differ much from that obtained for negatively stained particles. From side-view images of the nucleoprotein complexes a 3D reconstruction is currently in the process of being calculated. A very preliminary volume for these double hexamers showed an asymmetric particle in which each of the hexamers within the double hexamer seems to adopt different macromolecular architectures. These could correspond to two different binding modes of the T-Ag hexamer (one when bound to ss- and another one when bound to dsDNA). A marked vorticity in each of the hexamers was also observed. There was a twist between the two hexamers. And, most singularly, there is some density sticking out from the interface between the two hexamers that could be attributable to the single stranded DNA that would be unwound upon T-Ag binding to the cognate dsDNA (prior to any processive helicase activity of T-Ag).

Investigating the action of DEAD Box RNA helicase by single molecule visualization

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RNA helicases are motor proteins that use the energy of ATP hydrolysis to unwind RNA/RNA or RNA/DNA duplexes into their functional single-stranded intermediates. The precise molecular mechanism of unwinding by these essential enzymes has not been fully defined. Using Atomic Force Microscopy we show that addition of ATP to the pre-bound *Escherichia coli* helicase DbpA/RNA complex initiates unwinding. Visualization of the action of DbpA at single molecule resolution enabled us to define intermediate states during unwinding catalysis. Comparison the level of ATP hydrolysis of DbpA in the presence of duplex RNA to its native rRNA[1-3] substrate accounts for only 1/3 of the ATP consumed by DbpA when encountered with its specific rRNA substrate. These findings may suggest that this unique 'DEAD box' protein fulfill additional energy-dependent functions.

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Crystallographic studies of the bacteriophage T7 helicase-primase

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The phage T7 helicase-primase is a ring-shaped hexameric protein that unwinds DNA ahead of the replication fork and synthesizes RNA primers for lagging strand synthesis.[1,2] The helicase domain from the helicase-primase protein crystallized as a helical filament[3] that resembles the *Escherichia coli* RecA protein, an ATP-dependent DNA strand exchange factor. When viewed in projection along the helical axis of the crystals, six protomers of the T7 helicase domain resemble the hexameric rings seen in electron microscopic images of the intact T7 helicase-primase. Nucleotides bind at the interface between pairs of adjacent subunits where an arginine is near the γ-phosphate of the nucleotide in *trans*. The bound nucleotide stabilizes the folded conformation of a DNA binding motif located near the center of the ring. These and other observations suggest how conformational changes could be coupled to a DNA unwinding activity. A crystal structure determination of the intact helicase-primase protein is underway.

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A prototype for hexameric DNA helicases: crystal structure of plasmid RSF1010 encoded RepA

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Helicases engaged in DNA replication are ring-shaped hexamers which unwind double stranded DNA (dsDNA) vectorially in 5'→ 3' or in 3' → 5' direction under consumption of nucleoside triphosphates. Two structures of helicase domains have recently been reported: the N-terminal domain of E.coli DnaB (1) and the helicase domain of bacteriophage T7 primase-helicase (2).

Here we describe the first three-dimensional structure of an intact hexameric helicase, RepA, encoded by the plasmid RSF1010 and acting 5'→ 3' on ds DNA, at 2.4 Å resolution. RepA belongs to the family of helicases with 5 conserved motifs (H1, H1a, H2, H3, H4), of which H1 and H2 are comparable to the Walker A and B motifs in F1-ATPase. The monomer is globular with a central 9-stranded β -sheet covered by 7 α -helices. Its N-terminus binds to the adjacent monomer, thereby stabilizing the hexamer. The polypeptide fold of RepA is analogous to RecA (3) with comparable disposition of active site residues Lys43, Glu77, Asp140 and His179. They are located at the C-termini of conserved motifs H1, H1a, H2, H3 which are part of the central β -sheet and formed by β -strands β 1, β 2, β 4, β 5 respectively. In addition, Arg207 on H4 is located in the active site, acting as an "arginine finger" as it is part of the adjacent subunit. The segment 181-200 (part of H4) and the C-terminus, segment 263-279 are disordered and could not be modeled in the electron density. The complex between dATP and the T7 helicase domain (2) served to model ATP into the RepA hexamer. ATP is tucked into clefts between monomers, binding with triphosphate to the active site of one and stacking with adenine on Tyr243 of the adjacent monomer. The hexamer is annular with a ~ 17 Å wide entral hole, indicating that ss DNA but not ds DNA may be threaded and suggesting further studies into the yet unknown functional mechanism of helicases.

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Session 3: Mechanisms of DNA unwinding

Chair: Tom Ellenberger

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 surprisingly suggested 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. It also suggested that the enzyme uses a combination of two processes, duplex destabilisation and DNA translocation, that are coupled to produce helicase activity. In order to investigate this process biochemically, we have 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.

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Steps toward understanding the mechanism of the helicase

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Helicase proteins translocate on nucleic acids and separate the strands of the duplex form of the nucleic acid using NTP hydrolysis as the energy source. Bacteriophage T7 DNA helicase/primase and *E. coli* Rho proteins are members of a class of helicases that assemble into ring shaped hexamers. It has been shown that a single strand of a DNA binds in the central channel of the T7 helicase protein and it has been postulated that a single strand of an RNA both wraps around and binds in the central channel of the Rho protein. We have investigated the kinetic pathway of nucleic acid binding to preformed hexamers of these helicases to understand the mechanisms of nucleic acid binding and initiation of translocation and unwinding. Our studies show that nucleic acid binding is a multistep process in both helicases, and this process involves several intermediates and requires NTP binding but not its hydrolysis. Our studies show that initiation including NTP binding and nucleic acid binding are slow steps and DNA binding in the central channel involves a loading mechanism. After nucleic acid is bound in a competent manner, the six NTP binding sites show cooperativity in NTP binding and hydrolysis that provides a mechanism for sequential nucleic acid binding and release coupled to NTP binding and hydrolysis. The overall mechanism of NTP binding and hydrolysis by these helicases is very similar to the binding change mechanism of the F1-ATPase. We propose based on these studies a general mechanism for translocation of hexamer helicases on the DNA, wherein an F_1-ATPase-like rotational movement around one strand of the nucleic acid leads to unidirectional translocation and unwinding.

Helicase mechanisms and functional coupling in replication and transcription

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Nucleic acid helicases are enzymes that move "through" and separate or re-arrange complementary double-stranded sequences of DNA-DNA, RNA-DNA, or RNA-RNA. These enzymes are ubiquitous and comprise crucial components of macromolecular machines involved in the replication, transcription, recombination, repair, and splicing of the nucleic acids of the cell. Helicases are usually quite specific in terms of the structure and composition of the double-stranded nucleic acid "substrates" on which they will operate, and the overall helicase reaction is generally driven by the binding and hydrolysis of ATP or other nucleoside triphosphates. Helicase mechanisms are often difficult to study in isolation, since the uncoupled passage of a helicase through a double-stranded nucleic acid leaves the nucleic acid substrate unchanged, resulting only in the transformation of a certain amount of ATP into ADP and P_i. As a consequence helicases are often studied in the presence of other components of the relevant macromolecular machines to "couple" the helicase reaction to other cellular processes and permit the isolation of a trapped or changed helicase intermediate or product. Such coupling can also complicate the analysis of helicase reaction mechanisms.

In this talk I will attempt to describe and combine insights into helicase mechanisms from a number of sources, including studies on the hexameric DNA-DNA replication helicase (gp41) coded by bacteriophage T4 and the structurally very similar transcription termination helicase rho of *E. coli*. The helicase-like features of simpler proteins and protein model systems that interact with nucleic acids will also be considered in this context, as will the helicase activity of the transcription elongation complex itself. Such integrated approaches permit us to define some aspects of the steps involved in the helicase reaction cycle and how this cycle is coupled to the binding and hydrolysis of ATP. Some general principles governing the coupling and incorporation of helicase molecules and mechanisms into the overall functioning of macromolecular machines will also be discussed. (This work has been supported in part by NIH Grants GM-29158 and GM-15792, and by an American Cancer Society Research Professorship to PHvH.).

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E. coli DnaB helicase-DNA complexes: mechanism of recognition and structure

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The DnaB protein is the E. coli primary replicative helicase, i.e., the factor responsible for unwinding the duplex DNA in front of the replication fork. The enzyme is an essential replication protein in Escherichia coli which is involved in both the initiation and elongation stages of DNA replication (1-3). In spite of the central importance for our understanding of DNA replication processes, the mechanism of the action of the DnaB helicase, and the structural bases of the functioning of the enzyme are just recently being unraveled.

Quantitative studies of nucleotide binding to the DnaB helicase have established that the hexamer has six nucleotide binding sites, presumably one on each protomer (3-5). On the basis of thermodynamically rigorous fluorescence titrations, we determined that the binding process is biphasic, with the first three nucleotide molecules binding in the high affinity step, and the next three nucleotides binding in the low affinity step. The biphasic behavior results from the negative cooperative interactions among binding sites (4-7).

Analytical studies show that the DnaB helicase exists as a stable hexamer in a large protein concentration range stabilized specifically by multiple magnesium cations (8-10. The six protomers of the hexamer aggregate with cyclic symmetry in which the protomer-protomer contacts are limited to only two neighboring subunits (8-11). Negative cooperativity in nucleotide binding provides the first indication of the communication between the hexamer subunits. On the other hand, hydrodynamic and E. M. studies also provide direct evidence of the presence of long-range allosteric interactions in the hexamer, encompassing all six subunits (8-12).

Using the quantitative fluorescence titration method we determined the stoichiometry of the DnaB helicase - ssDNA complex and the mechanism of binding (9,10,13-15). In the complex with the ssDNA, the DnaB helicase binds the nucleic acid with a stoichiometry of 20 ± 3 nucleotides per DnaB hexamer. The DnaB hexamer has an effective single, binding site for the ssDNA, although potentially each of the six chemically indentical subunits can bind nucleic acids. Photo-cross-linking experiments indicate that the ssDNA binding site is located predominately on a single subunit of the hexamer (9,10,12-14). Studies of the enzyme-ssDNA complexes reveal large structural heterogeneity within the total ssDNA binding site. The total site is built of two subsites both occluding ~10 nucleotides. The ssDNA affinity for the strong subsite is ~3 orders of magnitude higher than that for the weak subsite (16).

Our data show that, in the complex with the replication fork DNA substrates, the DnaB helicase preferentially binds to the 5' arm of the fork (14). The 3' arm does not form a stable complex with the DnaB hexamer associated with the 5' arm, and the 3' arm is in a conformation which makes it accessible for the binding of another DnaB hexamer. Moreover, the duplex part of the fork substrate does not significantly contribute to the free energy of binding (10,11). The structure of the complex of the E. coli primary replicative helicase DnaB protein with ssDNA and replication fork substrates has been examined using the

fluorescence energy transfer method (16,17). We provide evidence that, in the complex with the enzyme, the ssDNA passes through the inner channel of the DnaB hexamer. In the stationary complex, the helicase does not invade the duplex part of the fork beyond the first 2 - 3 base pairs.

Dynamics of the DnaB protein binding to a ssDNA have been examined, using the fluorescence stopped-flow technique. Association of the DnaB helicase with the ssDNA occurs by the minimum three-step mechanism, where the bimolecular binding step is follow by two isomerization steps. These results indicate that the first step in the association reaction includes the local opening of the protein hexamer. The sequential nature of the mechanism indicates the lack of kinetically significant conformational equilibrium of the DnaB hexamer, as well as a transient dissociation of the hexamer prior to the ssDNA binding.

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Structure and Strand Separation Mechanism of the Hepatitis C Virus NS3 Helicase

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The structure of the Hepatitis C virus NS3 RNA helicase domain complexed with a single-stranded DNA oligonucleotide has been solved by Vertex Pharmaceuticals to 2.2 Å resolution (Kim et al., 1998 Structure 6: 89-100). The NS3 helicase consists of three structural domains. The first two domains have an adenylate kinase like fold, including a phosphate-binding loop in the first domain. The DNA oligonucleotide resides in the groove between the first two domains and the third. The observed orientation of the bound oligonucleotide is roughly perpendicular to the orientation proposed by two other groups, based on their modeling analyses of X-ray structures of apo HCV helicase.

Based on our unique structural findings, we have proposed a mechanistic model for HCV helicase catalyzed DNA/RNA duplex unwinding. The model proposes five salient features: 1) the 3'-single-stranded tail of a duplex nucleic substrate binds in the groove that separates domain 3 from domains 1 and 2; 2) ATP molecules bind to the HCV helicase through interactions with motifs I and II on domain 1; 3) the binding of ATP results in the closure of the cleft between domains 1 and 2 driven by ATP interactions with motif VI in domain 2; 4) simultaneously the closure of the ATP binding pocket leads to a translocation of domains 1 and 3 in a 3' to 5' direction along the bound nucleic acid strand; 5) key amino acid residues in the RNA binding channel (e.g. Trp-501) act as "gatekeepers" to prevent the bound polynucleotide strand from slipping in the opposite direction, resulting in a net 3' to 5' unidirectional movement.

We have used site-directed mutagenesis to investigate the roles of amino acid residues that are defined in our model as directly involved in polynucleotide binding, ATP binding or the coupling of ATP binding, and subsequent hydrolysis, to the processing of duplex RNA (Lin et al., In Press). Results from these mutagenesis studies invariably support our model for duplex unwinding, derived from the structure of the NS3 helicase:oligonucleotide complex.

Session 4: Helicases in chromosomal and phage replication

Chair: Roger McMacken

Mechanisms of DNA unwinding by *E. coli* Rep and UvrD helicases

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Our laboratory has been studying the mechanistic details of DNA binding, ATP binding/hydrolysis and DNA unwinding by the *E. coli* DNA helicases, Rep and UvrD (Helicase II), both 3' to 5' helicases and members of the SF-1 superfamily (1). Biochemical studies of DNA binding and ATP hydrolysis have implicated oligomeric forms of Rep and UvrD as important for helicase function, with such oligomeric forms thought to provide the functional helicase with multiple potential DNA and ATP binding sites (2). For example allosteric effects of nucleotides on DNA binding are observed within Rep dimers (3) and ATP hydrolysis stimulates the rate of ss-DNA exchange between subunits of Rep dimers (4). The two ATP sites within a Rep dimer also communicate (5;6). These observations have led to the proposal that unwinding by Rep dimers involves a mechanism in which subunits alternate binding to duplex DNA vs. single stranded DNA and unwinding occurs by an active mechanism in which the helicase binds to the duplex and unwinds multiple base pairs (3). UvrD, which shares ~40% sequence similarity with Rep also oligomerizes (7).

We have developed rapid kinetic methods to allow pre-steady state single turnover studies of DNA unwinding by Rep and UvrD using short oligodeoxynucleotide substrates (8;9) and have used these methods to investigate the mechanism of DNA unwinding by these helicases. These methods have enabled us to estimate the unwinding step-size for Rep, UvrD as well as for the *E. coli* RecBCD helicase. Although each helicase unwinds duplex DNA with very different rates, we measure similar step-sizes of 4-5 base pairs (8), suggesting that some aspects of the mechanisms of unwinding are the same for these helicases. We will discuss our recent rapid kinetic studies of DNA unwinding by these helicases that have led to insights into their mechanisms of DNA unwinding, including studies showing that oligomeric forms of Rep and UvrD are required for optimal helicase activity.

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Action of DnaB at the replication fork

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Interactions between DnaB, the replication fork DNA helicase, and other replication proteins are defining to both the architecture and action of the replisome. The core of the replisome is established by a protein-protein interaction between the τ subunit of the DNA polymerase III holoenzyme, the replicase, and DnaB. This interaction stimulates the DNA unwinding activity of DnaB by some 15- to 20-fold, allowing replication fork progression to proceed at nearly 1000 nt/sec (1) and determines which of the two polymerase cores (the heterotrimer of the α, ε, and θ subunits of the holoenzyme) becomes the leading-strand polymerase (2, 3). The transient association between DnaB and DnaG, the Okazaki fragment primase, is crucial for bringing the primase to the replication fork and also regulates the size of the nascent lagging-strand fragments (4, 5, 6). We have been probing the functions of these interactions by using mutant DnaB proteins that carry site-directed amino acid substitutions. The biochemical phenotypes of several interesting mutant proteins will be discussed.

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Loading of DnaB helicase at the λ replication origin

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The *E. coli* DnaB replicative DNA helicase, acting in conjunction with DNA polymerase III holoenzyme, is responsible for the rapid and processive replication of the bacteriophage λ genome (1). Studies of a 10-protein system that supports the initiation of λ DNA replication have identified the key steps required for establishment of the replication fork apparatus (2-4). The first step in this complex process is the binding of multiple copies of the λ O initiator protein to repeating sequences at the λ replication origin (*ori*λ), followed by self-assembly reactions that create a nucleosome-like structure, termed the O-some. λ P replication protein binds to each subunit of the hexameric DnaB helicase, displacing the host DnaC protein (5), and the resulting P•DnaB complex interacts strongly with the O-some to form a stable *ori*λ:O•P•DnaB second-stage nucleoprotein structure. Release of DnaB from its tight associations in this structure and initiation of DNA unwinding requires partial disassembly of the complex via the action of the *E. coli* DnaJ/DnaK/GrpE molecular chaperone system.

Our recent efforts have focused on improving our understanding of preinitiation complexes and of how the λ O and P replication initiators facilitate the initiation of DNA unwinding by DnaB. Using radiolabeled proteins and *ori*λ DNA templates, we have found that the typical *ori*λ:O•P•DnaB complex contains a single DnaB helicase molecule. Complexes containing two DnaB molecules are also present, but never represent the majority species even at excessive concentrations of P and DnaB.

Initiation of λ DNA replication is absolutely dependent on DNA supercoiling. Investigations of the molecular basis for this requirement have led to the surprising discovery that the second-stage preinitiation complex, the *ori*λ:O•P•DnaB nucleoprotein structure, exists in two separate states. The complex exists in a functionally inert form when the *ori*λ template DNA is relaxed (6). However, when sufficient negative superhelical tension is introduced into the template, the *ori*λ:O•P•DnaB complex is converted into an activated state. The activated state is distinguished by the capacity of the complex to trap the energy of DNA supercoiling. Thus, once the *ori*λ:O•P•DnaB complex is activated, its replication potential can survive for considerable periods after all superhelical tension is removed from the DNA template. Footprinting of activated *ori*λ:O•P•DnaB nucleoprotein structures with potassium permanganate revealed that the activated state of the complex is closely associated with the presence of an altered DNA structure in the *ori*λ A+T-rich region. Our data indicates that the 40 bp A+T-rich region adopts a non-B DNA structure with partial single-stranded character, but apparently is not fully unwound (melted) as concluded previously (7). The stabilities of both the activated state of the *ori*λ:O•P•DnaB complex and the non-B DNA structure are identical. Moreover, both are stabilized significantly and equivalently by DNA superhelical

tension. For example, introduction of a supercoil density of about - 0.045 into a relaxed, circular *ori*λ DNA template extends the half-lives of both the non-B DNA structure as well as the activated *ori*λ:O•P•DnaB complex from about 50 seconds to more than 35 minutes. We infer from the stabilizing effect of negative superhelical tension that both the activated state of this preinitiation complex and the non-B DNA structure are manifestations of a key replication intermediate involved with opening of the DNA duplex at the λ origin. Formation and maintenance of the activated *ori*λ:O•P•DnaB complex is ATP independent, which strongly suggests that DnaB helicase does not participate directly in the DNA-opening reactions at this stage. We have previously demonstrated that λ P initiator protein and its homologue, *E. coli* DnaC protein, each contains intrinsic, but cryptic, single-stranded-DNA (ssDNA) binding activities that are revealed when these proteins form a complex with DnaB (8). Additionally, the C-terminal domain of the λ O protein was found to contain a similar cryptic ssDNA-binding activity, which we find is elicited by interactions of O with the P•DnaB complex. Taken together, our observations suggest an initiation model in which the ssDNA-binding activities of O and P play an integral role in the activated *ori*λ:O•P•DnaB complex by trapping and holding nascent non-B DNA structures with partial ssDNA character that are formed when sufficient superhelical tension is present. This results in the formation of a "pre-open" complex at the origin which prepares the DNA template for the subsequent loading and entry of a hexamer of DnaB helicase.

Using short ssDNA fragments as a model system, we have recently discovered that transfer of DnaB helicase onto ssDNA templates via the λ O and P initiators, requires the C-terminal domain of O protein, but not its N-terminal origin-recognition domain. Transfer of DnaB onto the DNA template occurs rapidly and efficiently in *cis* following partial disassembly of a ssDNA:O•P•DnaB nucleoprotein structure by the DnaJ/DnaK/GrpE molecular chaperone system. Finally, we have identified and mutated a ssDNA-binding site in λ P. The mutant P protein fails to support the initiation of λ DNA replication even though it retains a normal capacity to form a complex with DnaB helicase. Replication reactions containing this mutant protein appear to be blocked at the step involving activation of *ori*λ:O•P•DnaB nucleoprotein structures.

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The *Bacillus subtilis* bacteriophage SPP1 G*39*P delivers and activates the G*40*P DNA helicase upon interacting with the G*38*P-bound replication origin

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Initiation of *B. subtilis* bacteriophage SPP1 replication requires the phage-encoded genes *38*, *39* and *40* products (G*38*P, G*39*P and G*40*P)[1]. G*39*P, which does not bind DNA, interacts with the replisome organiser, G*38*P, in the absence of ATP[2] and with the ATP-activated hexameric replication fork helicase, G*40*P[3]. G*38*P, which specifically interacts with the phage replication origin (*oriL*) DNA, does not seem to form a stable complex with G*40*P in solution[4]. G*39*P when complexed with G*40*P-ATP inactives the ssDNA binding, ATPase and unwinding activities of G*40*P and such effect is reversed by increasing amounts of G*38*P. Unwinding of a forked substrate by G*40*P-ATP is increased about 10-fold by the addition of G*38*P and G*39*P to the reaction mixture. The specific protein-protein interactions between *oriL*-bound G*38*P and the G*39*P-G*40*P-ATPγS complex are necessary for helicase delivery to the SPP1 replication origin. Formation of G*38*P-G*39*P heterodimers release G*40*P-ATPγS from the unstable *oriL*-G*38*P-G*39*P-G*40*P-ATPγS intermediate. G*40*P-ATPγS binds to the origin region, the uncomplexed G*38*P fraction remains bound to *oriL*, and the G*38*P-G*39*P heterodimer is lost from the complex. The results presented provide the first direct evidence of the delivery of a replication fork helicase of a Gram-positive bacterium near a replisome organizer bound to the replication origin without the need of any other factor (e.g., chaperone proteins, ATP-activation and ATP-hydrolysis by the origin binding protein, G*38*P, or the helicase loader, G*39*P).

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DnaA, DnaB and *oriC*: the complex for chromosomal initiation in bacteria

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The initiator protein DnaA binds to an asymmetric 9 bp binding site, the DnaA box. Replication origins have different structures and different numbers of DnaA boxes, e.g. 5 for *E. coli,* 12 for *T. thermophilus*, and 15 for *B. subtilis*. Nevertheless, all origins have an AT-rich region, and the sequence arrangement close to this region is similar in these origins. A special nucleoprotein structure is formed between DnaA and *oriC* that results in local unwinding of the AT-rich region, providing an entry site for DnaB helicase. The structural requirements for the unwinding reaction will be discussed.

DnaA protein has a modular structure with a conserved N-terminal domain. This is linked via a flexible loop to the central domain 3 that binds ATP. The C-terminal domain 4 is responsible for specific DNA binding. The N-terminal domain 1 (amino acid residues 1-86) is responsible for different protein-protein interactions. Aminoacids 1-76 are involved in DnaA-DnaA oligomerization. Oligomerization of DnaA is essential for open complex formation. DnaA-DnaA interactions resulting in the formation of DNA loops require aminoacids 1-86 and the DNA-binding domain, but not the central domain 3. Domain 1 is also involved in the interaction of DnaA with DnaB. However, for interaction with DnaB the N-terminal aminoacids 1-23 are dismissible. Interaction with DnaB can occur with nucleotide-free DnaA, but DnaB-helicase loading requires ATP- or ADP-DnaA.

The central domain 3 shows an open-twisted α/β-structure with a P-loop for nucleotide binding. ATP-DnaA, but not ADP-DnaA, binds to a 6 bp consensus sequence, ATP-DnaA box, in addition to binding to regular DnaA boxes. Several such sites are found in the AT-rich 13mers of *E. coli oriC*.

Session 5: Termination and regulation of replication by helicase inhibition

Chair: Juan C. Alonso

Interaction of replication initiator and terminator proteins with replicative helicase DnaB

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The interaction of three initiator proteins, namely DnaA, Pi and RepA of Escherichia coli and its plasmids and the replication terminator protein (RTP) *of B. subtilis*, with DnaB helicase of E. coli were investigated. The three replication initiator proteins recognize adjacent but distinct regions of DnaB and the interaction is absolutely essential for loading of the helicase to the replication origin. The plasmid encoded initiator proteins also interact with the host encoded DnaA and mutations that disrupt the interaction also block helicase loading. It appears that the interaction of DnaA with the plasmid encoded initiators is needed for optimal origin melting and thus indirectly also for DnaB loading.

The interaction of RTP with DnaB has been investigated by mutagenesis of RTP and by using variant forms of replication termini and by the use of crystallography and of a variety of DNA-protein and protein-protein cross linkers crosslinkers. It appears that in addition to RTP-Ter DNA interaction, DnaB-RTP interaction appears to be needed for replication fork blockage.

A genetic analysis of the funtional interactions between the bacterial toxin Kid and the DnaB helicase

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Kid is a bacterial toxin coded in the *parD* (*kis, kid*) system of plasmid R1 (1 and 2). This system belongs to the group of proteic toxin-antidote systems present in many bacterial plasmid and genomes (3). Biochemical analysis indicated that the purified preparations of the Kid toxin inhibit DnaB dependent initiation of plasmid DNA replication. Together with the observation that a plasmid that moderately overproduces DnaB can prevent the toxicity associated with this protein (4), this suggests that the Kid protein acts at the DnaB level. To further define the functional interaction between both proteins, we now report the identification of mutants of the DnaB protein that fail to neutralise the toxicity of Kid as well as the isolation and characterisation of mutants of the Kid protein that prevent or attenuate the toxicity of this protein.

A mutational analysis was carried out to define possible amino-acid residues of DnaB involved in the neutralisation of the toxicity of Kid. For this purpose a pBR322-dnaB recombinant was treated with hydroxyl-amine and transformed in OV2, a *supFts* strain, carrying pAB1120, which contains an amber mutation in the antidote of the *parD* system (the Kis protein). Inactivation of the SupF tRNA at high temperature result in an inactive antidote. This releases the action of the Kid toxin. *dnaB* mutants that fail to neutralize this toxicity should show a thermosensitive phenotype in this background. Analysis of nine *dnaB* mutants obtained in this way indicated that aminoacid residues of the protein that are spread in a wide C-terminal region, could play a role in neutralising the toxicity of Kid.

A mutational analysis was also carried out to define possible residues of Kid involved in the toxicity of this protein. Mutations that inactivate this toxicity were induced *in vitro* with hydroxyl-amine in the *parD* mutant plasmid pAB1120 and selected in OVG2 because they allow the growth of the cells at high temperature. Analysis of thirty such mutants, indicated

that the missense mutations found, affect aminoacids located in two two discrete regions of the Kid protein. Further analysis indicated that some of these mutations affected the auto-regulation of the *parD* system and were located in the same two regions. *parD* regulation is modulated by a complex of the Kid toxin and the Kis antidote (4,5) and therefore these data suggest a partial overlap of residues of Kid involved in interactions with its target and with its antidote.

The results obtained suggest that two regions of the Kid protein interact functionally, both with its antidote and with residues located at the C-terminal domain of DnaB. This DnaB helicase region is involved in binding to SS DNA, in ATP binding and in protein oligomerisation (6,7,8). Further characterisation of the DnaB mutants aiming to evaluate their activity and also to understand functional interactions between the Kid and DnaB proteins, will be presented and discussed.

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Plasmid and phage-encoded DNA helicases

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The RepA protein of the mobilizable broad host range plasmid RSF1010 has a key role in its replication. The replication of RSF1010 is independent of the host's replication initiation machinery of *E. coli*. This includes RNA polymerase, proteins DnaA, DnaB, DnaC, DnaG, and DnaT. RSF1010 replication proceeds asymmetrically from the vegetative origin *(oriV)*, each of the strands is copied continuously. The RepA protein, composed of 278 amino acid residues, is one of the smallest known replicative helicases. The protein forms a homohexamer of 29,869-Da subunits. A variety of methods were used to analyze the quarternary structure of RepA. Gel filtration and cross-linking experiments demonstrated the hexameric structure, which was confirmed by electron microscopy and image reconstruction (Scherzinger et al., 1997). These results agree with recent data obtained from X-ray crystallography (Röleke et al. 1997, and Abstract of W. Saenger). The RepA helicase unwinds double-stranded DNA with 5' to 3' polarity. As do most true replicative helicases, RepA prefers a tailed substrate with an unpaired 3'-tail mimicking a replication fork. Optimal unwinding activity was achieved at the remarkably low pH of 5.5. In the presence of Mg^{2+} (Mn^{2+}) ions, the RepA activity is fueled by ATP, dATP, GTP, and dGTP and less efficiently by CTP and dCTP. UTP and dTTP are pure effectors. Nonhydrolyzable ATP analogues, ADP, and pyrophosphate inhibit the helicase activity, whereas inorganic phosphate does not. The presence of *E. coli* single-stranded DNA-binding protein stimulates unwinding at physiological pH 2-3-fold, whereas the RSF1010 replicon specific primase RepB' protein has no effect, either in the presence or absence of single-stranded DNA-binding protein. Site-directed mutagenesis has been used to analyze the functional importance of defined residues in RepA *in vivo* and *in vitro*.

The phage P1 Ban protein is a hexameric replicative helicase that suppresses the thermosensitive phenotype of *E. coli dnaB* mutations by forming heterooligomers with DnaB protein. Its primary structure aligns with at least 44 sequences of proposed DnaB-like proteins indicating the existance of at least eight new well conserved motifs.

The multifunctional α protein of *E. coli* satellite phage P4 has four different activities, *ori1* recognition and binding ability, primase activity, helicase activity and it interacts with the copy number regulation protein (Cnr). Unwinding of DNA occurs in 3' to 5' polarity. The protein probably forms dimers in solution, hexamers have never been observed under various conditions (Ziegelin et al., 1997). The mutagenesis project was supported by EU grant BIO4-CT98-0106; the phage P4 project was supported by a grant of the Deutsche Forschungsgemeinschaft.

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DnaB from *Thermus aquaticus* Unwinds Forked Duplex DNA with an Asymmetric Tail-Length Dependence and 5'- to 3'-Polarity

DnaB helicase is a ring-shaped hexamer of ~300 kDa that is essential for replication of the bacterial chromosome. The *dnaB* gene was cloned from *Thermus aquaticus* and the gene product was purified to homogeneity. An unwinding assay was developed using a forked-duplex DNA substrate. Under conditions of single enzymatic turnover, the lengths of the 5'- and 3'- single stranded regions were varied, and 6-10 nucleotides of the 5'-single-stranded tail and 21-30 nucleotides of the 3'-single-stranded tail markedly stimulated the unwinding rate. Unwinding rates were also measured using oligonucleotides of mixed polarity. *T. aquaticus* DnaB unwound forked duplex DNA containing two 5' single stranded regions at an equal rate compared to a standard substrate. By contrast, the enzyme did not unwind forked duplex DNA containing two 3'- single-stranded tails. These data confirm that the protein unwinds DNA with 5'- to 3'- polarity. The data are also consistent with a model in which the 5'-single stranded tail regions passes through the central hole of the DnaB ring, and the 3'-tail passes outside of the ring. Crystallization studies are underway.

Functional Recruitment of Nuclear DNA Helicase II

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Nuclear DNA helicase II (NDH II), alternatively named RNA helicase A (RHA), unwinds both dsDNA and dsRNA in a reaction that requires a nucleotide cofactor and a divalent cation (Mg^{++}) (1-2). Molecular cloning of NDH II revealed motifs that are homologous to those found within the DEXD/H helicase superfamily (3-4). In addition to these helicase motifs in the center of the enzyme, NDH II contains auxiliary domains which may influence the affinity and specificity of substrate binding. These include two dsRNA binding domains (dsRBD) at the N-terminus and an RGG-box for single-stranded nucleic acid binding at the C-terminus (5). NDH II is highly conserved among higher eukaryotes, including *Drosophila*, *Caenorhabditis elegans*, mouse, cow and humans. An involvement of the NDH II homolog from *Drosophila*, i.e. the *maleless* (MLE) protein, in the enhancement of X-chromosomal transcription in males for the dosage compensation of X-chromosomal genes suggests a physiological role in transcription (6). In this case MLE has been shown by immunofluorescence at hundreds of discrete sites along the only male X-chromosome. In mammalian cells NDH II generally displays nucleoplasmic localization, but murine NDH II accumulates abundantly in the nucleolus in addition to a diffuse distribution in the nucleoplasm. The preferred nucleolar localization of murine NDH II allowed a morphological distinction of its association to specialized regions, such as the dense fibrillar components of the nucleolus, where active transcription of ribosomal genes takes place. Upon inhibition of transcription, e.g. by actinomycin D, DRB or heat shock, NDH II became dispersed in the nucleoplasm and partially diffused into the cytoplasm, suggesting that the steady subnuclear localization of NDH II is highly related to an on-going transcription activity. The dynamic change of the cellular localization of NDH II could also be correlated to the fluctuation of transcription during mitosis, where NDH II dissociated from ribosomal genes in prophase, just when ribosomal transcription activity was shut off. In telophase the protein returned to the ribosomal transcription sites, where RNA synthesis resumed increasingly with the decondensation of chromosomes. These results provide a scenario for the transcription-dependent recruitment of NDH II to its cellular nucleic acid targets, which may be directed by protein-nucleic acid interaction and/or protein-protein interaction in living cells.

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Helicases Displace Streptavidin from Biotin-Labeled, Single-stranded Oligonucleotides with a Directional Bias

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Translocation of enzymes along nucleic acids is a fundamental biochemical process necessary for replication, transcription, recombination, and repair. A new assay was developed for studying translocation of helicases on oligonucleotide substrates. The rate of dissociation of streptavidin from various biotinylated oligonucleotides was determined in the presence of helicase using an electrophoretic mobility shift assay. Thus far, three different helicases have been found to be able to significantly enhance the dissociation rate of streptavidin from biotin-labeled oligonucleotides in an ATP dependent reaction. For the bacteriophage T4 helicase, gp41, helicase-catalyzed dissociation of streptavidin from the 3'-end of a biotin-labeled 62-mer oligonucleotide occurred with a first order rate of 0.17 min^{-1}, which is over 500-fold faster than the spontaneous dissociation rate of biotin from streptavidin. Another bacteriophage T4 helicase, Dda, provides even faster displacement of streptavidin from the 3' end of the 62-mer. A first order rate of 7.9 s^{-1} was measured which is more than one million-fold greater than the spontaneous dissociation rate. There was no enhancement of streptavidin dissociation from the 5'-biotin-labeled oligonucleotide by either helicase. The difference in displacement between the 5' and 3'-ends serves to support the existing evidence for a 5'-to-3' directional bias in translocation on ssDNA for each helicase. In constrast, the SV40 Large T antigen is a 3'-to-5' helicase, and we find that this enzyme displaces streptavidin only from the 5' end of the oligonucleotide. Hence, these results suggest that helicases can use the energy derived from ATP hydrolysis to fuel translocation on single-stranded DNA with a strong directional bias.

Session 6: Helicases in replication-related processes, recombination and repair

Chair: Robert G. Lloyd

The Saccharomyces Pif1p and Rrm3p helicases have antagonistic effects on replication of ribosomal DNA

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Pif1p and Rrm3p are members of a subfamily of DNA helicases that is conserved from yeast to humans. These helicases affect the maintenance of telomeric, mitochondrial, and ribosomal DNA in baker's yeast. Unexpectedly, given their high degree of sequence similarity, the helicases have opposing effects on each of these DNAs. In my talk, I will focus on the role of these helicases in the ribosomal DNA (rDNA). Yeast rDNA consists of ~150 tandemly repeated 9 kb units. Replication begins and proceeds bi-directionally from origins of replication in a subset of these repeats.

However, progress of the leftward moving fork stops when it encounters the replication fork barrier (RFB), a polar block to replication fork progression, such that replication of rDNA is largely unidirectional. At the end of replication, the rightward moving and stalled forks converge at the RFB. This pattern of unidirectional replication of rDNA is widespread, seen in plants and mammals as well as in other single celled organisms. Using neutral-neutral two dimensional gel electrophoresis, we found that Pif1p and Rrm3p both affected the pattern of replication intermediates in the rDNA. Pif1p was needed for efficient maintenance of the RFB whereas Rrm3p was needed for fork progression throughout the rDNA and especially to resolve forks converged at the RFB. RRM3 alleles with point mutations in the ATP binding pocket that eliminate helicase activity in other helicases had the same phenotype as a deletion allele of RRM3, indicating that Rrm3p likely affects rDNA by virtue of its putative helicase function. In addition, Rrm3p inhibited and Pif1p promoted recombinational generation of rDNA circles, which we suggest is a secondary consequence of their effects on fork pausing. Chromatin immuno-precipitation demonstrated that Rrm3p and Pif1p are associated with rDNA chromatin in vivo, indicating that their effects on rDNA replication are likely to be direct. We found that SGS1, which encodes a RecQ subfamily DNA helicase, is synthetically lethal with RRM3. Our data suggest that Rrm3p and Sgs1p, two helicases that have no similarity to each other by the criterion of a BLAST search, cooperate in a late stage in rDNA replication whereas Rrm3p and Pif1p, two highly related helicases, have opposing effects on rDNA replication.

DNA helicase I: a helicase and a transesterase involved in bacterial conjugation

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Escherichia coli DNA helicase I (TraIp) is an F plasmid-encoded protein required for conjugative DNA transfer of the F plasmid from a donor to a recipient bacterium (1-3). The purified protein catalyzes a sequence-specific DNA transesterase reaction that provides the site- and strand-specific nick required to initiate DNA transfer (4,5) and a processive 5' to 3' helicase reaction (6) that presumably unwinds the F plasmid during DNA transfer. Sequence comparisons with other transesterases and helicases suggests that these activities reside in the amino- and carboxy-terminal regions of TraI, respectively. Computer-assisted secondary structure probability analysis identified a potential interdomain region spanning residues 305-309. Proteins encoded by segments of *traI*, whose N- or C-termini either flanked or coincided with this region, were expressed, purified, and assessed for catalytic activity. Amino acids 1-306 (N306) embody the transesterase activity, while amino acids 309-1756 (309C) constitute the helicase activity. Surprisingly, 309C was the minimal active DNA helicase; removal of an additional 40 amino acids abolished helicase activity. This is remarkable since the first helicase-associated motif (helicase motif I) is located approximately 650 amino acids further toward the C-terminal end of the protein. The region of the protein from position 306 to position 990 shares no homology with other helicases and has no known role. Genetic experiments using constructs designed to express either the transesterase domain or the helicase domain have shown that both domains of the protein are required for conjugative DNA transfer. In addition, we have made a point mutation in TraIp (K995M) that alters the essential lysine in helicase motif I to methionine. This protein, which catalyzes a transesterase reaction but lacks both helicase and ATPase activity, fails to support conjugative transfer of the F plasmid. Thus, transfer-associated unwinding of the F plasmid requires the TraIp helicase activity.

TraIp is an integral part of the F plasmid conjugative initiation complex which assembles at the origin of transfer (*oriT*) to initiate conjugative DNA transfer. The other known components of this complex, F-encoded TraYp and host-encoded IHF, are believed to modulate chromatin architecture by site-specific binding at *oriT*. In vitro studies have shown the *oriT*-dependent transesterase activity of native TraIp is stimulated in the presence of both IHF and TraYp, but not if one or the other is omitted (7). Using purified and catalytically active segments of TraIp, we have examined the contributions of the transesterase domain and/or the helicase domain to the formation of a functional initiation complex at *oriT*. We found IHF to be a potent inhibitor of *oriT* nicking by the N-terminal transesterase segment of TraIp, N306. Addition of TraYp did not relieve the inhibition. Stimulation of N306 transesterase activity, comparable to that achieved using native TraIp, was only observed in the presence of IHF, TraYp, and the catalytically active 309C helicase domain. 348C could not functionally substitute for 309C in this respect. The observation that IHF conditionally inhibits and stimulates functional initiation at *oriT* suggested a more complex role for this protein than had previously been supposed. Sequence analysis of the *nic*-proximal region of

oriT revealed the potential for two mutually exclusive and inverted IHF binding sites distinct from the previously assigned IHF binding site. These results have been considered in the context of previously published reports regarding protein-DNA interactions at *oriT* (8), as well as IHF in general (9), and the recent IHF-DNA crystal structure (10). A model will be discussed in which IHF is proposed to function as binary molecular switch that conditionally regulates the initiation of DNA transfer at *oriT*.

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RecG and PriA helicases underpin replication fork progression

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DNA replication complexes assembled at origins of replication rarely reach the terminus unhindered. They often stall, and may collapse to create new DNA ends that provoke recombination. If illegitimate, this recombination may prove harmful. However, fork collapse may also provide an efficient pathway for resuming replication. This apparent paradox arises from the ability of DNA ends to provoke recombination, and of strand exchange intermediates (D-loops) formed during this recombination to be targeted by replication proteins, at least in bacteria. The *E. coli* pathway emerged from the discovery that damage to DNA stimulates the initiation of replication at sites other than *oriC* by a mechanism that relies on the RecA and RecBCD recombination proteins, and on the primosome assembly factor PriA, but not on the normal replication initiator protein, DnaA.

A replicating cell may have to strike a balance between avoiding collapse of forks which could provoke mutagenic recombination events and promoting collapse of forks stalled at lesions in the DNA in order to promote replication by recombination. This balance is likely to be critical in rapidly growing cells when there is a heavy demand on both replication and transcription.RNA polymerase, like most DNA polymerases, can be blocked by many lesions that arise in DNA during normal metabolism. Unless removed, stalled RNA polymerase may block replication fork progression. We investigated this possibility by monitoring the effect on DNA repair of the stringent response regulators, (p)ppGpp, which modulate RNA polymerase.

We have shown that (p)ppGpp play a crucial role in promoting survival of UV-irradiated cells, probably by removing stalled RNA polymerase blocks to replication fork progression. Secondly we have demonstrated that blocked replication forks form Holliday junctions and that, although such junctions can be subsequently resolved by RuvABC, this enzyme cannot catalyse their formation. Thirdly, we have shown that RecG helicase catalyses the coupled unwinding of the leading and lagging strands of a fork structure to actively generate a Holliday junction, and demonstrate that RecG does this *in vivo*. Finally, we show that this novel RecG activity provides an important pathway for resuming replication that does not rely on cleavage of a Holliday junction formed from a stalled replication fork, thus avoiding the potential dangers of recombination. We propose that the formation of a Holliday junction from a stalled replication fork may provide a universal mechanism for promoting replication fork progression on an imperfect template.

Characterization of functional domains of the plum pox potyvirus CI RNA helicase

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Both experimental evidence and sequence data analysis indicate the existence of sequences potentially encoding helicases in a larger number of virus genomes. In particular, most of the plus strand RNA virus genomes encode at least one putative helicase protein. RNA helicase activity in an RNA virus protein was first demonstrated in the plum pox potyvirus (PPV) CI protein. This activity has also been found to be associated with other potyvirus CI proteins and with NS3 proteins of animal viruses of the family *Flaviviridae*, all of them belonging to the SF2 superfamily of helicase-like proteins. In addition, the nsP2 protein of Semliki Forest virus, a member of the SF1 superfamily, has also been shown to have RNA helicase activity.

The potyvirus CI protein aggregates in the cytoplasm of infected cells forming typical cylindrical inclusions, and is known to be involved in RNA replication and cell-to-cell spread. Self interaction of CI protein has been demonstrated to take place in the yeast Two hybrid system, and the domain involved in this interaction has been mapped at the N-terminal region of the protein. On the other hand, glycerol gradient centrifugation has shown different forms of aggregation of a fusion product of CI with the maltose binding protein (MBP-CI). Although the monomer has RNA helicase activity, aggregated forms showed higher RNA helicase/NTPase ratios. We have shown by deletion analysis that the 103 C-terminal amino acids of the protein are not required for RNA helicase activity. However, other deletions in the C-terminal part of the protein, although leaving intact all the region conserved in RNA helicases, drastically impaired the ability to unwind dsRNA and to hydrolyze NTPs. Two regions of the protein able to interact *in vitro* with RNA have been identified. Site-directed mutagenesis analysis have demonstrated that motif V and VI of the CI protein are involved in NTP hydrolysis, and that RNA helicase activity is essential for virus RNA replication. Our results are consistent with the X-ray crystallographic data of other RNA helicases of the superfamilies SF1 and SF2 that seem to indicate that the different conserved helicase motifs are closely connected in the tertiary structure of the protein, and that they may form a large functional domain, rather than several individual ones with strictly independent functions.

Translocation mechanism and recombination function of DNA helicases

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Escherichia coli RecQ helicase is a multifunctional helicase with homologues that include the *Saccharomyces cerevisiae* Sgs1 helicase, and the *Homo sapiens* Wrn and Blm helicases. In a coupled *in vitro* system, RecQ helicase permits the RecA- and SSB protein-dependent formation of homologously paired joint molecules between fully duplex DNA. The RecQ helicase initiates homologous pairing by unwinding a linear dsDNA substrate to produce ssDNA (that is first trapped by SSB protein) for use by RecA protein. Thus, RecQ helicase can act to initiate homologous recombination. In addition, RecQ protein unwinds a wide variety of DNA substrates, including joint molecules formed by RecA protein. Thus, RecQ helicase has two cellular functions: it can be 1) an initiator of homologous recombination or 2) a disrupter of joint molecules formed by aberrant recombination.

Second, we determined that RecQ helicase unwinds covalently-closed double-stranded DNA (dsDNA), and that this activity specifically stimulates *E. coli* topoisomerase III (Topo III) to fully catenate dsDNA molecules. Catenation is stimulated by SSB protein and requires ATP-turnover by RecQ helicase; inhibition of RecQ helicase results in complete decatenation by Topo III. We propose that these proteins functionally interact, and that their shared activity is responsible for control of DNA recombination. RecQ helicase has a comparable effect on the Topo III homologue of *S. cerevisiae*, consistent with other RecQ- and Topo III-homologues acting together in a similar capacity. These findings highlight a novel, conserved activity that offers insight into the function of the other RecQ-like helicases and that suggests helicase-topoisomerase combinations may also be important for control of recombination and decatenation of chromosomes in eukaryotic cells.

Finally, we determined the translocation step size of the RecBC helicase. The RecBC helicase is a processive DNA helicase that also functions in homologous recombination in *E. coli*. Using a series of gapped, oligonucleotide substrates, we determined that this enzyme translocates along only one strand of duplex DNA in the 3' ---> 5' direction. The translocating enzyme can traverse, or "step" across, a ssDNA gap if it is shorter than 22 nucleotides. This step is much larger than the amount of dsDNA that can be unwound using the free energy derived from one molecule of ATP hydrolyzed, implying that translocation and DNA unwinding are separate events. We propose that the RecBC enzyme both translocates and unwinds by a "quantized", inchworm mechanism, a mechanism that may have parallels for translocation by other motor proteins such as kinesin and myosin.

POSTERS

The loading channel of DNAB-DNAC complex revealed by 3-D cryoelectron microscopy

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DnaB, the major replicative helicase in *Escherichia coli*, has been extensively studied as a model system for the important group of DNA hexameric helicases. At the chromosomal replication initiation, DnaB needs to interact with another protein, DnaC, which after properly loading DnaB onto the DNA template is subsequently released from the complex. In solution, the DnaB-DnaC complex has been shown to have a stoichiometry of 6:6.

In this work we present a new 3-dimensional reconstruction of the complex between DnaB and its loading partner DnaC, obtained from cryoelectron microscopy of vitrified preparations. A considerable increase in the number of images used for this reconstruction as well as a new data acquisition strategy has lead to a remarkable improvement in both the quality and the resolution of the final reconstruction. This allows a more reliable assignment of DnaB and DnaC in the final volume based on considerations on relative masses, dimensions, and other structural characteristics. The six monomers of DnaC seem to form three dimers, which arrange in a ring-like shaped global structure that interacts with DnaB, interlocking into the monomers of the hexameric helicase.

The higher resolution achieved also reveals new interesting features of the complex. A prominent handedness of the hexameric helicase is noticeable in this new reconstruction. A tentative location of the DnaB domains in the volume is also feasible from these results. DnaB clearly appears as a trimer of dimers, structured as a ring with two quite different faces. The face that interacts with DnaC possesses 6-fold symmetry whereas the opposite face, where it is likely to be located the N-terminal domain of the protein, exhibits a 3-fold symmetry.

However, the most striking new feature revealed by this reconstruction is the fact that the channel running through the whole complex is not homogenous in diameter, but it narrows down considerably at the 3-fold side of DnaB to the point of being almost completely closed. This novel result might have important implications in the mechanism of loading of the helicase onto the DNA. The functional considerations stemming from this new feature are further discussed in this work.

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The translocation mechanism of a molecular motor revealed by step size measurement for the RecBC helicase.

The RecBC enzyme is a processive DNA helicase that functions in homologous recombination in *E. coli*. Using a series of gapped, oligonucleotide substrates, we determined that this enzyme translocates along only one strand of duplex DNA in the 3' → 5' direction. The translocating enzyme can traverse, or "step" across, a ssDNA gap if it is shorter than 22 nucleotides. This step is much larger than the amount of dsDNA that can be unwound using the free energy derived from one molecule of ATP hydrolyzed, implying that translocation and DNA unwinding are separate events. We propose that the RecBC enzyme both translocates and unwinds by a novel, "loaded-spring" mechanism, a mechanism that may have parallels for translocation by other motor proteins such as kinesin and myosin.

Structure and Functions of the N-Terminal Domain of *E. coli* DnaB Helicase.

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DnaB, the major replicative helicase in *E. coli*, uses energy from ATP hydrolysis to unwind the DNA duplex at replication forks. It is positioned in the replisome on the lagging strand, and moves in the 5' to 3' direction on the single-stranded (ss) DNA template.

Wild type DnaB is a hexamer of identical subunits, with electron microscopy (EM) studies[1,2] showing a toroidal structure. It is believed that the lagging strand template passes through the central 4 nm hole. The DnaB hexamer shows pH-dependent conversion between two distinct structures, one with 3-fold and one with 6-fold symmetry. It has been proposed that transition between these states reflect structural changes that occur during ATP-dependent translocation of DnaB on ssDNA.

CryoEM and proteolytic studies show that each DnaB monomer consists of two domains, a smaller N- and larger C-terminal, joined by a flexible hinge. The structured core of the N-terminal domain of DnaB has been determined to be residues 24-136[3], and its structure has been determined by multidimensional NMR[4,5] and x-ray crystallography[6]. At high concentrations, both in solution and in the crystal, it forms a symmetrical dimer, which implicates the N-terminal domain in the structural transition of the full-length hexamer.

DnaB interacts with several other replication proteins (such as DnaC and DnaG primase) to play a key role in coordinating the actions of the replisome. The formation of a complex of DnaB with its loading partner DnaC is required to load DnaB onto ssDNA at the *oriC* origin. NMR and BIACORE experiments have been used to map one region of DnaB that interacts with DnaC.

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Molecular biology of early steps in homologous recombination and the suppression of the SOS response

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While homologous pairing and strand exchange are the earliest contacts between two parental DNAs mediated by RecA and its eukaryotic homologues, Rad51 and Dmc1, homologous recombination is initiated by DNA double-strand breaks (DSBs). The protein that catalyzes DSB formation in meiosis in the budding yeast, *Saccharomyces cerevisae*, is the product of the *SPO11* gene. Disruption of this gene results in meiotic arrest, spore lethality and a lack of meiotic recombination. Spo11 homologues have been identified in other eukaryotes and archaebacteria resulting in the identification of a new family of proteins related to DNA topoisomerase IIs. We have identified and cloned Spo11 homologues in mouse and man. In mouse and man northern blot analysis revealed testis-specific expression of *SPO11*, but RT-PCR revealed expression is somatic tissues as well. The mouse dot blot showed expression in testis and thymus. Both the mouse and human transcripts undergo alternative splicing. Chromosome localization was performed for both mouse and human *SPO11*, and the human gene was localized to chromosome 20q13.2-13.3, a region amplified in some breast and ovarian cancers. Finally, using affinity-purified antibodies to the mouse protein we have visualized Spo11 only in pachytene of meiosis I in spermatocytes and only in those areas where the chromosomes are fully synapsed.

In all organisms, homologous recombination is inextricably related to DNA repair and replication, hence cell proliferation and its control. For example, in *E. coli*, RecA, the prototypical homologous recombination protein, is directly responsible for turning on the SOS response to genotoxic damage. The RecA-ssDNA-ATP filament, the active form of RecA, acts as a co-protease in the auto-catalytic digestion of the LexA repressor. Much less is known about how the SOS response is extinguished. DinI is the product of a damage-inducible, LexA-controlled gene. Previous work has shown that when this gene is over-expressed in mitomycin C-treated cells it prevent the cleavage of LexA and UmuD proteins. Furthermore, *in vitro* DinI prevents the cleavage of UmuD promoted by the RecA-ssDNA co-filament. Our experiments with purified RecA and DinI have revealed that they interact directly. While DinI does not bind to DNA it releases ssDNA from the active RecA-ssDNA co-filament. Furthermore, the C-terminal portion of DinI interacts with the DNA binding and homologous pairing of RecA, loop L2.

RNA helicases involved in ribosome biogenesis in *Saccharomyces cerevisiae*

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A variety of proteins, collectively called 'RNA chaperones', assure the correct folding of RNA molecules and mantain and/or modify their specific secondary and tertiary structures. Amongst these proteins are the RNA helicases. The most numerous class of *trans*-acting factors involved in ribosome biogenesis in *Saccharomyces cerevisiae* are the RNA helicases of the DEAD-box and relates families. To date, 16 putative RNA helicases have been implicated in ribosome biogenesis. Depletion or mutation of these RNA helicases lead to terminal phenotypes that are characteristic for the inhibition of specific steps during ribosome biogenesis. From these analyses, different functions can be envisaged: i) an RNA unwinding activity could be required to establish and/or dissociate snoRNA:pre-rRNA base pairings, which are in most cases mutually exclusive with the final folding of the rRNA in the mature ribosome; ii) putative RNA helicases may functionally assist endo- and exonucleases by rendering their substrates accessible for the processing reactions; iii) finally, they may recruit, rearrange or dissociate *trans*-acting factors and r-proteins within pre-ribosomal particles during the processing and assembly reactions by modulating specific intramolecular rRNA, rRNA:protein or even protein:protein interactions. Recent progress on the analyses of the RNA helicases studied in the laboratory and our current perspectives will be presented.

The DNA translocation and ATPase activities of restriction-deficient mutants of *Eco*KI.

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The type I restriction enzyme *Eco*KI specifies DNA methyltransferase, ATPase, DNA translocase and endonuclease activities. One subunit (HsdR) of the oligomeric enzyme contributes to those activities essential for restriction. These activities are thought to involve ATP-dependent DNA translocation and DNA cleavage.

The eight conserved sequences identified in HsdR subunits from all known type I restriction endonucleases include the seven characteristic of the DEAD-box family of proteins that comprise known, or putative, helicases. The remaining conserved sequence identifies an endonuclease motif similar to that found at the active site of type II restriction enzymes and other nucleases. We have examined the consequences of mutations in these eight motifs.

Mutations in each of the DEAD-box motifs impair or abolish restriction activity both *in vivo* and *in vitro*. Using *Eco*KI-dependent entry of T7 DNA as an assay, we have shown that the mutations within the seven motifs abolish translocation activity *in vivo*. The same mutations inactivate the ATPase activities of purified enzymes. These data provide direct evidence that the DEAD-box motifs are important in the coupling of ATP hydrolysis to DNA translocation but they do not provide evidence for strand separation activity.

Mutations that identify the endonuclease motif abolish restriction activity *in vivo* and both nicking and endonuclease activity *in vitro*. When conservative changes are made at these conserved residues, the enzymes lack nicking and nuclease activities, but retain the ability to hydrolyse ATP. *In vivo*, the restriction-deficient enzyme, like wild-type, can translocate 39kb of T7 DNA from the phage particle into the host cell.

It has been speculated that nicking may be necessary to resolve the topological problems associated with DNA translocation by type I R-M systems. Our experiments show that the nicking activity associated with the endonuclease motif is not necessary for translocation *in vivo*. Possible mechanisms of DNA translocation by *Eco*KI will be discussed in the light of current *in vitro* and *in vivo* experiments.

Operation of the *Eco*KI Type I DNA Restriction/Modification Enzyme.

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Type I DNA restriction-modification enzymes protect the bacterial cell from viral infection by cleaving foreign DNA which lacks N6-adenine methylation within a target sequence and maintaining the methylation of the targets on the host chromosome. They comprise three types of subunit, S (sequence recognition), M (modification/methylation) and R (restriction/cleavage) and can function solely as a M_2S_1 methylase or as a $R_2M_2S_1$ bifunctional methylase/nuclease. The subunits contain domains related to those in other smaller methylases, nucleases and helicases. The nuclease is a stable structure, whereas the methylase dissociates into nonfunctional forms M and M_1S_1 at the concentrations expected to exist *in vivo*. The restriction reaction relies upon extensive DNA translocation driven by ATP hydrolysis prior to endonucleolytic DNA cleavage. Cleavage occurs between two, widely-separated, target sites. This is consistent with the translocation process causing the collision of two enzymes on the DNA. However, atomic force microscopy has suggested that DNA binding induces dimerisation of the enzyme prior to the initiation of translocation and that cleavage occurs once the DNA loop bound between the two enzymes has been pulled in towards the enzymes.

Structure and Function of the *E. coli* RecQ DNA Helicase

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The RecQ family of enzymes are DNA helicases involved in replication and recombination. While the DNA unwinding mechanisms employed by this family are only now beginning to be understood, their cellular importance is already well recognized. Most notably, two human RecQ homologs (BLM and WRN) have been shown to be critical for genomic stability, and mutations in either of these genes leads respectively to Bloom's or Werner's syndrome. We have initiated an effort to study the structure and function of the *E. coli* RecQ homolog as a step toward uncovering the biochemical mechanisms utilized by the RecQ family of helicases.

Oligomerization and DNA binding:

Until recently, it was commonly believed that helicases would function as oligomers to provide the enzyme with multiple DNA binding sites. However, at least some helicases have been shown to be active as monomers and their three-dimensional structures have been solved. We have observed that *E. coli* RecQ is a monomeric protein in solution but binds to its helicase substrate as a dimer. The specificity of RecQ is such that it binds and unwinds duplex DNA provided there is a flanking single-strand with the correct polarity (a 3' overhang). Thus RecQ binds to DNA containing to DNA containing a 3' overhang as a dimer, whereas is binds to other DNA structures (e.g. ssDNA, dsDNA, DNA with a 5' overhang) as a monomer, suggesting that dimerization is important for helicase activity.

RecQ also exhibits a DNA-dependent ATPase activity. The structure of the DNA cofactor is not important indicating that dimerization of RecQ is not necessary for ATP activity. In addition, ssDNA stimulates ATPase activity in a length-dependent manner suggesting that RecQ can translocate along ssDNA in a monomeric form.

Domain structure:

A sequence homology search within the RecQ family of DNA helicases has revealed a number of regions of similarity shared among the family members. Besides the helicase domain, there is also a RecQ-homology domain of unknown function and an RNase D-like domain (Helicase and RNase D-like C-terminal domain, HRDC). The HRDC is conserved among several RecQ family members which including (the human BLM and WRN proteins) and has been proposed to function in substrate binding.

Limited proteolysis experiments on *E. coli* RecQ have revealed that the HRDC region is readily cleaved by proteases, implying that the region is relatively unstructured. RecQ variants lacking the HRDC retain DNA-stimulated ATPase activity comparable to that of the wild-type enzyme. However, stable binding to 3' overhang DNA substrates is abolished upon removal of the HRDC. Interestingly, however, loss of the HRDC region does not diminish helicase activity. These results suggest that the HRDC is required for stable binding to the helicase substrate but not for helicase activity *per se*.

Helicase delivery during the initiation of replication of the broad host range plasmid RK2

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Specific binding of the plasmid–encoded protein, TrfA, and the *Escherichia coli* protein DnaA to the origin region (oriV) is required for the initiation of replication of the broad-host-range plasmid RK2 (Konieczny, I., Doran, K. S., Helinski, D. R., Blasina,A. 1997). The TrfA protein in the presence of HU produces an opening of a set of 13-mers located in the A+T-rich region of RK2 origin. The DnaA protein enhances and/or stabilizes this open complex formation, but it cannot on its own form an open complex. In addition, DnaA protein has an indispensable role in the delivery and loading of DnaB helicase at the RK2 origin (Konieczny, I., Helinski, D. 1997). This has been supported by the finding that an oriV-DnaA-DnaB-DnaC prepriming complex can be isolated by gel filtration after incubation of the replication proteins with the supercoiled RK2 template. Moreover, a specific interaction between the DnaA and DnaB proteins is required for delivery of the DnaB. Both DnaA and TrfA proteins are required for DnaB-dependent template unwinding. Here, using SPR and EM techniques, we demonstrate that a oriV-DnaA-DnaB-DnaC complex is formed at DnaA-box sequences at the RK2 origin. Results clearly show that at the initial step the helicase is delivered at DnaA-box region located approximately 200bp upstream from opening.

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Functional interactions between the bacterial toxin Kid and the DnaB helicase: a mutational analysis

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The *parD* locus is a killer stability system located close to the origin of replication in the plasmid R1. This system is organized as a small operon containing two genes, *kid* and *kis*, which respectively encode a toxic component and its antidote (1,2). Purified preparations of the Kid toxin inhibit DNA replication of the ColE1 plasmid, but not of phage P4, a DnaA, DnaB, DnaC and DnaG independent replicon. Kid inhibits lytic induction of the lambda prophage, and this inhibition can be prevented by a multicopy plasmid carrying the *dnaB* gene. Moderate overexpression of *dnaB* can also neutralize the Kid-mediated killing of host cells (3). Taken together, these observations point to the DnaB helicase as the possible target of the Kid toxin in *E.coli* cells.

In order to further investigate the functional interactions between these two proteins we searched for mutants of *dnaB* which, when cloned into a multicopy vector, would not retain the capacity to neutralize the toxic effect of Kid. A pBR322-*dnaB* recombinant plasmid was mutagenized by using hydroxyl-amine and introduced into a strain in which Kid toxicity could be conditionally induced. Nine independent *dnaB* mutants unable to suppress the toxicity of Kid were isolated and sequenced at the nucleotide level. Four of the mutants present premature stop codons and encode different truncated DnaB proteins. The five remaining mutants show single amino-acid substitutions which are all located in the carboxy-terminal domain of DnaB, a domain which is involved in binding to SS DNA and ATP and in protein oligomerization (4,5). Introduction of each of the five mutant constructions into a *dnaB242*(ts) strain was able to restaure the cell viability at non-permissive temperatures, indicating that the *dnaB* mutants encode functional helicases. Independently, we observed that moderate overproduction of the carboxy-terminal domain of DnaB is sufficient to neutralize the toxicity of Kid. Taken together, our results suggest that the carboxy-terminal domain of DnaB may specify interfaces involved in contacts with the Kid toxin. An allele replacement approach designed to introduce the mutations into the *dnaB* gene on the chromosome is currently underway. The recombinant strains will then be tested for their resistance to the toxic effect of Kid. Preliminary data and experiments in progress will be presented and discussed.

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Crystal structure of the hexameric DNA helicase RSF1010 RepA at 2.4 Å resolution

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RepA is the DNA helicase required for replication of the broad host range plasmid RSF1010 which encodes its own replication system consisting of the three proteins RepA, RepB, and RepC [1]. In contrast to other hexameric helicases, hexamer formation of RepA does not require the presence of cofactors like Mg^{2+} or ATP. The 30kDA monomers (278 amino acids) are arranged in a ring showing 6-fold rotational symmetry [2] with an outer diameter of ~120 Å and a central channel of 17 Å. The monomer structure closely resembles that of RecA [3] and the T7 helicase domain [4]. Amino acids involved in ATP binding and hydrolysis are found in structurally conserved motifs equivalent to the five helicase motifs of the DnaB-like helicase family. Adjacent subunits of the hexameric ring are interacting at the catalytic/ATP binding sites, enabling cooperativity of the ATPase and DNA unwinding reactions.

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A mutation in *recF* suppresses the lethality of a *pcrA* null mutation

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PcrA was the first helicase for which the crystal structure was solved, in 1996, by Wigley and co-workers. It is the Gram positive counterpart of the Rep and UvrD helicases of *Escherichia coli*. It has been shown to be necessary for the replication of rolling circle plasmids in *Bacillus subtilis* and *Staphylococcus aureus*. When expressed in *E. coli*, this helicase was capable to restore the UV resistance of a *uvrD* mutant, suggesting its involvement in UV repair. No complementation of the *uvrD* mutator phenotype was observed.

Interestingly, in contrast to the *rep* and *uvrD* genes, whose function is not essential to the viability of *E. coli*, the *pcrA* gene is essential in *B. subtilis* and in *S. aureus*. This suggests that PcrA fulfills an essential, yet uncovered role in the cell. In order to understand this role, a genetic approach was used, consisting in isolating spontaneous *B. subtilis pcrA* suppressors.

One suppressor has been characterised so far. It maps in *recF* and the mutant allele, called *recF7*, consists in an in frame 9 bp duplication. RecF is mostly known from the genetic studies performed in *E. coli*. Together with RecO and RecR it participates in homologous RecA dependent recombination. *recF* mutants are UV sensitive and MMS sensitive, in *E. coli* as well as in *B. subtilis*. In contrast, the *recF7* mutant was not UV sensitive, nor MMS sensitive, suggesting that this allele confers a gain rather than a loss of function.

More recently, data accumulate suggesting that RecF also contributes to DNA replication. This second function of RecF may not be *recO* and *recR* dependent. To further understand whether the viability of a *pcrA* interrupted strain relies on the recombination or the replication function of RecF7, a *recO* null mutation was introduced into the *pcrA recF7* strain, and the resulting strain was viable. This suggests that the replicative, rather han the recombinogenic capacity of RecF7 is involved in suppressing a *pcrA* null mutation.

The T7 DNA helicase uses a multi-step DNA binding mechanism to bind ssDNA through the central channel

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Helicases are enzymes that unwind duplex DNA into single-stranded DNA using the energy derived from NTP hydrolysis. They are essential in DNA metabolic processes such as DNA replication, repair, and recombination. Many helicases function as ring shaped hexamers, composed of six identical subunits. Most of the ring-shaped helicases, including the T7 DNA helicase, have been shown to bind single stranded DNA through their central hole. This raises the question as to how DNA gets into the central hole of a ring-shaped helicase in order to form a topologically linked complex.

We have investigated the mechanism of single stranded DNA binding to a pre-formed hexameric T7 DNA helicase by measuring the kinetics of DNA binding. Rapid kinetic techniques were used to monitor DNA binding in real time, and our results support a multi-step DNA binding mechanism. Initial weak DNA binding occurred at a diffusion-limited rate, and this step was followed by several slower conformational changes of the protein, leading to tight DNA binding. We propose that the fast DNA binding step occurs at an easily accessible DNA binding site that serves as a loading site. This facilitates binding of the DNA in the central channel by increasing the effective concentration of the DNA. Nucleotide hydrolysis and DNA unwinding studies showed that the multi-step DNA binding mechanism was necessary and sufficient for the formation of a catalytically competent protein-DNA complex.

CHARACTERIZATION OF MUTATIONS AFFECTING THE ACTIVITY OF KID, A BACTERIAL TOXIN.

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parD (kis, kid) operon is a conditional killer system of plasmid R1. It is formed by a toxin, the Kid protein, and an antidote, the Kis protein (1,2). Transcriptional regulation of *parD* operon is modulated by the coordinated action of the two proteins (3) that form a strong complex (4). This complex is also required for neutralization of the toxic activity of Kid. *In vivo* and *in vitro* analysis strongly suggest that Kid functionally interact with the DnaB helicase (4). We have previously reported that two discrete regions of the antidote,Kis, located at the amino- (1,5 and 6) and carboxyl-ends (2), are required for its function. In this report we present data indicating that aminoacids located in two discrete regions of Kid are involved in its activities as a toxin and as coregulator of the *parD* operon. The data present suggest that aminoacid-residues of Kid involved in interactions with the antidote and the target, partially overlap.

The two regions in the Kid protein were identified by the analysis of mutants that inactivated its toxicity. For this purpose we used a mini-R1 plasmid, pAB1120, that carries an amber mutation in the *kis* gene (2). This mutant can be established in a *supFts* strain, OV2, at low but not at high temperature. After treatment with the mutagen hydroxilamine, we isolated thermo-resistant variants of pAB1120. Thirty mutants were selected and sequenced. All of them have single mutations in *kid* that are located in two regions: one close to the amino end (aminoacids 9-32) and the other close to the carboxyl-end (aminoacids 70-94). Six mutants were selected for a more detailed analysis: Val9Ile, Glu18Lys, Thr29Ile, Gly70Ser, Arg85Trp, and Pro94Ser. Some of them affect different elements of secondary structure as predicted by the PHD algorithm. In addition, they affect identical or conserved residues identified in the alignment of Kid with ChpAK and ChpBK, two homologous toxins of chromosomal origin (7).

We further analysed the effect of these mutations in the regulation of the system in order to test if some of them could also affect the functional interaction of Kid with its antidote. The transcriptional level of the *parD* operon in the six mutants was analysed by Northern blotting, primer extension and β-galactosidase assays. The analysis consistently indicated: (i) that the changes Val9Ile, Glu18Lys, and Arg85Trp do not affect the regulation of the system; (ii) a clear derepression was observed in Pro94Ser and Thr29Ile and; (iii) a poor derepression was observed in the Gly70Ser mutant. We hypothesize that the three first changes correspond to residues implicated in the interaction of the toxin with its bacterial target. Alternatively at least some of these mutations could have optimized interactions with the chromosomal antidotes homologous to Kis. The changes that lead to de-regulation could affect a protein-protein interface required to form an active repressor complex. As Kid is a dimer in solution and the dimerisation is probably required to form an active repressor, some of these mutations could affect the Kid-Kid interactions. Further analyses, that are being performed, are required to clarify these alternatives.

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Bi-directional DNA Tracking by the Type I Restriction Endonucleases Independent of DNA Nicking.

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The type I restriction endonucleases are large molecular weight, oligomeric proteins involved in the protection of bacterial DNA from invasive DNA particles. The endonucleases recognise specific asymmetric DNA sequences, but subsequently cleave the DNA at random, non-specific loci. These can be anywhere between 50 to 7,000 bp away from the site. DNA restriction relies on both ATP and Mg^{2+} cofactors and is stimulated by the methyl donor *S*-adenosyl methionine. ATP has a dual role; as a transferable source of chemical energy and as an allosteric activator of DNA binding. The link between site-specific recognition and non-specific cleavage is provided by one-dimensional motion along the DNA in an ATP dependent reaction. The enzymes are believed to remain bound to their recognition sites throughout the reaction whilst simultaneously translocating adjacent DNA, thus extruding an expanding DNA loop. The presence of DEAD-box helicase sequence motifs in the HsdR subunits suggests that DNA unwinding may play a role in protein motion on DNA.

The long-range communication between distant DNA sites was first examined on inter-linked rings of DNA (catenanes). The results demonstrated that the communication between recognition and cleavage cannot stem from random looping through 3D space, but must follow the 1D DNA contour between the sites. This result was substantiated by the observation that DNA tracking displaces a labelled oligonucleotide bound as a DNA triple-helix. The rate of displacement is dependent on the distance between the type I recognition site and the triple helix site, indicative of 1-D tracking. However, displacement does not require any DNA cleavage activity. This implies that tracking does not initiate from a free DNA end or nick site. Furthermore, any changes in DNA twist introduced during tracking must be released without accumulating significant amounts of nicked DNA.

To provide an unequivocal demonstration of motion along DNA, an optical tweezers assay for the restriction enzymes is being developed in collaboration with Dr. Justin Molloy (University of York, UK). DNA substrates suspended between two polystyrene beads are held in twin optical traps. Any DNA tracking that occurs will shorten the axial distance between the beads, pulling them out of the traps. In low-power mode, a transducer can follow displacement, whilst at higher powers, using a feedback loop, accurate measurements of force can be made.

Kinetic Step-size of DNA Unwinding for*E. coli* RecBCD Helicase

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The mechanism by which the *E. coli* RecBCD DNA helicase unwinds duplex DNA was examined by pre-steady state chemical quenched-flow kinetic methods. Single turnover DNA unwinding experiments, performed by pre-incubating RecBCD with a series of oligodeoxynucleotides with duplex regions ranging from 18 to 60 base pairs (bp) (in the presence of Mg^{2+}) showed distinct lag phases that increased with duplex length, reflecting the formation of partially unwound DNA intermediates during unwinding. Analysis of these kinetics indicates that RecBCD unwinds duplex DNA in discrete steps, with an average kinetic "step size" of ~4±0.5 bp and an unwinding rate of 122 ± 10 steps/sec, (488 ± 40 bp/s) (3 mM $MgCl_2$, pH 7, 25°C). This step size is the same as that determined for *E. coli* UvrD helicase [Ali, J. & Lohman, T. (1997) *Science* 275, 377], suggesting similarities between the DNA unwinding mechanisms for these two helicases. The fact that RecBCD enzyme covers ~20 bp when bound to the duplex end, yet unwinds in much smaller steps of 4 bp, suggests that it most likely functions by a type of "inch-worm" mechanism as previously suggested [Farah, J.A. & Smith, G.R. (1997) *J. Mol. Biol.* 272, 699-715].

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