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DNA STRAND EXCHANGE PROTEINS: A BIOCHEMICAL AND PHYSICAL COMPARISON

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Table 1A. Proteins Involved in Genetic Recombination

	ORGANISM		
General Function	***E. coli***	**Bacteriophage T4**	***S. cerevisiae***
Initiation	RecBCD, RecQ	gp46, gp47, gp41, gp59	Mre11, Rad50, Xrs2, Spo11
DNA strand exchange	RecA	UvsX	Rad51
ssDNA-binding	SSB	gp32	RPA
Accessory	RecF, RecO, RecR	UvsY	Rad52, Rad55, Rad57, Rad59
Branch migration	RuvA, RuvB	gp41, gp59	Rad54
	RecG	UvsW	
		Dda	
Holliday Junction cleavage	RuvC	gp49	-
Other proteins	DNA topoisomerase I and II	DNA topoisomerases	DNA topoisomerases
	DNA ligase	DNA ligase	DNA ligases
	DNA polymerase I	DNA polymerases	DNA polymerases

1. ABSTRACT

Homologous genetic recombination is an essential biological process that involves the pairing and exchange of DNA between two homologous chromosomes or DNA molecules. It is of fundamental importance to the preservation of genomic integrity, the production of genetic diversity, and the proper segregation of chromosomes. In *Escherichia coli*, the RecA protein is essential to recombination, and biochemical analysis demonstrates that it is responsible for the crucial steps of homologous pairing and DNA strand exchange. The presence of RecA-like proteins, or their functional equivalents, in bacteriophage, other eubacteria, archaea, and eukaryotes, confirms that the mechanism of homologous pairing and DNA strand exchange is conserved throughout all forms of life. This review focuses on the biochemical and physical characteristics of DNA strand exchange proteins from three diverse organisms: RecA protein from *E. coli*, UvsX protein from bacteriophage T4, and Rad51 protein from *Saccharomyces cerevisiae*.

2.INTRODUCTION

General genetic recombination involves the exchange of homologous regions between two chromosomes or double-stranded DNA (dsDNA) molecules. The resulting, recombinant DNA contains genetic information originally present in each of the parental molecules. Genetic studies demonstrated that, in *Escherichia coli*, there are several recombination pathways (1-4), with many proteins involved in the process (tables 1A-B) (5-7). One of these, the RecA protein, is essential to the primary pathways of homologous genetic recombination. The function of RecA protein is conserved from bacteriophage to humans and, thus, the focus of this review is to compare two other RecA protein homologues, the UvsX protein of bacteriophage T4, and the Rad51 protein of *Saccharomyces cerevisiae*, with the prototypic and the most well studied protein, the RecA protein of *E. coli*.

Clark and Margulies discovered the *recA* gene of *E. coli* by virtue of the strong effects of *recA* mutations on conjugal recombination (8). Subsequent to this discovery, the RecA protein was shown to be essential for homologous genetic recombination and for induction, following DNA damage, of both prophage and the SOS response (9-11). Included in the SOS response are LexA and phage repressor cleavage, amplification of RecA protein levels, induction of a mutagenic mode of DNA repair, filamentation, prophage induction, and Weigle reactivation (10,12). Not surprisingly, mutations in *recA* are pleiotropic, affecting not only recombination, but also DNA repair, mutagenesis, and cell division.

In vitro, the RecA protein (M_r 37,842) promotes the ATP- and single-stranded DNA (ssDNA)-dependent cleavage of the LexA repressor (13), which results in induction of the SOS response. In addition, it performs two reactions important to homologous recombination: ATP-stimulated DNA strand annealing (renaturation) between complementary single strands of DNA (14-17), and ATP-dependent DNA strand invasion (exchange) between ssDNA and a homologous sequence within dsDNA (18-26). Over the last 18 years, RecA protein has been the subject of intense study (relevant activities are listed in table 2), and as a result, has become the prototypic DNA strand exchange protein to which all other DNA strand annealing and DNA strand exchange proteins are compared. Homologous proteins have been identified in a variety of other organisms including bacteriophage, UvsX protein of T4 phage (27), at least 60 other bacteria (28-30), all eukaryotes examined (31), and, finally, the Archaea (32,33). Thus, RecA protein homologues comprise a family of universally conserved, homologous recombination proteins.

As stated above, a member of this family is the T4 phage-encoded UvsX protein. Harm first identified *uvsX* as a gene that controls UV-sensitivity in bacteriophage T4 (34). Subsequent to this work, a number of studies revealed that mutations in *uvsX* are remarkably diverse in their effect. Mutants which are defective in *uvsX*, 1) are recombination deficient *in vivo* (34-37); 2) have decreased mutagenesis (38); 3) display discontinuous T4 DNA replication *in vivo* (37); and 4) are deficient in repair of DNA damage caused by various chemical and physical agents (39). In addition, lethal mutations in *gene 49* are suppressed by mutation of the *uvsX* gene (37,40). *Gene 49* encodes an endonuclease that resolves recombination intermediates (branched DNA) into linear duplex DNA which can then be packaged into the phage capsid (41,42). *UvsX* is not absolutely required for T4 growth, however, since T4 phage has at least one additional recombination pathway which does not require the involvement of *uvsX* (see reference (43) for review).

Table 1B. Functions of Proteins Involved in Genetic Recombination

GENERAL FUNCTION	PROTEIN	BIOCHEMICAL FUNCTIONS
E. coli		
Initiating protein(s)	RecBCD	ATP-dependent dsDNA and ssDNA exonuclease, ATP-stimulated ssDNA endonuclease, DNA helicase, recombination hotspot χ-recognition
DNA strand exchange	RecA	DNA-dependent ATPase, DNA- and ATP-dependent coprotease, DNA renaturation, DNA strand exchange
ssDNA-binding protein	SSB	ssDNA binding, stimulates DNA strand exchange
Accessory protein(s)	RecF	ssDNA, dsDNA binding, weak ATPase, interacts with RecR protein
	RecO	ssDNA, dsDNA binding; interacts with RecR; RecOR prevents end-dependent disassembly of RecA filaments; interacts with SSB
	RecR	Interacts with RecF; RecFR complex attenuates RecA filament extension into dsDNA regions
Branch migration	RecG	DNA helicase, branch migration of Holliday junctions
	RuvA	Binds to Holliday-, cruciform-, and four-way junctions, interacts with RuvB protein
	RuvB	DNA helicase, branch migration of Holliday junctions, interacts with RuvA protein
Holliday Junction cleavage	RuvC	Binds to four-way junctions, cleaves Holliday junctions
Other proteins	DNA gyrase	Type II topoisomerase
	DNA topoisomerase I	Type I topoisomerase, ω protein
	DNA ligase	DNA ligase
	DNA polymerase I	DNA polymerase, 5'→3' exonuclease, 3'→5' exonuclease
Bacteriophage T4		
Initiating protein(s)	gp46	Interacts with gp47; endo- and exonuclease
	gp47	Interacts with gp46; endo- and exonuclease; stimulates gp46 action
	gp41	DNA-dependent NTPase, ssDNA binding, ATP- or GTP-dependent DNA helicase
	gp59	ssDNA binding, stimulates ATPase and helicase activities of gp41, interacts with gp32 and gp41
DNA strand exchange	UvsX	DNA-dependent ATPase, DNA renaturation, DNA strand exchange
ssDNA-binding protein	gp32	ssDNA binding, stimulates DNA strand exchange, interacts with UvsY and UvsX
Accessory protein(s)	UvsY	Stimulates DNA strand exchange, interacts with UvsX and gp32
Branch migration	Dda	DNA helicase, stimulates branch migration by UvsX protein
	UvsW	DNA helicase, branch migration of Holliday junctions, functional analogue of RecG
	gp41	DNA-dependent NTPase, ssDNA binding, ATP- or GTP-dependent DNA helicase
	gp59	ssDNA binding, stimulates ATPase and helicase activities of gp41, interacts with gp32 and gp41
Holliday Junction cleavage	gp49	Binds to and cleaves Y-junctions and Holliday junctions
S. cerevisiae		
Initiating protein(s)	Mre11	Forms complex with Rad50 and Xrs2 which is possibly responsible for resection of double-strand DNA breaks; with Rad50, ssDNA endo- and 3' to 5' dsDNA exonuclease
	Rad50	Forms complex with Mre11 and Xrs2 which is possibly responsible for resection of double-strand DNA breaks, ATP-dependent binding to dsDNA, contains ATP-binding Motif
	Xrs2	Forms complex with Mre11 and Rad50 which is possibly responsible for resection of double-strand DNA breaks
	Spo11	Binds DNA, likely catalytic subunit responsible for double-strand break formation
DNA strand exchange	Rad51	DNA-dependent ATPase, DNA strand exchange, interacts with Rad52, Rad54, and Rad55 proteins
ssDNA-binding protein	RPA	ssDNA binding, stimulates DNA strand exchange, interacts with Rad52 protein
Accessory protein(s)	Rad52	Stimulates DNA strand exchange, interacts with Rad51 and RPA proteins
	Rad54	Contains both ATP-binding, DNA helicase motifs, interacts with Rad51, hydrolyzes ATP, stimulates DNA strand exchange
	Rad55	Stimulates DNA strand exchange, contains Walker ATP-binding motif, interacts with Rad51 protein, forms stable heterodimer with Rad57 protein; shows homology to Rad51
	Rad57	Stimulates DNA strand exchange, contains Walker ATP-binding motif, forms stable heterodimer with Rad55 protein; shows homology to Rad51
Other proteins	Rad59	Shows homology to Rad52 protein, function is unknown

Table 2. Biochemical Activities of the Prototypic DNA Strand Exchange Proteins

	Activity	RecA protein	UvsX protein	Rad51 protein
ATPase				
	k_{cat} (min^{-1}) on ssDNA	18 - 30	145 - 240	0.6 - 0.7
	k_{cat} (min^{-1}) on dsDNA	19 - 22	5	0.05 - 0.1
	Length dependent	Yes	Yes	-
	Compositional preference	Yes	-	Yes
	Cooperative	Yes	Yes	Yes
	Reaction Products	ADP+P_i	ADP+P_i, AMP+PP_i	ADP+P_i
	Stimulated by	SSB	UvsY; gp32	RPA; Rad52
DNA Binding				
ssDNA	Stoichiometry (nt:monomer)	3 - 6	3 - 5	3 - 6
	Compositional Preference	Yes (GT>AC)	-	Yes (GT>AC)
	Length dependence	≥ 9 nt	≥ 16 nt	-
	Cooperative	Yes	Yes	Yes
	Stimulated by	SSB	gp32, UvsY	RPA
	Cofactor dependent	No	No	No
	Stabilized by NTP	Yes	Yes (ATPγS)	Yes
dsDNA	Stoichiometry (bp:monomer)	3	3	3
	pH-dependent	Yes	Not determined	Yes
	Cooperative	Yes	Yes	Yes
	Cofactor required	Yes	Yes	Yes
DNA Strand Exchange				
	Inhibited by Excess dsDNA	No	Yes	Yes
	ATP-binding required	Yes	Yes	Yes
	ATP-hydrolysis essential	No/Yes	Yes	No
	Supported by other NTPs	Yes	No	No
	Coaggregation	Yes	Yes	-
	Polarity	5' to 3'	5' to 3'	3' to 5'
	Stimulated by	SSB; RecF,O,R	gp32, UvsY	RPA; Rad52, 54, 55, 57

UvsX protein (M_r 43,760) has many of the same biochemical activities possessed by RecA protein (table 2): 1) ssDNA-dependent ATP hydrolysis activity (44,45); 2) binding to both ssDNA and dsDNA, with a preference for ssDNA (46,47); 3) formation of a nucleoprotein filament (46-48); 4) ATP-dependent renaturation of complementary ssDNA (45); and 5) ATP-dependent homologous pairing and exchange of complementary DNA strands (44,45,49,50), which, under the appropriate conditions, is stimulated by the two accessory factors, UvsY protein and gene 32 protein (gp32) (47,51).

The third member of the RecA-like proteins that will be discussed in this review is the eukaryotic Rad51 protein. Genetic analyses of the budding yeast *S. cerevisiae* identified many mutants deficient in homologous recombination and DNA double-strand break repair (DSBR). The genes defined by these mutations belong to the *RAD52* epistasis group. These mutants were isolated on the basis of their sensitivity to ionizing radiation and sensitivity to methyl methanesulfonate (MMS), and they display a characteristic array of pleiotropic phenotypes (52-54). Mutant strains are defective in meiosis, as evidenced by their inability to produce viable spores (54,55). Mutations in the *RAD51*, *RAD52*, and *RAD54* genes exhibit additional defects in mitotic recombination and also in mating type switching (54-56). In *RAD51*, *RAD52*, *RAD55*, and *RAD57* mutant strains, DNA double-strand breaks (DSBs) with processed tails, which are an early recombination intermediate in *S. cerevisiae* (57,58), form readily but are not further processed to produce viable recombinants (56,59). Thus, subsequent steps at least require these gene products (table 1). Genetic characterization of the *RAD52* epistasis group has identified *RAD51* as an important member of this group (52,56). The product of the *RAD51* gene, Rad51 protein, shows both sequence as well as structural homology to the RecA protein of *E. coli* (59-61). *In vitro*, the Rad51 protein from either *S. cerevisiae* (M_r 42,961; purified from *E. coli* or *S. cerevisiae*) (62-65) or humans (M_r 37,000; purified from *E. coli*) (66,67), possesses those activities which establish it as the eukaryotic homologue of the bacterial RecA protein (table 2). Based on sequence homology at the amino acid level, another RecA-like protein, the Dmc1 protein, is present in yeast (57,58,68). Though potentially another DNA strand exchange protein that is ubiquitious and which functions in meiosis, no biochemical characterization exists, and hence it will not be discussed herein. Like the RecA and UvsX proteins, Rad51 protein functions in concert with a number of accessory proteins that augment its limited DNA strand exchange activity (62,65,69-71).

3. BIOCHEMICAL MODELS FOR HOMOLOGOUS RECOMBINATION

3.1. Introduction

Biochemical mechanisms for homologous recombination can be separated into four distinct stages: initiation, homologous pairing and DNA strand exchange, DNA heteroduplex extension (branch migration), and resolution. Figure 1 depicts a generic model for homologous recombination. This model, the DNA double-strand break repair (DSBR) model (72,73), can be applied to mechanisms of homologous recombination in all organisms examined, since in most cases a DNA double-strand break (DSB) is necessary for initiation of the process (though similar models, involving initiation in ssDNA gaps, can also apply (see below)). The specific steps of this model are as follows. In

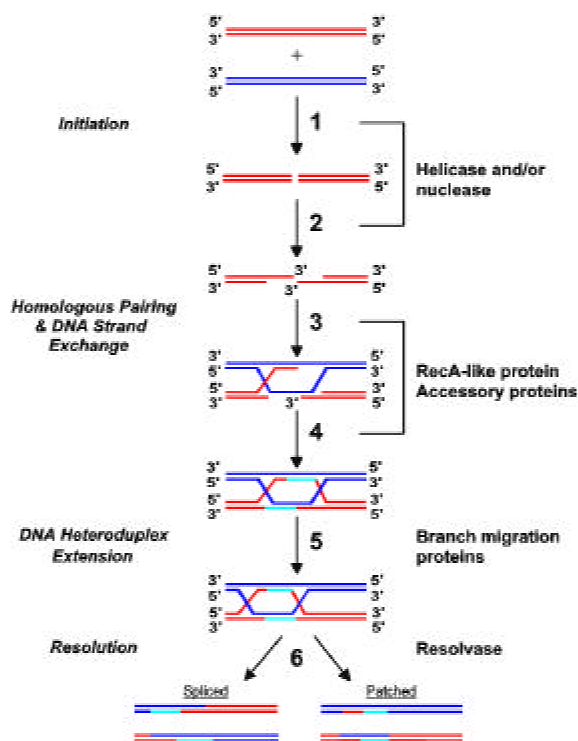

Figure 1. General model for homologous recombination. The model depicted is the double-strand break repair (DSBR) model (72,73). Since a double-strand break is involved in most initiation events, this model, in its most general form, likely can apply to mechanisms of homologous recombination in all organisms. Details are discussed in the text. Light blue lines indicate DNA that has been recently synthesized by a DNA polymerase.

step 1, a DSB is introduced into one of the DNA substrates. The formation of this DSB could be due to a programmed *in vivo* process or to environmental circumstances. More specific examples of these will be discussed below. In step 2, the DSB is processed by either a helicase, a nuclease, or a combination of both, to yield a free 3'-ssDNA overhang(s). In step 3, this free 3'-terminal end is homologously paired with its homologue by a RecA-like protein to form an intermediate known as a joint molecule. Following joint molecule formation, pairing of the displaced complementary strands results in the formation of characteristic intermediates known as Holliday junctions (step 4). In step 5, the Holliday junction(s) is extended unidirectionally by branch migration proteins. Finally, in step 6, the Holliday junction is cleaved by a resolvase to yield recombinant products (*e.g.* spliced or patched). Given some minor differences, each of these steps occurs in the major pathways of homologous recombination in *E. coli*, bacteriophage T4, and *S. cerevisiae* (figure 2). The key players in each of these pathways and their respective functions are listed in table 1.

3.1.1. *Escherichia coli*

In *E. coli*, recombination is readily observed following conjugation or transduction. As part of either process, the DNA is transiently linearized; consequently, most homologous recombination events are thought to initiate at the ends of linear dsDNA (74). In both instances, DNA strand invasion occurs between linear dsDNA (donor) and supercoiled DNA (recipient). A model for the major pathway (the RecBCD-pathway) of genetic recombination in *E. coli* is displayed in figure 2A. Participation of RecA protein in this pathway, as well as most other pathways (4), is absolutely essential as demonstrated by the fact that both conjugation (9) and transduction (75) are reduced by as much as 50,000-fold in *recA* mutant strains.

3.1.2 Bacteriophage T4

At least three major recombination pathways are described for T4 phage. Each pathway is distinguished by the structure of the DNA substrates that enter the recombination process. Two of the pathways occur in only certain regions of the T4 phage genome and are linked to DNA replication (43). The third pathway is the idealized general recombination reaction, which takes place in the absence of DNA replication, and occurs throughout the genome. A model for this pathway is depicted in figure 2B.

3.1.3. *S. cerevisiae*

The genes in the *RAD52* epistasis group of *S. cerevisiae* are required for homologous recombination and for the recombinational repair of DSBs (52-54). Though there are at least three separate *RAD52*-dependent recombination pathways (76), the major pathway for recombination in yeast defines the canonical DSBR model (figure 2C) (72,73). The *RAD51*-dependent pathway is responsible for the majority (95%) of recombination events; this pathway requires the *RAD51*, *RAD52*, *RAD54*, *RAD55*, and *RAD57* genes (76). A functional *RAD52* gene product is absolutely necessary in all pathways, since *rad52* mutants are significantly reduced for recombination (>3,000-fold) (77). It is unclear exactly where the Rad52 protein functions in the pathway but it appears to act after the Rad50 protein (78), and with the Rad51 protein (70,71,79).

3.2. Initiation

Generally, homologous recombination is initiated at a dsDNA end. Subsequently, the linear dsDNA is processed by organism-specific proteins to produce ssDNA suitable for a RecA-like protein function. A dsDNA end can be formed in two general ways: 1) *via* environmental factors (*e.g.* UV light, free radicals, γ-rays, X-rays, and chemical mutagens), and 2) *via* intended *in vivo* processes (for example, in *E. coli*, conjugation and transduction produce dsDNA ends; in *S. cerevisiae*, a protein (Spo11) cleaves DNA to yield dsDNA ends (80,81)). For all of the organisms discussed here, a dsDNA end can also potentially form when the replication machinery encounters a single-strand interruption in the template DNA, causing a collapse of the replication fork (82). The subsequent repair of the interrupted replication fork will then take place *via* the homologous recombination mechanisms discussed below.

3.2.1. *Escherichia coli*

In *E. coli*, formation of the DNA break is a consequence of conjugation, transduction, or direct damage to the DNA. RecBCD enzyme initiates recombination by acting

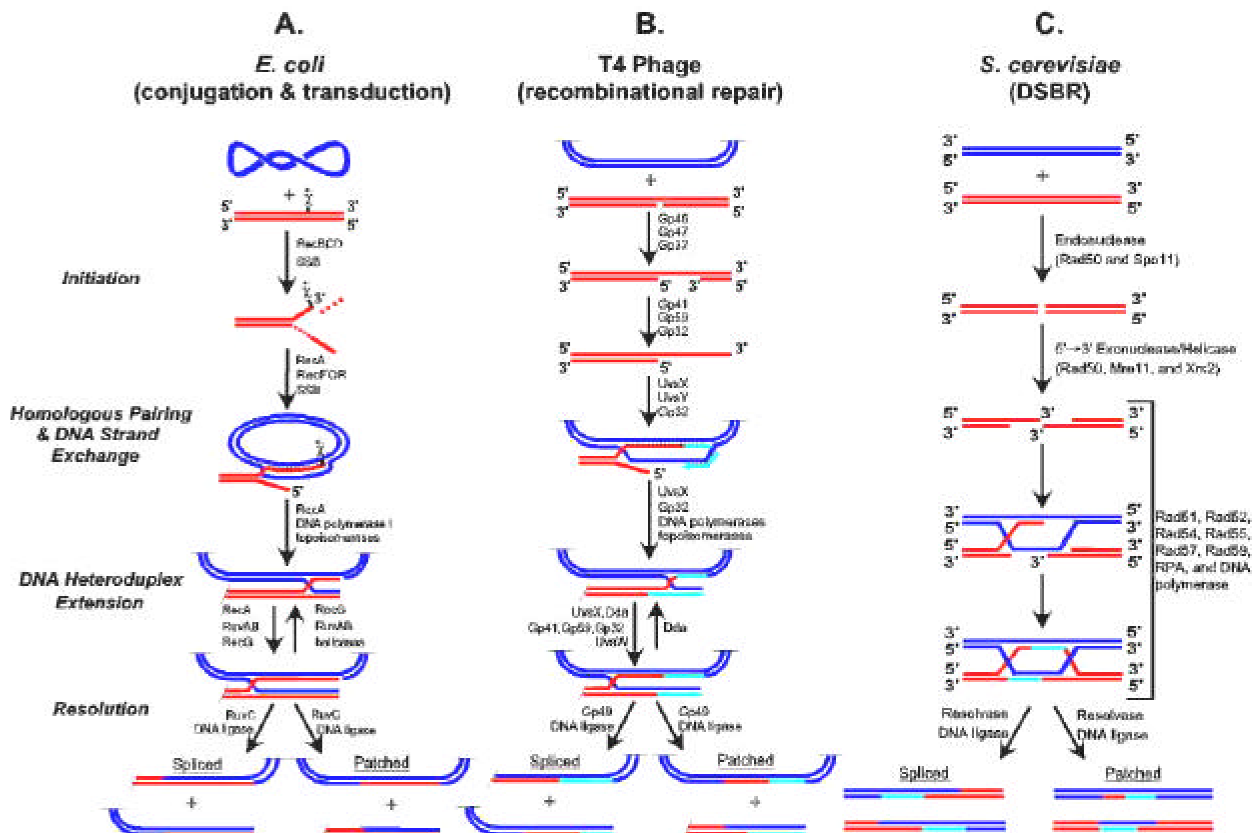

Figure 2. Biochemical models for homologous recombination. (A) *Escherichia coli*; (B) Bacteriophage T4; (C) *Saccharomyces cerevisiae*. Details are discussed in the text. Dashed lines indicate DNA degraded by a nuclease/helicase and light blue lines indicate DNA recently synthesized by DNA polymerase. The model in panel A is modified from reference (306). The model in panel C is modified from reference (307).

on the break to create a ssDNA substrate suitable for RecA protein activity (4,83,84). The RecBCD enzyme is a combination nuclease/helicase that binds to the end of linear dsDNA, initiates unwinding of the DNA, and simultaneously degrades preferentially, the 3'-terminal strand of the DNA (85,86). These concurrent activities continue until RecBCD enzyme interacts with a DNA sequence (5'-GCTGGTGG-3') (87,88) known as Chi (χ; crossover hotspot instigator (89)). At χ, the nuclease activity of the enzyme is altered so that degradation of the 3'-terminal strand is down-regulated and the nuclease activity of the 5'-terminated strand is up-regulated, but the helicase activity remains unaltered (85,90,91). The effect of this χ-mediated modification is a recombinationally active RecBCD enzyme that retains helicase activity, but has a diminished nuclease activity with an opposite (5'→3') polarity of degradation. After unwinding and degradation, the χ-modified RecBCD enzyme produces resected dsDNA with a 3'-terminal ssDNA tail terminating at the χ sequence (86,88,90-93). During translocation and unwinding, RecBCD enzyme loads RecA protein onto this χ–containing ssDNA tail (94) which, in fact, is the preferred substrate for RecA protein-dependent strand invasion of χ-containing supercoiled DNA (85,90,94,95). The single-stranded DNA binding (SSB) protein also plays an important role at this step by trapping the unwound ssDNA, preventing it from reannealing and protecting it from other nucleases. Recently, it was shown that another helicase, the RecQ helicase, is also able to function in the initiation of recombination by providing RecA protein with a suitable DNA substrate (96).

3.2.2. Bacteriophage T4

The initiation step in T4 phage recombination also requires processing of at least one DNA partner to produce ssDNA appropriate for UvsX protein function. The recombinogenic ssDNA is believed to be generated *via* cooperation between two reactions. Genetic data suggested that the gene 46 protein (gp46) and gene 47 protein (gp47) form an exonuclease which creates a ssDNA gap in a substrate containing a nick (97); recent *in vitro* results demonstrate that gp46 is both an endo- and an exonuclease, and that these activities are stimulated by gp47 (S. Morrical, personal communication). Subsequently, the gapped substrate is acted upon by a 5'→3' helicase, the gene 41 protein (gp41) (98), and its enhancer, the gene 59 protein (gp59) (99), to produce a free 3'-end that is used by UvsX protein for DNA strand invasion. Gp32 (the analogue of *E. coli* SSB protein) also functions in this unwinding reaction, since it

appears to form a protein complex with gp41 and gp59 (99,100).

3.2.3. ***S. cerevisiae***

In the budding yeast *S. cerevisiae*, homologous recombination commonly initiates at DSBs (101). DSBs are assumed to occur spontaneously in mitotically growing cells, but in meiosis the DSBs may be programmed, and they are associated with meiotic recombination hotspot activity (57,102-104). The current view is that DSBs are a consequence of cleavage by a nonspecific endonuclease at sites in the chromatin where the DNA is readily accessible (101,103,105,106). Formation of the DSBs requires at least the products of the *RAD50* and *SPO11* genes (table 1), since their formation is abolished by null mutations in either of these genes (57,107). Recently, it was shown that the Spo11 protein is likely to be the catalytic subunit responsible for formation of the DSBs (80,81). Also, it has become clear that additional gene products are involved in DSB formation (*e.g. MEI4* (108); *MER2* (109); *MRE11* (110,111); *REC102*, *REC104*, *REC114* (112); and *XRS2* (113,114)), since null mutations in any of these genes prevents DSB formation. Following DSB formation, 3'-single-stranded tails of about 600 nucleotides are produced *via* 5'→3' exonuclease activity (57,58,68). Resection of the 5'-strand termini at DSB's appears to require at least the Rad50, Mre11, and Xrs2 proteins since mutations in the corresponding genes result in cells that cannot process DSBs (106,107,114-116). Recently, all three of these proteins were shown to form a complex with one another (111), further supporting the possibility that they act together to form the nuclease responsible for resection of the DSB's.

3.3. Homologous pairing and DNA strand exchange

Following the production of a free 3'-ssDNA overhang, the following steps occur: 1) the RecA-like protein cooperatively binds to the free 3'-ssDNA end to form a nucleoprotein filament; 2) a quick and efficient search for homology occurs within the dsDNA recipient that results in the formation of a joint molecule; and 3) once the two DNA molecules are homologously aligned, the RecA-like protein then catalyzes the nascent exchange of strands to produce a Holliday junction.

3.3.1. ***Escherichia coli***

In *E. coli*, the RecA protein binds ATP, and then in combination with SSB protein, forms a continuous presynaptic filament on the ssDNA (a filament formed on dsDNA is shown in figure 5; discussed in Section V). The presence of SSB protein permits the formation of a continuous filament of RecA protein by destabilizing secondary structure (117-122). This nucleoprotein complex acts as the functional unit to perform a rapid and efficient search for homology within the dsDNA recipient, resulting in the formation of a joint molecule or D-loop. In the presence of SSB protein, the RecF, RecO, and RecR proteins (table 1), stimulate formation of the joint molecules, by helping RecA protein overcome the inhibition of binding by the SSB protein (123,124). The 3'-terminal end is more invasive in RecA protein-dependent pairing reactions because RecA protein polymerizes along ssDNA in a 5'→3' direction; thus, the 3'-end is more likely to be covered (125,126). Following joint molecule formation, RecA protein, perhaps with the aid of other proteins (for example, DNA polymerase I and/or a topoisomerase, table 1), catalyzes pairing of the remaining two DNA strands to produce a Holliday junction (for further details on the potential roles of DNA polymerase I and topoisomerases in homologous recombination, see reference (4)).

3.3.2. Bacteriophage T4

In T4 phage, UvsX protein forms a nucleoprotein complex with ssDNA (a filament formed on dsDNA is shown in figure 5). This nucleoprotein filament subsequently pairs with dsDNA in the presence of ATP (44,45). Homologous pairing is stimulated by the presence of the accessory factors, UvsY protein and gp32 (47,51) (figure 2B). The gp32 protein is envisioned to bind ssDNA first. Then, the UvsY protein binds to the ssDNA and helps load UvsX protein onto the gp32-ssDNA complex by assisting UvsX protein in displacing the bound gp32 (127). UvsY protein possesses two important properties that are important to this process: its intrinsic affinity for ssDNA is greater than that of gp32 (127) and it exists predominantly in hexameric form, the form which binds ssDNA (128). It was proposed that binding of ssDNA by the UvsY hexamer changes the conformation of the ssDNA (perhaps by wrapping the ssDNA around the hexamer). This change either permits nucleation of the UvsX protein presynaptic filament formation and/or destabilizion of gp32-ssDNA interactions so that gp32 is more readily displaced by UvsX protein (128). In addition, interactions between UvsY protein, gp32 and UvsX protein are important, but not essential, for optimal UvsX activity (129).

Once UvsX protein begins to cooperatively bind to the ssDNA, gp32 is gradually displaced. Following homologous pairing, joint molecules are converted to crossover intermediates (Holliday junctions) when UvsX protein exchanges strands between the two homologously paired substrates (44,45,49). The interactions between the gp32, UvsX, and UvsY proteins are a prototype for those between the SSB, RecA, and RecFOR proteins described above for *E. coli*, and for the interactions between the heterotrimeric replication protein A (RPA), Rad51, Rad52, Rad55, and Rad57 proteins described below for *S. cerevisiae*.

3.3.3. ***S. cerevisiae***

It is becoming clear that a number of proteins are involved at this step in *S. cerevisiae* (table 1 and figure 2C). Less obvious, however, is the precise role for each protein in this process. The yeast Rad51 protein has considerable homology to the *E. coli* RecA protein (59-61) and, recently, it was demonstrated that Rad51 protein, along with yeast RPA (the yeast ssDNA-binding protein), can promote DNA strand exchange *in vitro* (62-65). Similar to the RecA and UvsX proteins, Rad51 protein forms a nucleoprotein filament, which is the active species in DNA strand exchange (a filament formed on dsDNA is shown in figure 5; discussed in Section V). Therefore, it is likely that *RAD51* functions at this step of recombination. However, also important to this step, are the products of the *RAD52*, *RAD54*, *RAD55*, *RAD57*, and *RAD59* genes. Using a chromosomal inverted-repeat assay to study mitotic recombination in haploid yeast cells, Symington and coworkers showed that mutations in these genes reduce recombination efficiency by >3,000-, 25-, 20-, 33-, and 5-fold, respectively (76,77,130). In addition, the products of the

RAD51 and *RAD59* genes appear to act synergistically, since double mutants reduce recombination by 1200-fold (130). Finally, mutations in the gene of the large subunit of RPA (RPA1) produce a defective recombination phenotype (131,132). Recent *in vitro* studies begin to define the specific function of these accessory proteins: RPA stimulates formulation of the Rad51 nucleoprotein filaments (65); Rad52 protein, in combination with RPA, stimulates DNA strand exchange by the Rad51 protein (70,71,133); and, a heterodimer composed of the Rad55 and Rad57 proteins also stimulates Rad51-protein dependent homologous pairing, particularly when RPA must be displaced from the ssDNA (69). Finally, Rad54 protein interacts directly with the Rad51 protein and, in the presence of RPA, produces a significant stimulation in the Rad51 protein-dependent pairing rate, to a level comparable to that of the RecA and UvsX proteins (134).

Several studies provide direct evidence for interactions between a number of these proteins. First, the Rad51 and Rad52 proteins interact both *in vitro* (59) and *in vivo* (135). Second, interactions between the Rad51 and Rad55 proteins, and between the Rad55 and Rad57 proteins, were demonstrated (69,136). Third, there is now both *in vivo* and *in vitro* evidence that the Rad51 and Rad54 proteins directly interact (134,137,138). Finally, there is genetic evidence that the large subunit of RPA interacts with Rad52 protein (131,132). Existence of these interactions lead to the proposal that a complex of these proteins (termed a "recombinosome" (136) or "protein machine" (137)) is responsible for enacting homologous pairing and DNA strand exchange; though, it is also possible that there is not a stable complex *per se*, but rather a sequential progression of interactions between groups of proteins and/or individual proteins, similar to that described for the T4 system.

Though the manner by which they are formed and resolved is unclear, recently, double Holliday junctions were identified as the *in vivo* products of the pairing stage (139), lending additional support for the double-strand break repair model proposed earlier (see figures 1 and 2), since the model predicts such structures as intermediates (72,73).

3.4. DNA heteroduplex extension

Subsequent to Holliday junction formation, the nascent region of DNA heteroduplex undergoes branch migration. Although each of the RecA-like proteins discussed in this review can catalyze DNA heteroduplex extension, it is now appreciated that a class of specialized DNA helicases is more efficient than the RecA-like proteins in catalyzing this reaction (140-143). Therefore, it is possible that the RecA-like proteins play a limited role in branch migration.

3.4.1. *Escherichia coli*

Figure 2A shows that in *E. coli* there are several alternative means to extend the heteroduplex DNA. First, RecA protein has branch migration activity which is unidirectional and proceeds at 2-10 bp/sec (18). Second, a complex of the RuvA and RuvB proteins comprises a unique motor protein that also has branch migration activity (144,145), but which is approximately five-fold faster than that of the RecA protein (146,147). However, in contrast to RecA protein, the RuvAB proteins catalyze branch migration in either direction (146). Finally, the RecG protein, which is both a DNA and DNA/RNA hybrid helicase, can bind to and migrate Holliday junctions (148,149). Curiously, the RecG protein appears to antagonize the heteroduplex extension activities of RecA protein and the RuvAB proteins (150).

3.4.2. Bacteriophage T4

In T4 phage, DNA heteroduplex extension can be mediated in several different ways (figure 2B). Just as with RecA protein, the UvsX protein itself promotes polar branch migration at a rate of 12-15 bp/sec (45,50). In addition, the Dda protein can stimulate branch migration mediated by UvsX protein (151). The Dda protein is an ATP-dependent DNA helicase (152) which, like the RecG protein, has the ability to both extend and disrupt nascent joint molecules (153). Recently, however, the significance of this stimulation by Dda protein in the physiological process was brought into question when it was demonstrated that UvsX protein-mediated branch migration is strongly inhibited by the UvsY protein under physiological conditions (154). Under these conditions, branch migration is driven by the combined actions of gp41 and gp59, rather than by UvsX protein (154). The gp59 loads gp41 onto the strand exchange complex *via* direct interactions with gp32 (100,155). Finally, recent *in vitro* evidence shows that another protein, UvsW, has the potential to act at this step (156). UvsW protein, partially purified as a GST-fusion protein, displays ssDNA-dependent ATPase activity, and is a DNA helicase that acts on a branched DNA substrate. These observations support for the physiological function of UvsW protein being involved in DNA branch migration.

3.4.3. *S. cerevisiae*

In yeast, the Rad51 protein also possesses polar branch migration activity. Interestingly, heteroduplex extension occurs 3'→5' relative to the displaced strand, which is a polarity opposite to that of the RecA protein (63). If indeed a "recombinosome" exists, then one might expect that at least some of the other proteins are also involved at this step. One candidate is the Rad54 protein, which possesses both ATP binding and DNA helicase motifs (137,138); recently, Rad54 protein was shown to stimulate Rad51 protein-mediated DNA strand exchange (134). For the same reasons, the other candidate is the Rad55/57 heterodimeric complex. Though this complex appears to act at an early step of the recombination process (69), a later function remains a possible, additional role.

3.5 Resolution

The final step of recombination requires resolution of the Holliday junctions *via* nucleolytic cleavage. Symmetric cleavage of the junction produces recombinant progeny that are either patched products (exchange of ssDNA strands between the two starting substrates) or spliced products (exchange of flanking markers) (figure 2).

3.5.1. *Escherichia coli*

In *E. coli*, resolution of the Holliday junctions is performed by the RuvC protein (157,158). Since RuvC protein cleaves Holliday junctions symmetrically, both patched and spliced recombinants are produced. RuvC acts in concert with the RuvAB proteins to coordinate the steps of

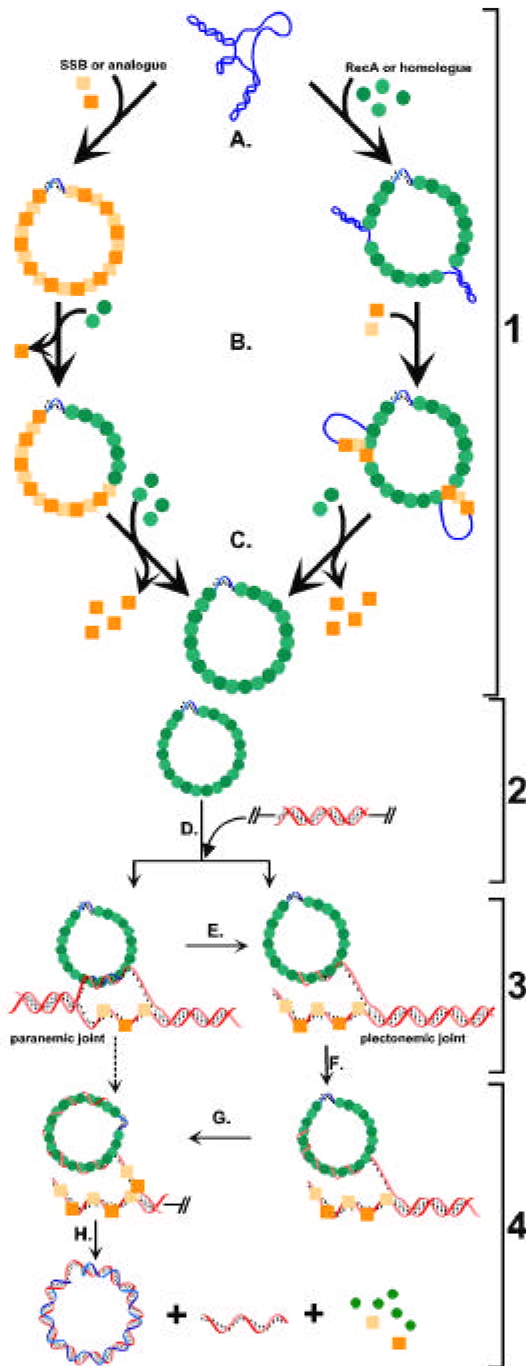

Figure 3. Kinetic steps of DNA strand exchange. The reaction between circular ssDNA and linear dsDNA is shown. The product is a nicked circle and a displaced single strand of DNA. A similar reaction scheme likely applies to all of the DNA strand exchange proteins discussed but reaction polarity is not indicated; the illustration is adapted from Kowalczykowski (185). The steps shown are: 1-presynapsis; 2 and 3-synapsis; and 4-DNA heteroduplex extension. The green spheres represent the strand exchange protein; the orange squares represent ssDNA binding proteins.

branch migration and Holliday junction resolution. Recently, the late stages (DNA heteroduplex extension *via* the RuvA and RuvB proteins, and resolution by the RuvC protein) of recombination in *E. coli* were reconstituted *in vitro* (159).

3.5.2. Bacteriophage T4

The most likely candidate for Holliday junction resolution in T4 phage is endonuclease VII (gp49). This enzyme cleaves branched DNA molecules (41) and four-way Holliday junctions (160). Cleavage of synthetic four-way junctions is symmetrical and is organized around the junction point on opposite sides (161,162). While both T4 endonuclease VII and RuvC protein perform the same activity, there is no strong homology shared between the two proteins (163). This could help explain the difference in the specificity of cleavage of the two proteins. RuvC protein is specific for four-way junctions, while T4 endonuclease VII will cleave not only four-way junctions, but also Y-junctions (164) and heteroduplex loops (165) as well.

3.5.3. *S. cerevisiae*

Resolution of the intermediate with two Holliday junctions in yeast would yield either spliced or patched products depending on how the junctions were cleaved (figure 2C). If the two junctions are cleaved in opposite orientations, this would generate spliced products. However, if they are cut in the same orientation, patched products would be produced. At this time, there is no obvious candidate for this activity. Two partially purified enzymes, termed endo-X1 (166) and endo-X2 (167), were shown to cleave cruciform-containing DNA; however, neither enzyme was purified to homogeneity. Subsequently, another gene, *CCE1* (Cruciform Cutting Endonuclease 1), was identified during a screen of yeast mutants which were unable to cleave cruciform structures (168). The product of the *CCE1* gene was shown to be the same as endo-X2 (168). Though Cce1/endo-X2 displays the ability to cleave Holliday junctions *in vitro*, the activity is mitochondrial, rather than nuclear, in origin (169).

4. MECHANISM OF DNA STRAND EXCHANGE

As is evident from the preceding, RecA protein and its analogues mediate a reaction, DNA strand exchange, that is central to homologous recombination. The enzymatic properties of the in vitro strand exchange activity of RecA protein have been studied in great detail (4,28,170) and a summary of the biochemical properties of the DNA strand exchange proteins discussed herein is shown in table 2. Below, we detail the steps of protein-promoted DNA strand exchange.

DNA strand exchange catalyzed by RecA protein occurs by a number of kinetically distinct phases that can be subdivided into at least three experimentally distinguishable steps: (1) presynapsis, (2) synapsis and (3) DNA heteroduplex extension (figure 3). Presynapsis is the step where RecA protein assembles onto the ssDNA to form the nucleoprotein species that is active in the homology search. Synapsis is characterized by initially random non-homologous contacts occurring between the presynaptic complex and naked dsDNA (termed coaggregates), the search for homology, homologous pairing, and finally conversion to either

paranemic or plectonemic joint molecules (discussed below in the synapsis section). DNA heteroduplex extension, as discussed earlier, is the polar migration of the nascent DNA heteroduplex joint.

DNA strand exchange occurs between two DNA molecules provided that the following criteria are met: (1) one of the DNA molecules contains a region of ssDNA; (2) the ssDNA region occurs at a site homologous to the other dsDNA molecule; and (3) for topological reasons, one of the substrate molecules has an end. A reaction that has become a model for mechanistic studies involves the exchange between circular ssDNA (ϕX174 or M13 phage) and linear dsDNA (figure 3). The products are nicked, circular dsDNA and linear ssDNA (171,172).

4.1 Presynapsis (figure 3, Step 1)

Presynapsis is the ordered assembly of the RecA protein homologue on ssDNA to produce a nucleoprotein complex that is the active species in DNA strand exchange (filaments formed on dsDNA are shown in figure 5 and discussed in Section V). This process occurs by either of two pathways: first, the single-stranded DNA binding protein (SSB; RPA; UvsY and/or gp32) binds to ssDNA (figure 3, step 1A); this is followed by the subsequent binding of the DNA strand exchange protein (RecA; UvsX or Rad51) and displacement of the single-stranded DNA binding protein to form the presynaptic filament (figure 3, steps 1B and 1C, left panel). In the second pathway, the DNA-strand exchange protein binds to the ssDNA substrate, forming an incomplete nucleoprotein filament due to limitations imposed by DNA secondary structure (step 1A, right panel). The single-stranded DNA binding protein binds, removes the secondary structure and is then displaced by further binding of the DNA strand exchange protein (steps 1B and C). The net result of either of these two pathways, is the formation of a complete nucleoprotein filament that involves direct contact by the primary DNA-binding site of the strand exchange protein, and results in the generation of a contiguous secondary DNA-binding site that is essential for homologous pairing and DNA strand exchange (173,174).

4.1.1. *Escherichia coli*

Filament formation by RecA protein is dependent on DNA length, DNA composition, and the amount of secondary structure within the ssDNA substrate (95,175-178). The ATP-bound form of the protein is needed to constitute the active form of the nucleoprotein filament. ATP greatly stabilizes the complex, and transforms RecA protein into a DNA-binding state with high affinity for ssDNA (120). The RecA nucleoprotein filament is capable of hydrolyzing ATP with a k_{cat} of 18-30 min^{-1}, a value that is affected by ssDNA length and sequence (117,177-179). However, DNA serves only as a scaffold to promote filament formation since RecA protein can be activated by high salt concentrations to form both filaments and to hydrolyze ATP (180-182). Though RecA protein is an ATPase, ATP hydrolysis is not essential for DNA strand exchange (64,183,184). Stoichiometric amounts of RecA protein, relative to ssDNA concentrations, are required for filament formation, with maximum DNA strand exchange rates occurring when filaments are formed at a ratio of at least 1 RecA monomer to 3 nucleotides of ssDNA. This stoichiometric requirement indicates the importance of a saturated, contiguous RecA protein-ssDNA filament in the DNA strand exchange process (185).

In addition to ATP, ATP-γ-S and dATP also support presynaptic filament formation and are capable of transforming RecA protein into the high affinity ssDNA-binding state, the active form of the protein (179,186). This complex, the presynaptic filament, is the physiologically relevant species in RecA protein-mediated cellular processes. Filament assembly onto ssDNA occurs in a 5'→3' direction (187), which is the same direction as branch migration. The resulting filament has a regular structure and the prominent feature is a large helical groove, with 6.2 monomers per helical turn and each monomer interacting with 3 bases of DNA (figure 5) (188).

The SSB protein effects the binding of RecA protein to ssDNA, greatly stimulating the DNA strand exchange process (117,189). The role of SSB protein in presynapsis is to remove secondary structure from ssDNA, which is inhibitory to the formation of the saturated presynaptic complex (117,119-122). This is consistent with the role of the protein as a helix-destabilizing protein and the observation that other helix destabilizing proteins can substitute for SSB protein in DNA strand exchange *in vitro* (119).

4.1.2. Bacteriophage T4

Presynaptic complex formation by the T4 phage proteins is similar, but features an added complexity, the UvsY protein. During presynapsis, UvsX binds to ssDNA cooperatively with a stoichiometry of one UvsX monomer for every 3 to 5 nucleotides of ssDNA to form a nucleoprotein filament (46,47). In contrast to RecA protein, the binding of ATP does not stabilize the UvsX protein-ssDNA presynaptic filament under steady-state conditions for hydrolysis of ATP; however, the binding of ATP-γ-S does (190). This suggests that ATP-γ-S increases the equilibrium binding affinity of UvsX protein for ssDNA which is very similar to what is observed for RecA protein (179,191). However, the UvsX protein-ssDNA complex is more vigorous in hydrolyzing ATP (the rate of ATP hydrolysis is 8 to 10-fold higher) (44), making its presynaptic filament more dynamic; this may explain why ATP does not stabilize the UvsX-presynaptic complex, (*i.e.*, the UvsX-ssDNA-ATP complex is short lived) and why an accessory protein like UvsY protein is needed. In addition, UvsX protein is unique among RecA protein analogues studied to date, in that it produces both ADP + P_i and AMP+ PP_i (44). The significance of the AMP production is presently unknown, however.

The formation of the UvsX protein presynaptic filament is facilitated by the presence of both gp32 and UvsY protein (a UvsX-dsDNA nucleoprotein filament is shown in figure 5B and is discussed in Section V). Gp32 enhances filament formation by removing secondary structure native to the ssDNA (45,51). UvsY facilitates filament formation by stabilizing the presynaptic complex itself (51,192,193). Although gp32 is the T4 phage counterpart to the *E. coli* SSB protein, there is no structural homologue of the UvsY protein in the *E. coli* recombination system; however, the RecF, O, and R proteins may comprise a functional homologue (123,124,194-198).

4.1.3. *S. cerevisiae*

Presynaptic filament formation by Rad51 protein results in a stoichiometric nucleoprotein filament that resembles the filament formed by both the RecA and UvsX proteins (figure 5C, discussed in Section V) (63). This complex is the active species in DNA strand exchange (63). Optimal reaction rates are achieved with a stoichiometry of 3 nucleotides per Rad51 monomer (63,199). Binding to ssDNA is stabilized by either ATP (59) or the non-hydrolyzable analogues of ATP, ATP-γ-S and AMP-PNP (64). Rad51 protein filament formation is stimulated by the RPA heterotrimer (63,65), which is the eukaryotic analogue of the *E. coli* SSB and T4 gp32 proteins. In contrast to both the RecA and UvsX proteins, very little ATP is hydrolyzed by the Rad51 nucleoprotein complex (k_{cat} = 0.73 min^{-1}) during presynapsis or during DNA strand exchange (64); interestingly, some of the enzymatic characteristics of Rad51 protein more closely parallel those of a mutant RecA protein, RecA K72R, which has a reduced level of ATP hydrolysis (~ 600-850-fold reduced to a k_{cat} of 0.032min^{-1}) yet nonetheless, is able to promote DNA strand exchange.

4.2 Synapsis (figure 3, Steps 2, 3)

Once the presynaptic filament has assembled on ssDNA, synapsis ensues. In this stage of the reaction, a dsDNA molecule must be bound to the filament, homology to the ssDNA within the filament located within the dsDNA, and a plectonemic heteroduplex joint formed. For this phase of DNA strand exchange to occur, a second DNA molecule must bind in a sequence-independent fashion to the secondary DNA binding site of the nucleoprotein filament; presumably, the close approach of this second DNA molecule is facilitated by shielding of the negatively charged phosphodiester backbones of the two DNA molecules by the DNA strand exchange protein. Once dsDNA is bound, the search for homology takes place. The search is rapid, and requires that the binding of dsDNA to the secondary site be both weak and transient. The recognition of homology takes place when the ssDNA within the presynaptic filament hydrogen bonds, *via* non-Watson-Crick base pairing, to either the major or minor groove of the bound dsDNA, in a mechanism that does not require triplex intermediate formation. This alignment provides a signal to the strand exchange protein that homology has been located; base switching occurs, and the heteroduplex dsDNA occupies the primary site whereas the displaced strand occupies the secondary site. If the location of homology occurs in the center of the dsDNA, a paranemic joint forms; if this occurs at the dsDNA molecule ends, a plectonemic joint forms. Due to their inherent instability, paranemic joints must be converted to plectonemic joints in order to survive and become recombinant DNA structures.

4.2.1. *Escherichia coli*

Once an active presynaptic complex forms, the complex can then pair the ssDNA within the filament to dsDNA. On the basis of probability, this initial contact must be at a site of non-homology. Indeed, RecA protein is capable of forming a complex between ss- and dsDNA, or between ssDNA molecules with little or no sequence complementarity (173,200-203). These complexes, called coaggregates, contain many DNA molecules arranged together in a three-dimensional network that can be sedimented in a low speed centrifuge (204,205). They form rapidly, and kinetically precede joint molecule formation. Thus, it has been inferred that coaggregates, or a related complex, of RecA protein and ss- and dsDNA are intermediates on the pathway of the strand exchange reaction (204,205).

Although RecA protein binds ssDNA in a sequence-independent manner (though compositional preferences exist (95,176,206,207)), the problem of the homology search is analogous to the problem that sequence-specific DNA binding proteins face in locating their target sequence (208). However, for the case of RecA protein and its analogues, the scale of the search problem is much larger: the binding protein is the entire nucleoprotein filament (which can consist of thousands of protein monomers, depending on the ssDNA length), and the target is the unique complementary sequence within the entire genome. Although the entire nucleoprotein filament is involved in the search for homology, the minimum length of homology required for recognition is as low as 15 nucleotides *in vitro* (95), which is somewhat less than that needed *in vivo*, where homologous recombination requires minimally, about 23-40 bp of homology (209,210).

For homologous alignment to occur, a second DNA molecule must bind to the secondary DNA binding site of the RecA protein filament. This binding, although not sequence specific, does have a hierarchy: optimal binding occurs with oligopyrimidinic DNA while poorer binding is observed with oligoadenylic DNA (173). This hierarchy of binding is not due to the sequence of the ssDNA present in the primary site, but rather is due to an intrinsic property of RecA protein (173). Thus, the proposal that homologous recognition occurred by a novel form of non-Watson-Crick base-pairing called "self-recognition" (211,212) is not substantiated (173,174).

How then does RecA protein "sense" when homology has been located? Though many details of the homology search remain unknown, several steps are clear. During the search, the binding of a second DNA molecule to the nucleoprotein filament must, by necessity, be both weak and transient, to facilitate a rapid search. When the homologous locus is found, the dsDNA is unwound, and one of the strands of the duplex pairs with its complement (which is bound to the primary, original DNA-binding site of the pairing protein). The other strand is displaced into the secondary DNA-binding site, where it is bound more tightly than the duplex DNA. During the search for homology, the DNA duplex remains base-paired; it is only when homology is located, that strand switching occurs. The recognition of homology between the ssDNA within the filament and either the minor groove of dsDNA (213-215), or the major groove of the dsDNA, provides the signal to RecA protein that homology has been located (173). Immediately after base-pair switching, the heteroduplex dsDNA product would occupy the primary site, whereas the displaced ssDNA would occupy the secondary site; there is evidence to support this scenario (174,214,216,217).

Once the two DNA molecules are homologously aligned, RecA protein then catalyzes the nascent exchange of

strands. In the prototypical *in vitro* reaction depicted in figure 3, this requires a local denaturation of the dsDNA molecule and the subsequent exchange of the identical single strands of DNA. These steps may be simultaneous or separated in time, but the result is the production of an intermediate known as a joint molecule. Two types of joint molecules may form, depending on the topological constraints of the DNA: either paranemic or plectonemic. A paranemic joint is one in which the individual complementary strands do not intertwine, producing a molecule that is base-paired but not topologically linked; whereas, a plectonemic joint is one in which the incoming single strand is intertwined around its complement as in native dsDNA. In the reaction displayed in figure 3, paranemic joints will form in the interior of the duplex substrate, and plectonemic joints will form at the ends of the duplex substrate. An experimental distinction between paranemic and plectonemic joints is that paranemic joints require RecA protein (or its counterpart) for stability whereas plectonemic do not. Since paranemic joints are statistically more probable than plectonemic, paranemic molecules are likely intermediates on the reaction pathway to the formation of more stable plectonemic joint molecules (218,219).

Both the type and efficiency of joint molecule formation are affected by SSB protein. If SSB protein is omitted, the displaced ssDNA is used by RecA protein to form a second joint molecule with another dsDNA molecule; this results in the formation of complex, homology-dependent networks of joint molecules. In the presence of SSB protein, RecA protein does not form these networks, since SSB protein prevents re-invasion events by binding to the ssDNA displaced from the joint molecules; in addition, the yield of joint molecules is greater (220). However, under reaction conditions where RecA protein is better able to compete with SSB protein (*i.e.* in the presence of the volume excluding agents polyethylene glycol or polyvinyl alcohol; or when dATP is used as cofactor), networks are readily formed (186,221,222). Thus SSB protein acts both at the pre- and post-synaptic steps of DNA strand exchange.

4.2.2. Bacteriophage T4

Some aspects of synapsis are similar for UvsX protein and RecA protein: 1) UvsX protein coaggregates non-homologous ssDNA and dsDNA (47); and 2) UvsX protein catalyzes formation of approximately equal numbers of paranemic and plectonemic joint molecules (223). Differences also exist between the two proteins. First, while both RecA protein and UvsX protein form homology-dependent DNA networks in the absence of a single-stranded DNA-binding protein, UvsX protein also forms networks even in the presence of gp32, but this may simply reflect an increased ability of UvsX to displace gp32 (50). Second, since UvsX protein, unlike RecA protein, binds readily to either ssDNA or dsDNA under normal strand exchange conditions (46), joint molecule formation is reduced due to UvsX protein binding to the dsDNA (223); this behavior more closely resembles that of Rad51 protein (see below). This limitation is overcome *in vitro* by using limiting amounts of UvsX protein relative to the DNA (45).

4.2.3. *S. cerevisiae*

As for UvsX protein, joint molecule formation is inhibited if Rad51 protein is allowed to bind dsDNA and can be avoided by assembling presynaptic filaments using exactly stoichiometric amounts of Rad51 protein, or by using excess dsDNA (63). This inhibition of joint molecule formation results from the rapid and kinetically stable binding of Rad51 protein to dsDNA, a situation that is very different from the behavior of RecA protein.

Rad51 protein initiates joint molecule formation if the linear dsDNA contains a single-stranded overhang at least 2 nucleotides in length that is complementary to the ssDNA within the presynaptic filament (199). Both joint molecules and nicked circle products are reported to form more efficiently with dsDNA containing a 3'-overhang than with a 5'-overhang (199).

4.3 Branch migration (figure 3, Step 4)

Once the plectonemic joint has formed, the branch migration phase of DNA strand exchange commences. During this phase, the nascent, heteroduplex joint is extended until complete exchange of single strands of DNA occurs, resulting in a nicked, double-stranded circle. Though kinetically distinct, branch migration may not be a mechanistically separate step, but rather may represent a continuation of plectonemic joint molecule formation (219,224).

4.3.1. *Escherichia coli*

For RecA protein, branch migration proceeds in a 5'→3' direction relative to the incoming single strand (the same direction as RecA protein polymerization), at a rate of 2-10 bp sec^{-1} (171,225), requires ATP hydrolysis (19,226) and induces torsional stress in the dsDNA (227,228). Branch migration is relatively tolerant of nucleotide sequence mismatches (229,230) and DNA lesions (231,232), although the reaction is inhibited 60% by heterologous insertions of 140 bp within the dsDNA substrate and by insertions of 1000 nucleotides within the ssDNA substrate (230,231).

The ability of RecA protein to not only promote DNA heteroduplex extension, but also to extend the DNA heteroduplex beyond limited regions of sequence non-homology (heterology), may serve a valuable biological function, despite the existence of specialized branch migration helicases like the RuvAB proteins (233). For this reason, the mechanism by which RecA protein facilitates the bypass of DNA sequence heterology is an important issue. Several studies established the key requirements for this heterology-bypass (234,235) (D.A. Kitchell and S.C.K., unpublished observations). RecA protein can facilitate bypass through a heterology as large as 140 bp within the dsDNA substrate (230,231), provided that these mismatched sequences are beyond the point at which DNA strand exchange initiates (*i.e.*, the heterology needs to be beyond the 5'-end of the strand being displaced from the dsDNA). However, RecA protein is unable to bypass an insert of only 22 base pairs that is at the distal end of the dsDNA (234). In all cases, the ability of RecA protein to promote DNA heteroduplex extension progressively diminishes as the length of homology distal to the heterology decreases. Furthermore, when the length of the heterology increases, the amount of distal homology required to bypass this heterology increases, apparently to compensate for the longer heterologous distance that must be traversed.

Thus, to bypass a heterology, DNA strand exchange must initiate in a region of DNA sequence homology, and there must be additional homology beyond the heterology. ATP-γ-S could not substitute for ATP in these bypass experiments, demonstrating that ATP hydrolysis was necessary, presumably to permit some dissociation/redistribution of the RecA nucleoprotein filament.

Several mechanisms were proposed to explain these important characteristics. One envisioned that once the heterology was encountered, lateral slippage of the exchanging strand within the RecA nucleoprotein filament would permit re-alignment of the homologous sequences (234). A second mechanism envisioned that facilitated rotation of the DNA substrates about each other mediated by the RecA protein filament in an ATP-hydrolysis coupled mechanism, provided the "motor" that rotated the DNA past the heterology; the specific details of this model were elegantly elaborated previously to which the reader is referred for details (235). Here we would like to present an alternative idea, based on these collective data and unpublished considerations (D.A. Kitchell and S.C.K., unpublished observations).

Figure 4 shows how topological stress, resulting from local unwinding of dsDNA that is constrained in a closed topological domain, can facilitate bypass through a heterology. The basic premise of the topological model is that, homologous pairing involves intermediates (the synaptic complexes) in which the dsDNA is highly unwound. Furthermore, in the presence of ATP, the RecA nucleoprotein filament that participates in DNA strand exchange is dynamic, and it is frequently binding and releasing dsDNA in its quest for proper DNA sequence alignment. Electron microscopy shows that the RecA nucleoprotein filament can envelop kilobase pair-lengths of homologous dsDNA (216), and that the binding of dsDNA by the RecA nucleoprotein filament produces a topological unwinding of the dsDNA from the canonical 10.4 bp/turn to 18.6 bp/turn (236-241,242 (figure 4)). Thus, in the static situation, when the RecA nucleoprotein filament envelops dsDNA, the dsDNA including the heterology is extensively unwound by up to 8.2 bp/turn resulting in 18.6 bp of dsDNA/filament turn versus 10.4 bp turn in B-form dsDNA (figure 4C). However, in the dynamic case, in the presence of ATP, if the RecA nucleoprotein filament were to release a medial region of the dsDNA while the two flanking regions of the dsDNA remained bound to the filament, then the binding energy that maintained the bound dsDNA in its unwound form would be absent, and the released dsDNA segment would be both topologically constrained and highly underwound (figure 4D, left side); in fact, the release of dsDNA from just 2 turns of the filament would result in 16.4 bp of unwinding. Thus, the transient release of dsDNA from 20 turns of the filament would produce a domain that is unwound by 164 bp relative to B-form DNA. If this unwinding occurred in a region of dsDNA heterology, then there would be sufficient, locally trapped unwinding to induce transient strand separation of the dsDNA (figure 4D, right side), and to bypass the heterology in a kinetically stochastic way. With the region of dsDNA heterology locally melted, bypass would readily occur upon subsequent re-polymerization of the filament (figure 4E); however, if a nick were located in the region of heterology, then the superhelical strain is released and no bypass occurs. This model explains the key experimental requirements for bypass (the requirement for increasingly longer lengths of homology distal to the heterology, the need for ATP, and the inhibitory effect of a dsDNA nick), and it lends insight into how RecA protein, and presumably all of the other DNA strand exchange proteins (since they all extend dsDNA when bound) can mediate bypass of short regions of DNA sequence heterology without the need for specialized branch migrations proteins.

4.3.2. Bacteriophage T4

UvsX protein, like RecA protein, catalyzes branch migration such that DNA strand displacement is 5'→3' relative to either the incoming presynaptic filament or the displaced strand (45,50). However, UvsX protein catalyzes branch migration at a rate of 12-15 bp sec^{-1} (50,151), which is faster than the rate catalyzed by RecA protein (see above).

4.3.3. *S. cerevisiae*

In contrast to RecA and UvsX proteins, Rad51 protein promotes strand exchange with the opposite polarity (i.e. 3'→5' relative to the incoming single strand (63)) at a rate of 0.5 bp sec^{-1}, and has no requirement for ATP hydrolysis at any stage (64). In addition, the rate of formation of both joint molecule intermediates and products in the reaction is significantly reduced compared to that of either the RecA (2-10 bp sec^{-1}) or UvsX proteins (12-15 bp sec^{-1}) (62-64). Also, this stage of the reaction is inhibited by a 458 bp heterologous insertion in the dsDNA substrate (63).

4.4. ATP hydrolysis in DNA strand exchange

DNA strand exchange requires the presence of a nucleoside triphosphate cofactor, usually ATP. Under standard conditions, ATP hydrolysis coincides with the pairing and exchange of strands of DNA. Initially, it was thought that ATP hydrolysis was a requirement for DNA strand exchange. It is now known that neither the hydrolysis of ATP (183,226) nor the presence of a high-energy phosphate bond (184) are necessary for DNA strand exchange to occur. It is the formation of the high-affinity ssDNA-binding state of RecA protein that is necessary for DNA strand exchange; the formation of which is brought about by ATP binding to RecA protein bound to DNA. The binding of the non-hydrolyzable analogue of ATP, ATP-γ-S, or the non-covalent complex of ADP-AlF^{-}_{4}, can also induce the high-affinity DNA-binding state of RecA protein. Although the hydrolysis of ATP is not required for the exchange of DNA strands, it is required at phases of DNA strand exchange that require the dissociation of RecA protein that is induced by ADP, the product of ATP hydrolysis.

4.4.1. *Escherichia coli*

RecA protein is a DNA-dependent ATPase with a single active site for the binding and hydrolysis of ATP and other nucleoside triphosphates (17,206,243-245). The enzyme is able to hydrolyze ATP with either ss- or dsDNA as cofactor, resulting in a k_{cat} with ssDNA of 18 - 30 min^{-1} and with dsDNA of 19 to 22 min^{-1} (178,246,247). The active species in ATP hydrolysis is the nucleoprotein filament, with ATP being hydrolyzed uniformly throughout the filament and

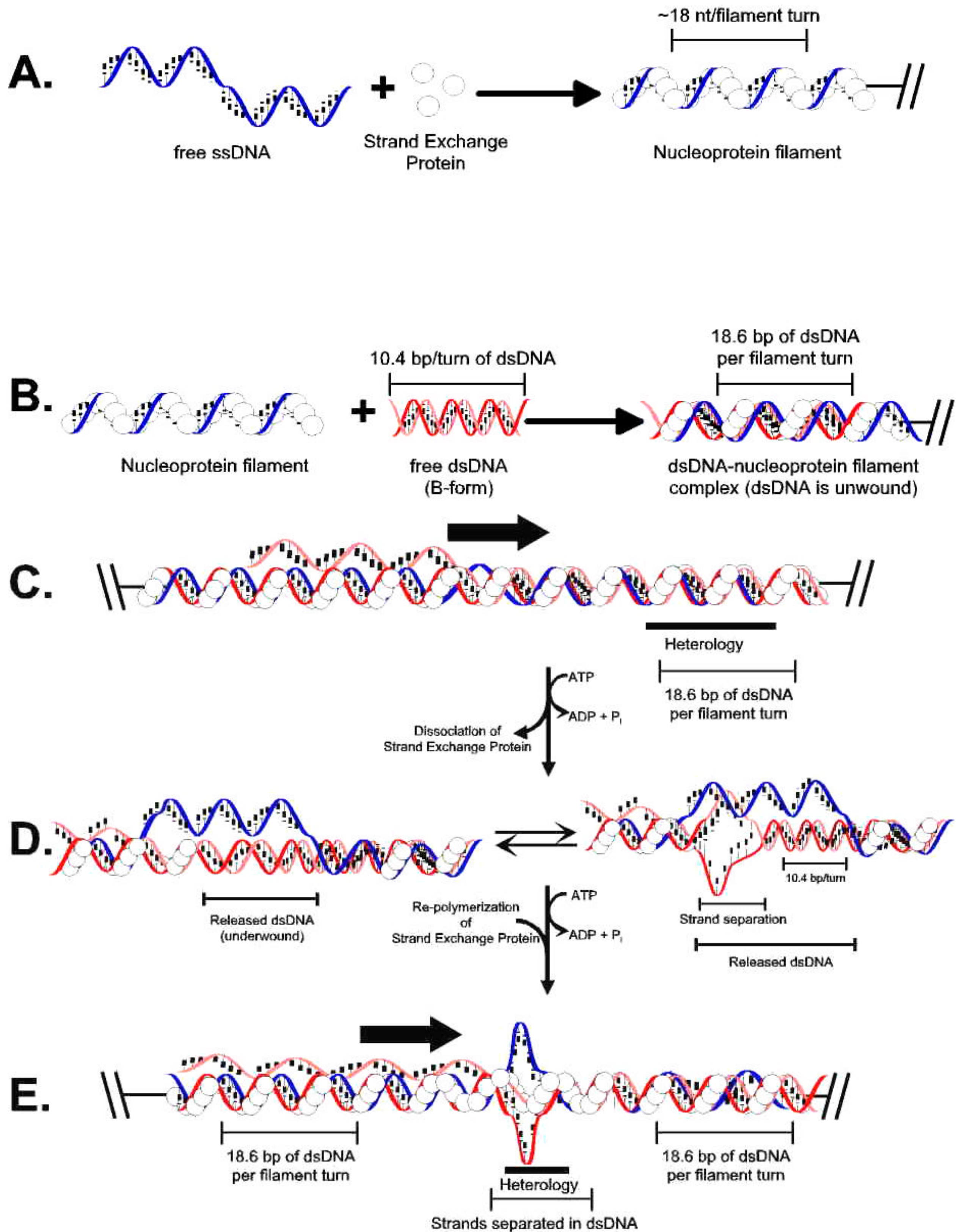

Figure 4. Model for heterology bypass by RecA protein. A pairing event involving ssDNA (drawn in blue) and dsDNA (drawn in red/pink) containing a region of heterology, is shown. The spheres represent RecA protein; the large black arrow indicates the direction of migration of the heteroduplex joint. Details of this model are discussed in the text.

with no detectable enhancement at filament ends (117,177,248). Hydrolysis of ATP results in one or more conformational changes in RecA protein. ATP and ADP serve to modulate RecA between the "high-affinity" DNA-binding and "low-affinity" DNA-binding states, respectively (179,191). It may not be not the hydrolysis *per se* that modulates the two states, but rather the release of P_i, which is extremely rapid (249). This proposal is supported by experiments utilizing the ground state analogue, ADP-AlF_4^-, which maintains RecA protein in the high-affinity ssDNA-binding state in the absence of a high-energy bond (184,250).

Although ATP hydrolysis occurs throughout DNA strand exchange promoted by RecA protein, it is not required for all of the stages (19,184,219,226,251-254). ATP hydrolysis is not required for the exchange of DNA strands (184,250,255). However, ATP hydrolysis is required to dissociate RecA protein from the heteroduplex products once the reaction is complete (226,252,256), to facilitate the bypass of structural barriers such as heterologous sequences (253,254), and to maintain reaction polarity (125,257). In addition, ATP hydrolysis is required for DNA strand exchange involving four strands (258).

Wild-type RecA protein is able to promote strand exchange using the non-hydrolyzable analogue ATP-γ-S (226). In addition, a mutant RecA protein, K72R, in which ATP hydrolysis is reduced by approximately 600-850-fold, is able to promote homologous pairing and exchange of up to 1.5 kilobase pairs of DNA (183). In both of these situations, extension of the heteroduplex is blocked (*i.e.* joint molecule intermediates form but are not converted to gapped or nicked dsDNA products) and the reaction is bi-directional (257), which is in contrast to that of the ATP reaction, which has a distinct polarity (171). However, a nucleoside triphosphate is not an essential component of the pairing and exchange process, because a complex of ADP and AlF_4^- activates all of the activities of RecA protein (184,250,255).

4.4.2. Bacteriophage T4

UvsX protein, in contrast to RecA protein, has an absolute requirement for ATP hydrolysis in DNA strand exchange (259). The rate of ATP hydrolysis by UvsX protein is 8- to 10-fold higher than that of RecA protein, with a k_{cat} of 145 - 240 min^{-1} (44), and this activity is inhibited by both ADP and ATP-γ-S (259). In the presence of 2 mM ATP-γ-S, UvsX protein is unable to promote strand exchange; however at 16 and 80 μM ATP-γ-S, UvsX protein can form intermediates (259). This may simply reflect the situation that existed for RecA protein prior to the realization that the ATP-γ-S reaction had a unique reaction optimum (184). Substitution of ATP-γ-S for ATP allows UvsX protein to bind ssDNA, unwind dsDNA (259), and promote DNA strand annealing (45). Addition of ATP-γ-S to an ongoing DNA strand exchange reaction, initiated with ATP, causes a transient increase in the rate of branch migration (50). This transient stimulation, which occurs due to inhibition of ATP hydrolysis, is attributed to a kinetic stabilization of the active filamentous form of UvsX protein. The high turnover of the UvsX protein nucleoprotein filament appears to limit the steady-state rate of branch migration (259).

4.4.3. *S. cerevisiae*

Positioned at the opposite end of the ATP hydrolysis spectrum is the eukaryotic Rad51 protein. Although Rad51 protein does not require ATP hydrolysis at any step of DNA strand exchange *in vitro* or *in vivo* (64), it does hydrolyze ATP with a k_{cat} of 0.73 min^{-1} (64). Rad51 protein is able to promote both intermediate formation and directional DNA strand exchange using either ATP-γ-S or AMP-PNP, two non-hydrolyzable analogues of ATP (64). In addition, a Rad51 mutant protein, K191R, (the analogue of the RecA K72R mutant protein), is defective for ATPase activity *in vitro*, yet it can promote polar DNA strand exchange (64). Interestingly, the mutant Rad51 protein is phenotypically normal when expressed in yeast cells, demonstrating that ATP hydrolysis is unnecessary for Rad51 protein function (64).

This raises the question of why such a large difference exists between these three proteins in the rates of ATP hydrolysis during DNA strand exchange? Since ATP hydrolysis is not needed for DNA strand exchange, at least two possible explanations exist. First, there may be a relationship between the requirement for ATP hydrolysis and the genetic complexity of the organism. Such a relationship would exist if the presynaptic filament was required to persist for the longer times needed to find DNA sequence homology in the more complex organism: those with the most complex genome would require the more stable presynaptic complex which would exist if the ATP turnover were lower. In accord with this idea, T4 phage which has the least complex genome, has the highest rate of ATP hydrolysis; *E. coli* which has an intermediate complexity, has a corresponding intermediate rate of ATP hydrolysis; and *S. cerevisiae*, which has a more complex genome, has little or no ATP hydrolysis. Alternatively, the requirement for ATP hydrolysis may be related to the length of the organism's cell cycle: T4 undergoes rapid growth after infection; *E. coli* has a doubling time of 20 to 30 minutes; whereas *S. cerevisiae* has a doubling time ranging from 90 to 140 minutes. Since disassembly of the presynaptic complex is also presumably important to a cell's metabolism, the cells with the fastest growth rate should have a pairing protein with the highest ATP turnover. Both views may be correct: since it should take a longer time to identify homology in a more complex genome, it is reasonable to expect that the presynaptic filament should be kinetically more stable. Furthermore, since ATP hydrolysis is also used to remove the DNA strand exchange protein from the DNA heteroduplex product, the more slowly growing organism may simply have the luxury of waiting for a slower dissociation process.

5. STRUCTURAL COMPARISON

In this section, we compare the form of the nucleoprotein filaments for each protein. We also compare the location of conserved amino acid sequences within the context of the crystal structure of RecA protein, using homology-dependent structural modeling for the Rad51 and UvsX proteins. Although the three proteins do form structurally similar nucleoprotein filaments (figure 5), and they do have significant homology at the amino acid level, they are significantly different in key regions, making each protein unique.

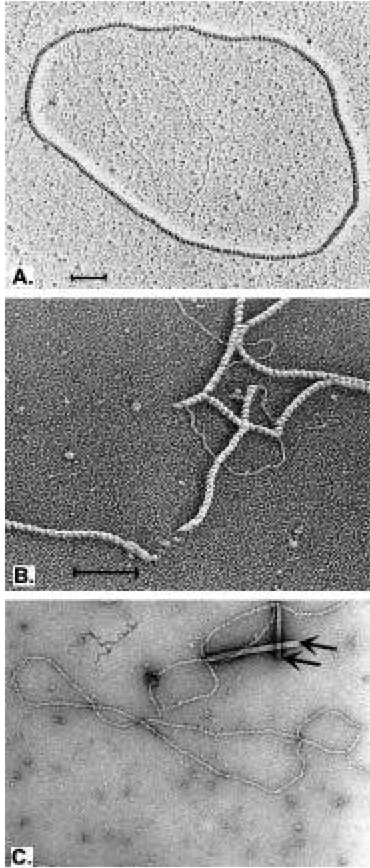

Figure 5. Electron micrographs of nucleoprotein filaments formed on dsDNA. (A) RecA nucleoprotein filament assembled on nicked, circular dsDNA. Filaments were formed by incubating RecA protein with the dsDNA substrate in the presence of ATP for 20 minutes, followed by the addition of ATPγS for an additional 60 minutes to stabilize the complex. The bar in this figure represents 0.1 nm. The striations visible are due to the helical nature of the filament, with the darker bands corresponding to groove in the filament where dsDNA and repressor binding occurs (237). Reprinted with permission from Nature 299, 185-186, Copyright (1982) Macmillan Magazines Limited. (B) UvsX nucleoprotein filament assembled on linear M13 dsDNA. The complexes were formed by incubating UvsX protein with the DNA substrate in the presence of ATP for 8 minutes, followed by fixation and staining with uranyl acetate (46). The scale is not the same as in (A); though the bar represents 0.1 nm. Reprinted with permission from the Journal of Biological Chemistry 260:4484-4491, Copyright (1985) The American Society for Biochemistry and Molecular Biology. (C) Rad51 nucleoprotein filament assembled on nicked, circular ϕX dsDNA. Filaments were formed in the presence of ATP for 60 minutes, followed by the addition of ADP-AlF^-_4 for an additional 30 minutes to stabilize filaments. The filament shown was negatively stained with uranyl acetate (263). The tobacco mosaic virus particles (indicated by the arrows), are approximately 200 Å in diameter. Reprinted (abstracted/excerpted) with permission from Science 259:1896-1899. Copyright (1993) American Association for the Advancement of Science.

5.1. Filament structure

5.1.1. *Escherichia coli*

In the presence of either ATP, ATP-γ-S, or ADP-AlF^-_4, RecA protein polymerizes onto either ss- or dsDNA in a highly cooperative, polar fashion (187,236,260,261). On dsDNA, the resulting helical nucleoprotein filament (figure 5A) contains 6.2 RecA protein monomers per turn, has a pitch of 95 Å and is approximately 110 Å in diameter (188). The stoichiometry, as determined either by mass per unit length measurements (188), or by fluorescence measurements and sedimentation analysis (262), is one RecA monomer per three base-pairs.

The RecA protein filament is arranged so that a deep helical groove exists (the striation in figure 5A). Although it is not visible in the figure shown, one side of the groove is smooth, while the other is penetrated by the protrusion of the individual monomers (263). These protrusions, or lobes, arise from residues 270 to 328, which corresponds to the highly acidic, carboxyl-terminus of the protein. A similar groove exists for Rad51 protein although no corresponding lobes appear to exist, and this is most likely due to the fact that the Rad51 protein sequence, when aligned against that of RecA protein, terminates at amino acids 266-267. This would suggest that RecA protein should possess additional functions, which may be attributed to this lobe region. Truncated RecA proteins, lacking from 20 to 45 C-terminal residues, are capable of promoting LexA repressor cleavage and DNA strand exchange, which suggests that this region is not involved in these activities (264-266). This region was suggested to be involved in filament-filament interactions that may be involved in keeping RecA protein in an inactive form (267). A more recent study presents evidence that the conserved basic amino acid residues within this region have a direct role in binding dsDNA, and that they form part of a “gateway” for homologous recognition (268).

Within the nucleoprotein filament, the conformation of both ss- and dsDNA is significantly different from that of native B-form DNA. Both DNA types are extensively stretched (approximately 150% of B-form) and, in the case of dsDNA, the helix is also unwound (237). For dsDNA, there are approximately 18.6 base-pairs per turn and the axial rise per base-pair increases from 3.4 Å to 5.1 Å (236,269) while the twist changes from 35° to 20° per base-pair (237,238,262). Electron micrographic analysis and neutron scattering reveal that the DNA is located near the axis of the nucleoprotein filament (270,271). This means that in order for DNA strand exchange to occur, the second DNA molecule must somehow find its way into this complex.

5.1.2. Bacteriophage T4

Similar to RecA protein, UvsX protein also forms helical filaments on both ss- and dsDNA in the presence of a nucleotide cofactor (*e.g.* ATP, ATP-γ-S, or Br^8-ATP) (46,48). Also, resembling RecA protein, UvsX protein forms a helical filament on ssDNA with a distinctly different structure in the absence of ATP (46,48). Figure 5B shows an electron micrograph of UvsX protein on linear dsDNA in the presence of ATP. The helical pitch of the extended UvsX protein-DNA filaments depends on the nucleotide cofactor present. When ATP is present, the pitch is about 120 Å (46).

However, when ATP-γ-S is employed, the mean pitch is 88.6 Å (48). This is regarded as similar to the helical pitch of 95 Å obtained for RecA protein-DNA complexes in the presence of ATP-γ-S (272). Within the UvsX protein-DNA filament, the DNA is stretched such that the axial rise is 5 Å per base or base-pair and untwisted to about 19 bases or base-pairs per helical turn (48). These DNA parameters are very similar to those of the DNA within the RecA protein filament (272). Although there are many similarities between the two filaments, there is one significant difference: when the filaments are formed in the absence of a nucleoside triphosphate cofactor, UvsX protein forms an extended filament (48), whereas RecA protein does not (272).

5.1.3. *S. cerevisiae*

The Rad51 protein from *S. cerevisiae* forms helical filaments on DNA similar to those of RecA and UvsX proteins, and it induces a similar conformation in the DNA (263). On dsDNA, Rad51 protein forms helical nucleoprotein filaments with a helical pitch of 99 Å and 6.2 subunits per turn (figure 5C). The binding stoichiometry is three base-pairs per Rad51 protein monomer, which is consistent with the value for RecA protein (188). In addition, the rise per base-pair induced by the binding of Rad51 protein also increases from 3.4 Å to 5.1 Å; thus, the DNA is also stretched by a factor of 1.5 while having its helicity reduced from 10.5 to 18.6 base-pairs per turn. Sung and Robberson obtained similar results, although they obtained a helical pitch of 80 Å, 17.6 bp per helical repeat and a predicted DNA length that was extended to only 134% that of B-form DNA (63). Although Rad51 protein is able to form filaments on duplex DNA, this nucleoprotein filament is not active for DNA strand exchange (63).

In the presence of RPA, Rad51-ssDNA nucleoprotein filaments resembled those of RecA protein assembled on ssDNA, having a right-handed helical appearance with a pitch of 94 Å (63). Within the complex, the ssDNA is stretched to approximately 154% of native B-form DNA. Filaments formed between Rad51 protein and DNA are less regular than those of RecA (263) and individual lobes of Rad51 monomers are also less visible than those of RecA protein. This is due to structural differences between the Rad51 and RecA protein filaments and not due to disorder within the Rad51 protein filament (263).

5.2. Primary amino acid sequence and crystal structure

There is considerable similarity at the amino acid level between these proteins (29,30,59,79,273-275). We divided the primary amino acid sequence of the three proteins into three sections: an amino-terminal domain, a central "core" region, and a carboxyl-terminal domain; the proteins were then aligned at the highly conserved A and B Walker motifs (figure 6) (274). This alignment is colored; the colors correspond to the 8 highly conserved motifs identified by the Karlin laboratory (29,274). The same colors are used in both the RecA protein structure and the theoretical structures presented for the UvsX and Rad51 proteins (figure 7). The models for Rad51 and UvsX proteins are not intended to imply genuine 3-dimensional structures; instead they show the potential positions of the conserved domains within the crystal structure scaffolding of the *E. coli* RecA protein. In figure 8A, we highlight the residues that are identical in UvsX and RecA proteins, and in figure 8B, we highlight the residues that are identical in Rad51 and RecA proteins (29,30).

5.2.1. *Escherichia coli*

RecA protein is a 352 amino acid (aa) protein (276). The sequence is highly conserved in prokaryotes (43 to 98% at the amino acid level) (28,30), but less well conserved across genera (29,274). The amino-terminal domain consists of aa 1 through 33; the central "core" region extends from aa 34 to 240, and the carboxyl-terminal domain contains residues 241 through 352 (Figures 6 and 10).

The crystal structure for RecA protein was solved to 2.3 Å resolution in the absence of DNA and in the presence of ADP that was diffused into the crystal (figure 7A, 8 - 10; and references (267,277)). This structure contains a major "core" domain with regions that bind ADP and are proposed to bind both ss- and dsDNA. In addition, it contains two smaller domains at the amino- and carboxyl-termini that protrude from the structure and stabilize the helical polymer (267); the amino-terminal domain is important in forming the monomer-monomer interface in the nucleoprotein filament and also in regulating the self-assembly process (278), whereas the carboxy-terminal domain is involved in controlling dsDNA binding (265,268).

The "core" region of RecA protein extending from aa 34-240 (figure 6, 7A), consists of 8 sub-domains, designated B1, B2, C1, C2, D1, E1, E2 and F1 (274). These sub-domains are highly conserved among eubacterial RecA proteins and also among eukaryotic RecA protein analogues (29). This region contains residues which constitute the ATP binding domain (224), the two highly conserved Walker motifs A and B, which correspond to residues 66-73 and 140-144, respectively (279), and the proposed DNA binding regions, designated Loops 1 (aa 157-164) and 2 (aa 195-209) (figure 9) (267). In the crystal structure, this domain contains eight β-sheets flanked by α-helices. By diffusing ADP into crystals, it was possible to identify residues involved in nucleotide binding (267,277). Amino acids D100, Y103 and G265 all make contacts with the base of the nucleoside. The two highly conserved Walker motifs, designated A and B, also make contact with the nucleoside diphosphate (figure 9). Motif A interacts with the PP_i moiety of ATP (277) and is essentially invariant among eubacterial RecA proteins (29), but is less well conserved among the three proteins compared here (figure 6). Residue K72 (RecA protein numbering) is an invariant residue, which when mutated to arginine, abolishes ATP hydrolysis (but does not affect ATP binding), indicating that Motif A is part of the catalytic domain for hydrolysis (183). A similar result was observed for Rad51 protein where mutation of the equivalent residue to arginine, producing the Rad51 K191R protein, abolished ATPase activity completely (64). Motif B, which corresponds to the N-terminal portion of sub-domain E1, is also highly conserved across all RecA protein sequences. This Motif consists of four successive hydrophobic residues followed by an aspartic acid at position 144, which is the key residue in Motif B (267,277) and is identical in all RecA protein sequences (29,30). This residue coordinates the Mg^{2+} ion at the active site between the β and γ phosphates (277). The

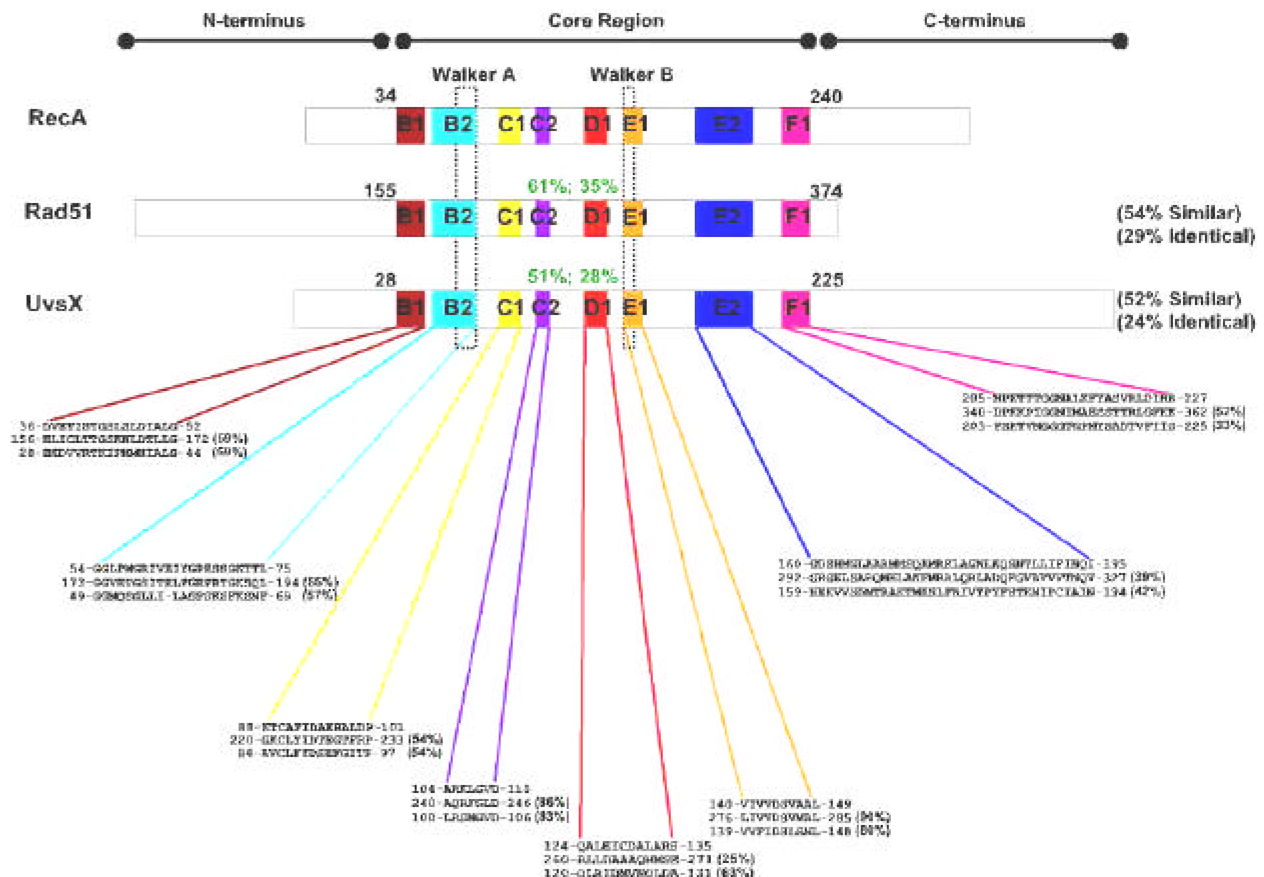

Figure 6. Sequence alignment of RecA, Rad51, and UvsX proteins. The three proteins are aligned at the conserved ATP binding motifs A and B (dotted lines). Numbers immediately to the right of the Rad51 and UvsX proteins are the sequence similarity and identity, respectively, when the entire protein sequence is compared to RecA protein. Numbers in green above the core domain of Rad51 and UvsX proteins are the sequence similarity and identity as compared to RecA protein, for the core regions only. The assignment of the 8 sub-domains (B1, B2, C1, C2, D1, E1, E2, and F1) is the result of an alignment by Karlin and coworkers (274). The sequence for each sub-domain is shown. The order of the sequences presented is RecA protein, Rad51 protein, and UvsX protein. The numbers flanking each sequence block indicate the residue numbers for the RecA, Rad51, and UvsX protein sequences, respectively. The numbers immediately to the right of each block of sequences indicate the percent similarity as compared to *E. coli* RecA protein (obtained using the program Bestfit, which is part of the GCG software package). The numbers in black immediately above sub-domains B1 and F1 are the first and last residues respectively, of the core domains of each protein.

region immediately C-terminal of Motif B (aa 145 to 149) is invariant among eubacterial sequences and less conserved among other RecA analogue sequences; the serine at 145 and leucine at 149 are invariant, however (figure 6 and reference (29)). With regard to ATP hydrolysis, other important residues are E96 (invariant in all RecA protein analogues) which may be involved in the activation of a water molecule for the attack on ATP leading to hydrolysis, and Y264, which cross-links to 8-azido-ATP (280).

For RecA protein and its analogues to function in DNA strand exchange, they need to accommodate at least three DNA strands: the first strand bound to the primary site, and the second and third bound as dsDNA to the secondary (and perhaps tertiary) binding sites. Although the precise location of these sites remains unclear, two are proposed to be in this central core domain, and the third is proposed to be within the C-terminal domain (268). The two regions within this core domain that are proposed to form the DNA binding sites, are the disordered regions in the crystal structure which are designated loops L1 and L2 (figure 9) and which are comprised of residues 157-164 and 195-209, respectively (267). These loops lie close to the polymer axis, the location of the DNA substrate (267). L1 forms the inner, upper lip of the polymer and was proposed to be involved in duplex DNA binding, the secondary site for RecA protein (267). L2 projects outward from the carboxyl end of β-strand 5 and lies toward the innermost face of the filament, directly above the ATP binding site. L1 is strongly conserved in eubacterial sequences, but only at the first five amino acids of the region (29). L2 is substantially conserved, but only among eubacterial RecA proteins (29,274). There is little sequence homology with either Rad51 or UvsX proteins in this region (29,273,274). Several lines of evidence, however, suggest that Loops 1 and 2 are involved in DNA binding, though the data for Loop 1 are conflicting. The isolated L1 loop peptide

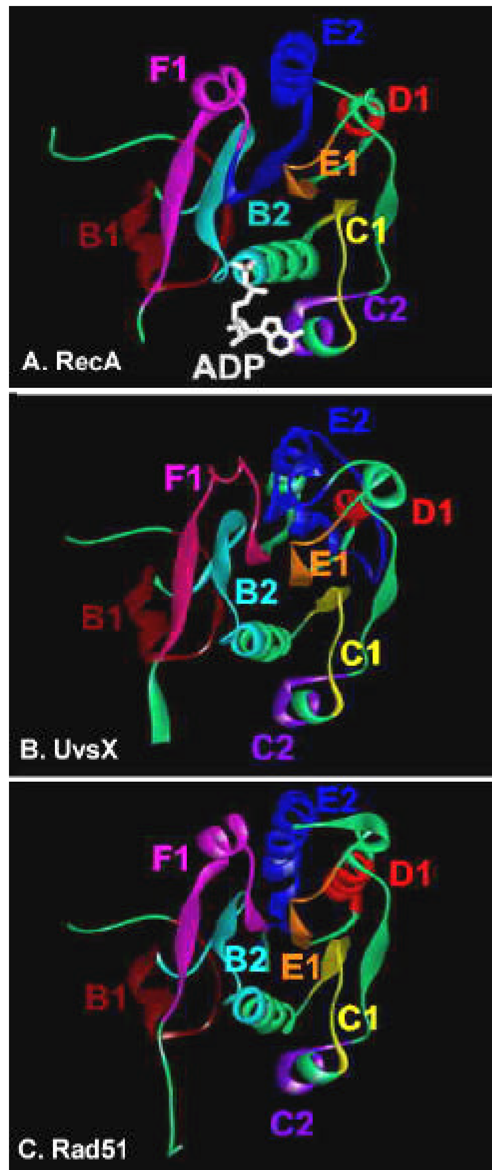

Figure 7. Structures for the core regions of RecA, Rad51, and UvsX proteins. (A) The core domain for RecA protein. The position of ADP as a reference point is shown in white. (B) The expected structure for the core region of UvsX protein. Note that the sub-domain E2, the most poorly conserved region of UvsX, is incapable of forming an α-helix and also disrupts the sub-domain D1. (C) The expected structure for the core region of Rad51 protein. The two model structures were obtained by modeling the RecA protein structure with each of the 8 sub-domains in the RecA protein sequence replaced by either UvsX or Rad51 protein sequences (296-298). Details of the modeling procedure are discussed in the text. The colors used for each of the sub-domains match those used in figure 6. For each predicted structure, the regions shown in light green/aquamarine are residues from the α-carbon backbone of the RecA protein sequence that was used as a scaffold for the homology modeling. The letters for each sub-domain, described previously (274), are indicated in the same color used for figure 6.

cannot bind DNA (281); it exhibits a large mutational flexibility (282); and chemical modification data show that H163 is protected more by dsDNA than by ssDNA (283), suggesting involvement in dsDNA binding. In contrast, the isolated Loop 2 peptide can bind ssDNA (281) and, surprisingly, a mutant variant of the Loop 2 peptide can promote DNA strand invasion of supercoiled DNA by a single-stranded 53-mer (284), suggesting that Loop 2 may be responsible for binding ssDNA providing charge neutralization, and facilitating the close approach to dsDNA. Further, the effects of mutations in Loop 2 (residues 195-209) and the adjacent helix (helix G, residues 210-221) are consistent with this region being involved in DNA binding (285).

Using photochemical cross-linking, Wang and Adzuma demonstrated that when RecA protein was in excess over ssDNA, cross-linking occurred to M164, suggesting that this region is part of the primary site involved in ssDNA binding (286). Also under conditions of RecA protein excess, Mallkov and Camerini-Otero were able to cross-link to both Loops 1 and 2 using d(IU)-containing substrates, suggesting that both loops comprise the primary site responsible for ssDNA binding (287). Finally, Morimatsu and Horii showed that with a 55-mer oligonucleotide, cross-linking occurred at Y65, Y103, Loop 2 and Y264 (288). In cross-linking experiments using conditions of DNA in excess and d(IU)-containing substrates as the second DNA to probe for the second DNA binding site, the majority of cross-linking occurred to M202 and F203, located in Loop2 (286). In separate studies, cross-linking experiments also using conditions of DNA in excess, which should identify both DNA-binding sites 1 and 2, revealed additional positions that may be involved in, or are close to, the DNA binding sites: aa 61-72 (Motif A involved in ATP binding) (289), aa 178-183 (289,290), and aa 233-243 (partially outside the core region) (289). Finally, in a study using stoichiometric amounts of RecA protein and ssDNA, cross-linking to Y103 was observed (290); this amino acid is well outside Loops 1 and 2. The positions of the residues discusses above are shown in figure 9 (maroon side-chains). The discrepancy between the observed sites of DNA cross-linking and the proposed DNA binding sites in the crystal structure may be due to the fact that the structure determined is of the inactive, ADP-bound, DNA-free RecA protein. Thus in the DNA-bound form of the protein, amino acids removed from the DNA binding site may be in close proximity. Additionally, cross-linking may not have occurred with residues in real contact with the DNA, but rather with residues proximal to the binding site. This characteristic was observed for residue Y264 which cross-links to 8-azido-ATP (figure 9 and (280)) but which is not directly involved in ATP contact in the crystal structure, although it is located within the ATP binding cleft (277).

The carboxyl terminus of RecA protein, which spans aa 241 through 352, is highly acidic, containing ~14% of the acidic residues of the protein and is considerably variable, even among prokaryotic RecA protein analogues (29). This region was proposed to be involved in filament-filament interactions (267), in the interaction with repressor

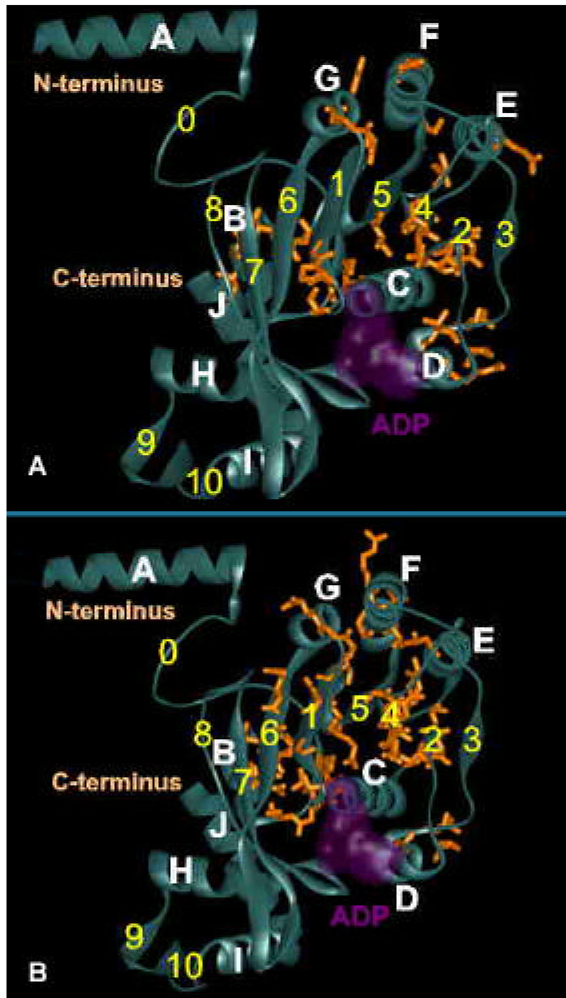

Figure 8. Conserved residues. (A) The crystal structure of RecA protein is shown in grey with the side chains of the residues that are identical to those in the UvsX protein sequence shown in orange. (B) The crystal structure of RecA protein shown in grey with the side chains of the residues that are identical to those in the Rad51 protein sequence shown in orange. ADP is shown in transparent purple. Labels for the α-helices (white letters) and β-strands (yellow numbers) are from Story *et al.* (267).

proteins (29,291,292), in the control of dsDNA binding (265), and in the formation of the secondary DNA binding site (267,292,293). Partial deletion of this region produces truncated RecA proteins 300 amino acids in length that are able to cleave LexA protein and function in DNA strand exchange (264-266). Mutagenesis of a sub-section of this C-terminal domain (aa 270-328) shows it is involved in binding of dsDNA to nucleoprotein filaments formed on ssDNA (268). The replacement of 9 basic amino acid residues in this region showed that mutations in the two conserved residues, K286 and K302, caused deficiencies in recovery following UV-irradiation, whereas mutations in the non-conserved basic residues had little or no effect (268). Purified K286 and K302 mutant RecA proteins were able to form presynaptic complexes but these complexes, once formed, were defective in binding to and pairing with dsDNA (secondary DNA binding). These results are consistent with the involvement of this region in dsDNA binding, and its proposed role as a "gateway" for homologous recognition where the dsDNA interacts with the ssDNA inside of the nucleoprotein filament (268). In addition to dsDNA binding, the binding of a secondary DNA molecule (which is critical to DNA strand exchange) and LexA repressor cleavage are competitive processes; these findings are consistent with the dual role for this region in dsDNA and repressor binding (291,294).

5.2.2. Bacteriophage T4

The UvsX protein sequence is 390 amino acids in length. When the amino acid sequences of *E. coli* RecA protein and T4 UvsX protein are compared, the two proteins are 52% similar and 24% identical (Figures 6, 8A and reference (295)). The sub-division of the primary amino acid sequence of UvsX protein is as follows: the N-terminus from aa 1-27; the core region from aa 28-225, and the C-terminus from aa 226 to 390 (figure 6). The most significant difference between the sequences takes place in the carboxyl-terminal region (273), while the most similarity exists within the core region where there is 51% similarity and 28% identity (figure 6). The core region can also be divided into 8 sub-domains (figure 6 and 7B). However, there is poor alignment at sub-domains E2 and F1; alignment only becomes possible when large gaps are introduced into the RecA protein sequence, as shown in the alignment of Story *et al.*(273). Even though there is low sequence similarity between UvsX and RecA proteins in these two sub-domains, we included them in the modeling process using the sequence alignment shown in figure 6. Modeling of the 8 sub-domains, using the RecA protein crystal structure as a scaffold, was conducted as follows: the positions of the 8 sub-domains were located in the primary amino acid sequence of RecA protein; these residues were then replaced with the corresponding sequence blocks from the UvsX protein primary sequence. This hybrid sequence was submitted to Swiss-Model, which is an Automated Protein Modeling Server running at the Geneva Biomedical Research Institute, Glaxo Wellcome Research and Development S.A., Switzerland (available at http://expasy.hcuge.ch/swissmod/SWISS-MODEL.html) (296-298). The structure for this hybrid protein, showing only the core region, with conserved blocks in color and with RecA protein residues in aquamarine/green is presented in figure 7B.

Modeling of UvsX protein on the RecA protein scaffold shows that the sub-domains used in the modeling assemble into a structure which closely resembles that of the *E. coli* RecA protein. These include the Walker binding motifs A (sub-domain B2) and B (sub-domain E1), located at aa 60-68 and aa 139-143, respectively. As for the RecA and Rad51 proteins, the invariant lysine is present in Motif A at position 66 and the four hydrophobic residues followed by an aspartic acid at position 143 are also present in Motif B (273). The proposed DNA binding domains in RecA protein, Loops 1 and 2, are not well conserved in the UvsX primary sequence, with 50% similarity in Loop 1 and 37% similarity in Loop 2. Interestingly, in Loop 2, residues V207 and M208, which are identical to those of RecA protein at positions 201 and 202, respectively, are conserved. This suggests that the region containing this amino acid may be in close proximity to one of the DNA binding sites.

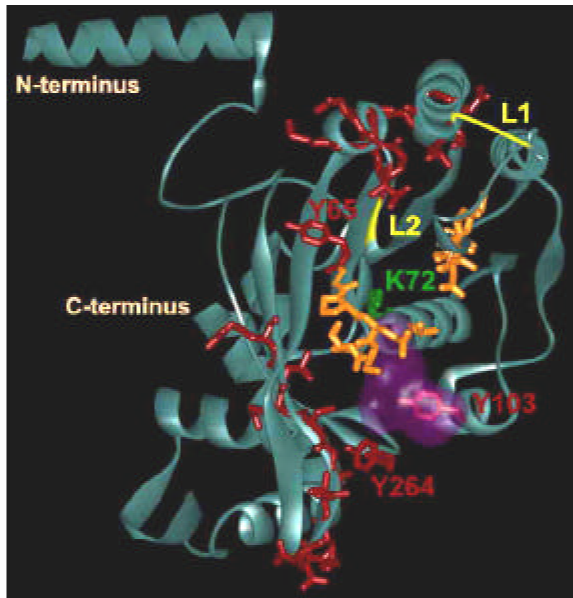

Figure 9. Crystal Structure of RecA protein monomer. The crystal structure of RecA protein showing the side chains of relevant residues as discussed in the text. The potential location of Loops 1 and 2 (which are described in the crystal) are shown in yellow; the amino acid side chains of the Walker motifs A and B are shown in orange, with the side-chain of residue K72 highlighted in green; amino acid side-chains that are in close proximity to DNA, as identified by photochemical cross-linking, are indicated in maroon.

5.2.3. ***S. cerevisiae***

The various Rad51 proteins range in length from 339 (human, chicken and mouse) to 400 (*S. cerevisiae*) amino acids in length (31,59,66,299-302). All of the eukaryotic analogues have extended N-terminal domains and lack the extended C-terminal domain present in their prokaryotic counterparts. Specifically, the *S. cerevisiae* Rad51 protein is 120 residues longer at the amino terminus and 90 amino acids shorter in the carboxyl terminus than RecA protein (figure 6 and references (31,59,61)). Our division of the primary sequence of *S. cerevisiae* Rad51 protein into three regions is as follows: aa 1-154 constitute the amino-terminal portion; the core extends from residues 155 to 375 and the carboxyl domain comprises aa 376 to 400 (figure 6). Alignment of the complete sequences of the two proteins shows that they are 54% similar and 29% identical (59). Within the core region, RecA and Rad51 proteins are even more alike, with 61% similarity and 35% identity (figure 6 and reference (59)).

The amino-terminal domain (which is unique to each eukaryotic species) was proposed to be involved in monomer-monomer interactions, and in forming species specific interactions with Rad52 protein (303,304). Rad51 and Rad52 proteins from either *S. cerevisiae* or humans interact both *in vitro* (59,305) and *in vivo* (135). In *S. cerevisiae*, the amino acids responsible for this interaction map to a region that includes aa 4 through 187 in Rad51 protein (303); in the case of the human proteins, the domain was not mapped. The *S. cerevisiae* Rad51 protein interacts *via* the carboxyl-terminal one third of the Rad52 protein (135), whereas the human Rad51 protein interacts *via* aa 270-330 of Rad52 protein (305).

As for RecA and UvsX proteins, the Rad51 protein core region also contains 8 sub-domains; there is significantly more homology within the 8 sub-domains than across the entire sequence (274) (figure 6). We have also modeled the Rad51 core domain on the crystal structure of RecA protein. This modeling was done in the same way as for UvsX protein. The model is presented in figure 7C and shows that the sub-domains used in the modeling assemble into a structure, which closely resembles that of both the *E. coli* RecA protein and the T4 UvsX protein.

The core region contains the two Walker motifs: Motif A is located at aa 185 to 192 and Motif B is located at aa 276 to 280. This region may also be involved in interaction(s) with other members of the *RAD52* epistasis group that were demonstrated using the two-hybrid system (136). It was noted that the core region may contain a leucine zipper motif (L-X_6-L-X_6-L-X_6-F) at aa 296-317, present in sub-domain F1 (63). The RecA and Rad51 proteins share the most homology within sub-domain E1, which contains Motif B (274) (figure 6 and 8B). Mutation of the conserved lysine at position 191 to alanine results in deficiencies in DNA repair and recombination (59). In contrast, conservative mutation of the same residue to arginine, produces the Rad51 K191R protein which is the analogue of the K72R RecA mutant protein, and which retains biological activity *in vivo*, is defective in ATPase activity *in vitro*, but is still able to promote DNA strand exchange *in vitro* (64). The core region also contains the proposed DNA binding sites of RecA protein, Loops 1 and 2. The modeling of this region using *E. coli* RecA protein residues as spacers between the conserved sub-domains, demonstrates that this region is able to assemble into a similar structure to that of RecA protein. Those regions where sequence similarity is low (sub-domains B2, E2 and F1), have dissimilar predicted structures (figure 7C), while those where sequence similarity is high (sub-domains B1, C, D and E1), have a more similar 3D structure which is almost identical to that within RecA protein (figure 7C).

The C-terminus extends from aa 375 to 400. Within this region, the RecA and Rad51 proteins share very little sequence similarity. The absence of the extended C-terminal domain that is found in the RecA and UvsX proteins may account for the relatively high dsDNA binding activity of Rad51 protein that distinguishes the eukaryotic family from its prokaryotic counterparts. Though inhibitory to DNA strand exchange *in vitro* with naked substrates, this enhanced dsDNA binding may facilitate homologous pairing with dsDNA bound by nucleosomes *in vivo*.

6. PERSPECTIVE

The three proteins reviewed here exhibit many similarities and differences. They show significant homology at the amino acid level; they form similar nucleoprotein filaments on both ss- and dsDNA, and they assume similar structures in their highly conserved central core regions. All three proteins promote DNA strand exchange *in vitro*, which is consistent with the conservation of a central core domain containing all of the sub-domains required for this activity.

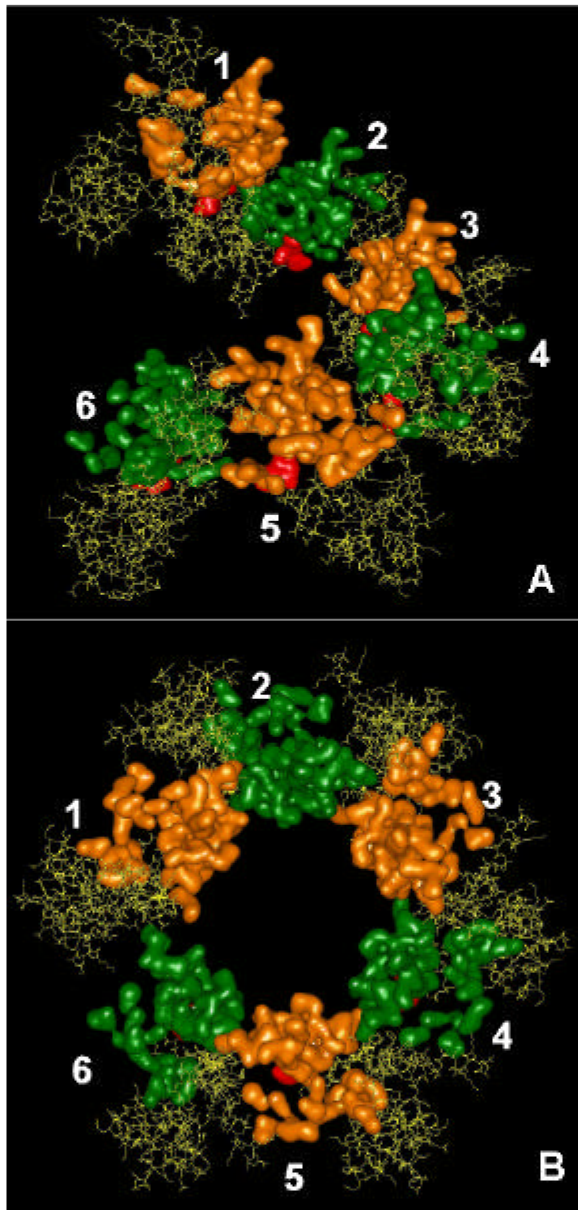

Figure 10. Location of the highly conserved core domain within the RecA protein hexamer. Two views of a single turn of a RecA filament are presented. The orientation in (A) is similar to that of Story *et al.* (267) and shows a single turn of the RecA protein filament from the side. (B): The same turn of the RecA filament viewed from the top. For this view, the hexamer in (A) was rotated 90 degrees toward the viewer. The numbers in white indicate the six monomers making up the turn. The core domain of each of the monomers is represented as a solvent surface; these are coloured alternately in orange and green. The α-carbon backbones of the non-conserved regions, the N- and C-termini, are displayed as stick diagrams in yellow. The position of ADP within each monomer is shown as a reference point (red solvent surface).

The differences between these proteins exist in their NTP requirements in DNA strand exchange, and their rates of ATP hydrolysis and their polarity of DNA strand exchange. From a comparison of the amino acid sequences and homology modeling, it is apparent that the N- and C-terminal domains of these proteins are significantly dissimilar. This likely reflects the differences in the additional functions that these proteins perform in their respective hosts, and in the unique protein-protein interactions required for these functions. Thus, though defining the ubiquitous class of DNA strand exchange proteins, these three representative proteins nevertheless display an intriguing diversification of biochemical functions that will require further scrutiny to unravel fully.

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