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ENZYMES OF GENERAL RECOMBINATION

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CONTENTS

HISTORICAL PERSPECTIVES AND SUMMARY 229
REC BCD ENZYME 231
REC A PROTEIN 234
Structure of recA Protein 234
Basic Activities of recA Protein 235
DNA Strand Exchange 242
Role of SSB in Strand Exchange 254
RESOLUTION OF HOLLIDAY STRUCTURES 257
RECOMBINASES IN OTHER CELLS 257

HISTORICAL PERSPECTIVES AND SUMMARY

General recombination is the process by which DNA sequences are exchanged between homologous chromosomes at essentially any site. The first molecular model to account for such exchanges was suggested some 20 years ago by Robin Holliday to explain the patterns of gene conversion in the smut fungus *Ustilago maydis* (1). This model [and its subsequent variations (2)] has formed the conceptual framework for much of current research on the molecular mechanisms of general recombination (Figure 1). An important and

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distinctive feature of the Holliday model is the covalent association of the two duplex DNA molecules that are to engage in recombination via a heteroduplex joint (the Holliday structure) that by virtue of its capacity to branch migrate can generate long regions of heteroduplex (3, 4). Such heteroduplex regions can account for the heterozygosity that is frequently observed in recombinant chromosomes (5, 6). Where mismatches result, their correction causes the unequal segregation of closely linked markers, i.e. gene conversion (7, 8). Because of its symmetry, the Holliday structure can be processed along either its horizontal or vertical axes to yield recombinant molecules in which the parental alleles bordering the potentially heterozygous regions are either conserved in their original linkage or reciprocally exchanged.

The isolation by Clark & Margulies (9) of strains of *Escherichia coli* defective in general recombination permitted for the first time the application of the powerful tools of genetics to a study of its molecular mechanism. Three loci were discovered initially: *recA*, *recB*, and *recC* (9). Additional genes, including *recE*, *recF*, *recG*, *recJ*, *recK*, *recL*, and *recN*, were subsequently identified (10–15). At the present time, only the products of the *recA*, *recB*, *recC*, and the recently discovered *recD* genes have been isolated in a functional form. *RecA* codes for the recombination enzyme of *E. coli*, the recA protein or recombinase (12–19); *recB*, *recC*, and *recD* (12, 20, 21) specify the three subunits of a multifunctional enzyme whose specific role in general recombination has yet to be established. By virtue of its nuclease activity, the recBCD enzyme could catalyze the processing of the DNA product of recA protein action to the final products of the recombinational exchange. [The *recF* and *recE* gene products probably catalyze processing by alternative pathways (22)]. The recBCD enzyme may also be involved at earlier stages in recombination. Another gene, *ssb*, codes for the single-stranded DNA-binding protein (23, 24). This protein, which plays a crucial role in DNA replication, also provides an important adjunctive function in recombination (25).

This review will primarily consider the products of the *recA*, *recB*, *recC*, *recD*, and *ssb* genes. The analysis of the structure and mechanism of these proteins has reached the stage where at least the outlines of an enzymatic pathway of homologous recombination can be formulated. It will not cover the enzymology of site-specific recombination exemplified by the bacte-

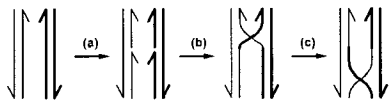


Figure 1. The Holliday model for general recombination. (*a*) Two homologous DNAs are aligned and the apposing strands nicked. (*b*) The Holliday recombination intermediate is formed by reciprocal strand invasion followed by covalent connection of the two duplex DNA molecules. (*c*) Migration of the crossover point (branch migration) generates a long heteroduplex region.

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riophage lambda–induced *int-xis* proteins, which has reached a high level of molecular resolution. For reviews of this subject, the reader is referred to Nash (26) and Sadowski (27).

REC BCD ENZYME

The recBCD enzyme (exonuclease V) of *E. coli* is a complex, multifunctional protein that catalyzes the hydrolysis of both linear duplex and single-stranded DNA, coupled to the hydrolysis of ATP (28–31). It is also an ATP-stimulated endonuclease that acts specifically on single-stranded DNA. In addition to its nuclease activity, exonuclease V is a DNA helicase, that is, it can use the energy of ATP hydrolysis to unwind linear duplex DNA (32–34). An enzyme with analogous activities has been purified from *Hemophilis influenzae* (35, 36). Exonuclease V had until recently been thought to consist of two subunits with molecular weights of approximately 140,000 and 130,000, the products of the *recB* and *recC* genes, respectively (31, 37, 38), although there were indications that it might possess a third subunit (39). It is now clear from the work of Smith and his colleagues (21) that the fully functional enzyme does indeed contain a third subunit with a molecular weight of 58,000, the product of the *recD* gene, hence its present designation as the recBCD enzyme.

A major impediment to an understanding of the mechanism of action of exonuclease V has been the availability of only very small amounts of pure enzyme, due in turn to its exceedingly low cellular concentration (31, 33, 37). However, cloning of the *recB* and *recC* genes either individually or together in high copy number vectors has permitted substantial overproduction of the enzyme (40–42), so that milligram quantities of quite pure enzyme are now readily available for analysis. Because *recD* is immediately adjacent to the *recB* gene, and, in fact, the *recB, C,* and *D* genes may constitute an operon, existing clones of the *recB* or *recB* and *recC* genes very likely contain the *recD* gene as well (21, 43).

The function of the individual subunits in the various activities associated with the recBCD enzyme has not yet been entirely clarified. However, a class of mutants, *recB*‡, has been shown to lack nuclease activity, and correspondingly, to lack the recD subunit (44). Despite the absence of nuclease activity, *recB*‡ mutants are recombination proficient, suggesting that it is the DNA helicase activity of the recBCD enzyme that functions in homologous recombination, although this has not yet been firmly established. The finding that the purified recB subunit retains DNA-dependent ATPase activity, but is lacking exonuclease, and that the recC subunit has neither of these activities (43), is consistent with the assignment of helicase activity to the recB and/or C subunits and nuclease to the recD subunit. Careful enzymatic analysis of each of the subunits together with the appropriate reconstitution experiments

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is obviously needed to establish with certainty the structure-function relationships of the three components of the recBCD complex.

In considering what role(s) the recBCD enzyme plays in homologous recombination, detailed information regarding its nuclease and helicase activities is obviously essential. In acting on linear duplex DNA, the recBCD enzyme initiates its attack at the ends of the DNA molecule and generates duplexes with long single-stranded tails and single-stranded DNA fragments ranging in size up to 1000 nucleotides (45). These are ultimately hydrolyzed to small oligonucleotides, with an average chain length of 4–5 residues (31, 46). However, in the presence of single-stranded DNA-binding protein (SSB), the amount of single-stranded fragments generated is greatly reduced (33). Similarly, exonucleolytic hydrolysis of linear single strands is inhibited by SSB. The generation of duplex DNA molecules with tails and long single-stranded fragments is also enhanced at high, but physiologically significant, ATP concentrations (3–5 mM) (31, 37, 45). Thus, under conditions similar to those to be expected in vivo, i.e. high concentrations of ATP and in the presence of SSB, the exonuclease activity of the recBCD enzyme is largely suppressed (although not eliminated) and the enzyme acts primarily as a helicase, whose activity is largely unaffected under these conditions. Exonuclease but not helicase activity is also inhibited completely by 1-mM Ca^{2+} in the presence of 1-mM Mg^{2+} (47). In this case, however, Ca^{2+} appears to act as a competitive inhibitor of Mg^{2+} and the inhibition of nuclease activity can be relieved by simply increasing the Mg^{2+} concentration (D. Julin, I. R. Lehman, unpublished).

The recBCD enzyme is most active on duplex DNA molecules with flush ends (49, 50). However, it will also attack circular duplexes containing gaps, provided that the gaps are $\geq$5 nucleotides in length (48, 49). This attack, which occurs at a 10-fold lower rate than at linear duplex ends, is very likely a consequence of endonucleolytic cleavage of the single strand at the gap to generate a linear duplex, which is then a substrate for exonucleolytic attack (48, 49). The requirement for gap sizes in excess of 5 residues presumably reflects the requirement by the enzyme for access to the cleavage site.

A detailed analysis of the helicase activity of the recBCD enzyme has shown that the optimal substrate is a linear duplex with nearly flush-ended 3' and 5' termini (50). Duplex molecules with single-stranded tails, particularly those in which the tails are >25 nucleotides in length, are unwound poorly, presumably because of the inability of the enzyme to bind such molecules. Circular duplex circles containing gaps are not unwound; however, the single-strand endonuclease activity of the recBCD enzyme can cleave the single-stranded region within the gap to produce linear molecules that can then be unwound at a rate dependent on the length of single-stranded tail generated by the cleavage (49).

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Electron microscopic examination of the products formed from linear duplex DNA by the recBCD enzyme has revealed structures containing a single-stranded loop with two single-stranded tails, one emanating from each strand, and double loops of equal size, one on each strand of the duplex (34, 48). A plausible interpretation of the mechanism by which these structures are generated is that the recBCD enzyme binds to the ends of the duplex and unwinds the DNA to form a single-stranded loop and two single-stranded tails. These then give rise to a double loop structure by annealing of the two tails. The double loops are propagated along the duplex by continued unwinding and threading of one of the two strands past the enzyme. During this unwinding, which is coupled to ATP hydrolysis, the enzyme, by virtue of its nuclease activity, generates single-stranded DNA fragments.

As noted above, the recBCD enzyme possesses endonuclease activity that is specific for single-stranded DNA (31). Unlike the exonuclease activity, the endonuclease is not absolutely dependent on ATP; however, it is stimulated by ATP (some 7-fold). Since coating of single strands by SSB renders them largely insusceptible to the endonuclease (33), it is unlikely that the endonuclease represents an important activity of the recBCD enzyme in vivo. Although duplex DNA is not, in general, cleaved by the recBCD-associated endonuclease, it will catalyze the endonucleolytic cleavage of linear duplex DNA containing *chi* sites (51, 52). *Chi* sites are recombinational hot spots in *E. coli* and bacteriophage lambda DNA that enhance general recombination in their vicinity (53). *Chi*-dependent cleavage by the recBCD enzyme occurs on only one of the two strands of the duplex, that containing the *chi* sequence 5' G-C-T-G-G-T-G-G 3' (54). Cleavage does not occur within the *chi* sequence, but rather at sites located four to six nucleotides to the 3' side of *chi,* and may be the basis for the stimulatory effect of this sequence (52). Thus, mutations in the sequence that diminish *chi* activity in vivo also reduce cleavage by the recBCD enzyme in vitro. Similarly, mutants with enzymes that are defective in the recD subunit show no *chi* activity in vivo, and crude preparations of such enzymes do not show *chi*-dependent cleavage of duplex DNA (21, 44). Presumably, *chi*-specific endonuclease activity is associated with the recD subunit of the recBCD enzyme.

These findings have led to a model for the action of the recBCD enzyme in homologous recombination in which the helicase and *chi*-specific endonuclease activity of the recBCD enzyme act in the steps preceding strand exchange to generate a single strand, which can then serve as a substrate for recA protein–promoted D-loop formation (52, 55). According to this model the enzyme binds the flush ends of a duplex DNA molecule, and by virtue of its ATP-driven helicase activity unwinds it to produce first the single-stranded loop and then the double-looped structure described above. The double loop is then propagated along the duplex until the recBCD enzyme encounters a *chi*

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sequence in the correct orientation. Cleavage of the strand bearing the *chi* sequence followed by continued unwinding then generates a 3' terminated single strand, which can then be assimilated by an adjoining duplex, leading ultimately to the formation of a Holliday intermediate. Inasmuch as the recBCD enzyme requires the flush ends of a duplex DNA molecule to initiate its action, a double-strand cleavage of the circular *E. coli* chromosome is presumably required to provide an entry site for the recBCD enzyme. In the case of bacteriophage λ, the terminase enzyme that introduces a double-strand break at *cos*, the packaging origin, may provide the entry site (56).

Although the nuclease activities associated with the recBCD enzyme could in principle act in the resolution of Holliday intermediates, there is no evidence that this is the case (see below).

REC A PROTEIN

If one were to point to a single advance that triggered the very rapid development of our current understanding of the molecular mechanism of general recombination in *E. coli,* it would clearly be the construction of a lambda-transducing phage carrying the *recA* gene and the concurrent demonstration, nearly 20 years after the *recA* gene had first been identified, that its product was a protein with a molecular weight of approximately 40,000 (57, 58).

The discovery of the role of the recA protein in regulating the so-called SOS response of *E. coli* to DNA-damaging agents, and, in particular, autoregulation of the *recA* gene, led to a model that unified a mass of seemingly disparate data (59, 60). Knowledge of the way in which the *recA* gene is regulated also permitted the construction of strains of *E. coli* in which the *recA* gene was expressed constitutively at very high levels (61–63). Thus, gram quantities of homogeneous recA protein rapidly became available for the analysis of its structure and function. For a detailed treatment of the central role of recA protein in the regulation of SOS functions, the reader is referred to several excellent reviews (for example, 64). Activities of recA protein related to its role as a recombinase were reviewed in detail several years ago by Radding (65), McEntee & Weinstock (66), and by Dressler & Potter (67).

Structure of recA Protein

The recA protein is composed of 352 amino acids and has a molecular weight of 37,842 (68, 69). Other than a relatively low tyrosine and tryptophan content (seven and two residues, respectively), its amino acid content is unremarkable (68, 69). The amino terminal half of the recA protein contains sequences that are similar to peptide sequences at the active site of several of the serine proteases (69). However, the significance of this finding has

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diminished with the recent discovery that the recA protein enhances the intrinsic capacity of the lexA and phage λ repressors to undergo cleavage, but does not itself catalyze peptide bond hydrolysis (70).

Both tetragonal and hexagonal crystals of recA protein have been obtained at low pH (5 to 6). The hexagonal form is of appropriate size for X-ray crystallographic examination, and in a preliminary analysis the diffraction extended to 3.5 Å. In both forms the recA protein showed a space group of $P6_1$, indicating a helix of six recA protein monomers per unit cell (71). Predictions of secondary structure and the approximate tertiary folding of recA protein (72) suggest that it possesses a "nucleotide binding fold" and an alternating β strand–α helix pattern. Solution of the three-dimensional structure of recA protein, which hopefully is imminent, is an obvious prerequisite for a detailed understanding of how recA protein functions in general recombination.

Basic Activities of recA Protein

FORMATION OF REC A PROTEIN FILAMENTS A striking feature of recA protein is its tendency to form aggregates or higher order polymers. This property of recA protein may be related to its action in stoichiometric rather than catalytic amounts in the DNA strand exchange reactions that will be considered later. In the presence of the nonhydrolyzable ATP analogue, ATPγS, and at pH 6.2, recA protein forms long filamentous structures easily visible in the electron microscope (73). More recent light-scattering studies have shown that recA protein can aggregate and self-assemble into filaments at neutral pH and in the absence of nucleotides (74–76). Formation of recA protein filaments is exceedingly sensitive to monovalent and divalent cation concentration. As an example, aggregation of recA protein as judged by light scattering is optimal at 10-mM Mg^{2+}, but is only marginally detectable at 5-mM Mg^{2+}, and virtually disappears at 40-mM Mg^{2+} (74). Similarly, the extent of aggregation declines precipitously between and 20- and 35-mM Tris (75). Nucleotides (ATP, ADP, GTP) have been observed to disrupt recA protein filaments in both electron microscopic and light-scattering studies (74).

A significant concern is the possibility that contaminating DNA in recA protein preparations may in some way potentiate filament formation. In fact, it has been observed that the very long filaments formed upon addition of Mg^{2+} and ATPγS to solutions of recA protein contain RNA, which is either present as a contaminant or generated by the action of a polynucleotide phosphorylase activity that appears to contaminate some preparations of recA protein (77). It is at present unclear to what extent, if any, RNA (or DNA) contamination can account for recA protein filamentation (75, 77).

The finding that various nucleoside triphosphates, i.e. ATP, at physiologi-

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cally significant concentrations, can disrupt recA protein filaments would tend to suggest that these structures are not of significance in vivo. In fact, recent studies have indicated that recA protein filaments are structurally different from the filamentous complexes of recA protein formed on single-stranded DNA (see below). Indeed, formation of large, free recA protein filaments and binding of recA protein to single-stranded DNA to form nucleoprotein filaments that are active in strand exchange appear to be competing reactions (75). It is, however, possible that smaller aggregates (dimers, tetramers, etc) are intermediates in DNA binding. The uniform appearance of recA filaments in the electron microscope suggests that monomers within the filament are equivalent (76, 78–81). Whereas a monomer may be the active unit in the formation of filaments on DNA, the complexity of the aggregation reactions of recA protein has so far impeded efforts to determine if some other multimeric form of the protein plays a significant role.

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BINDING OF REC A PROTEIN TO SSDNA At neutral pH, recA protein polymerizes onto single-stranded DNA (ssDNA) in a highly cooperative manner (73, 75, 76, 78), coating the DNA completely and extending it significantly. The nucleoprotein filaments formed in this way are 12 nm in diameter and have contour lengths that are 60% that of protein-free DNA duplexes. The contour lengths of the recA protein-ssDNA complexes vary depending on factors such as the ionic strength and presence or absence of nucleotides. In the presence of ATP, the contour length can be as much as 150% of the length of the corresponding recA-free duplex DNA (81). These recA protein–ssDNA complexes are very likely the active species in DNA strand exchange promoted by the recA protein.

Measurements of the stoichiometry of binding of recA protein to ssDNA have yielded values that range from 3 to 6 nucleotides per recA monomer. A stoichiometry of 1 recA monomer per 4 nucleotides was reported by West et al based on the ratio of recA protein to single-stranded ϕX174 DNA in saturated recA protein–ssDNA complexes isolated by ultracentrifugation (82). A similar value was obtained by measurements of the amount of ssDNA protected from nuclease digestion by recA protein (83) and by light scattering (75). A value of 1 recA monomer per 4 nucleotides was also obtained in titrations using ATP hydrolysis as a measure of recA protein binding (84). In contrast, a value of 1 recA monomer per 6 nucleotides has been reported on the basis of titrations of the fluorescent etheno-derivative of ssDNA with recA protein (85, 86). The higher value for the size of the binding site may be related to the greatly enhanced affinity of recA protein for the modified residues in the etheno-DNA. The lower estimates for the binding site size (3 nucleotides per recA monomer) have been obtained with duplex DNA (see below).

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Binding of recA protein to ssDNA is relatively stable as judged by its rate of equilibration with a challenging DNA ($t_{1/2}$ of approximately 30 min) (83). The binding is, however, strongly influenced by the type of anion present (87) and by the addition of nucleotide cofactors (83, 87). Addition of ATP to recA protein–ssDNA complexes stimulates the rate of equilibration so that the $t_{1/2}$ is approximately 3 min. In the presence of ADP, equilibration is even more rapid ($t_{1/2} \approx 0.2$ min). The slower equilibration with ATP suggests that a slow step leading to or including the ATP hydrolysis step precedes a rapid ADP-induced release from the DNA. Even in the presence of ADP, however, the overall equilibrium still favors almost complete association of recA protein with the ssDNA. In contrast to ATP, addition of ATPγS to recA protein–ssDNA complexes completely prevents movement of recA protein from the DNA (83). It would therefore appear that one function of the ATPase activity associated with the recA protein is to permit it to cycle on and off ssDNA, ATP being required for binding and ADP potentiating its dissociation (83, 87).

Transfer of recA protein from one DNA molecule to another appears to proceed via a ternary complex with no free recA protein intermediate (88). However, ATP hydrolysis is not tightly coupled to the transfer (89). Moreover, it is not tightly coupled to association or dissociation of recA monomers (at least when single-stranded DNA-binding protein is present), although exchange again occurs between adjacent recA-ssDNA complexes (90).

In contrast to the stoichiometry of 1 recA protein per 4 nucleotides of ssDNA observed in the absence of nucleotide, the stoichiometry in the presence of ATPγS is 1 recA protein per 8 nucleotides (83). The significance of the exact twofold increase in the stoichiometry of binding in the presence of ATPγS is not clear; however, one possibility is that a cryptic second DNA-binding site on the recA monomer is activated upon binding ATPγS. Some support for two binding sites for ssDNA per recA monomer has come from studies of recA protein–catalyzed annealing of complementary DNA strands described below.

The mutant recA protein (recA1) in which a glycine at position 160 is replaced by an aspartic acid residue (91) binds ssDNA cooperatively with a stoichiometry similar to the wild-type recA protein (1 monomer per 3.5 nucleotides). However, the mutant protein, which is completely lacking in ATPase activity (82, 91), is dissociated from ssDNA upon addition of ATP or ADP. A similar effect has been noted with wild-type recA protein in the presence of dTTP, a nucleotide not normally hydrolyzed by the recA protein (F. R. Bryant, I. R. Lehman, unpublished).

BINDING OF REC A PROTEIN TO DUPLEX DNA Binding of recA protein to duplex DNA differs from the binding to ssDNA in two ways: (*a*) it is highly pH-dependent, with a pH optimum near 6.0 (73, 92), and (*b*) it occurs only in

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the presence of a ribonucleoside triphosphate cofactor (73, 93–96). The two binding reactions also differ in their sensitivity to ionic strength and in the extent of inhibition by ADP (73, 92).

Binding of recA protein generally results in unwinding of the duplex DNA (94–99). Unwinding in the presence of ATPγS produces a doubling in the number of base pairs per twist in the DNA helix (100). Although the unwinding observed with ATP appeared initially to occur to only a limited extent (94), it has recently been found that duplex DNA can be unwound by at least 28–30% in the presence of ATP (without a ssDNA cofactor), and that binding and unwinding are inseparably linked in this process (101).

All of the nucleoside triphosphates that are hydrolyzed by recA protein in the presence of ssDNA (see below) are also hydrolyzed in the presence of duplex DNA; a pH optimum of ~6.0 is observed in each case. Especially suitable cofactors include ATP, ATPγS, and UTPγS (73, 94), with both binding and unwinding observed in the presence of each. At pH 7.5, binding to duplex DNA in the presence of ATP is nearly undetectable; however, binding is facilitated by ATPγS (94–96, 101–103) if either homologous ssDNA or a low concentration of Mg^{2+} (1 mM) is added (94–99, 102).

In the presence of ATPγS, recA protein condenses on duplex DNA as thick rodlike filaments (73, 76, 78, 79, 104, 105). Close examination of these complexes by Koller and his colleagues (79, 100, 105) has shown the recA protein to be arranged along the DNA duplex as a helical structure. They observe that one helical turn with a pitch of approximately 100 Å and a diameter of 100 Å is formed upon interaction of 6.3 recA protein molecules with 18.6 base pairs of DNA. As a consequence, the DNA is unwound from the 10.5 base pairs per turn characteristic of the B-form to 18.6 base pairs per turn, i.e. 15° per base pair, and the DNA is stretched to 150% of its usual length (79, 100).

In contrast, Griffith and his collaborators (106) have found that binding of recA protein to supercoiled duplex DNA in the presence of Mg^{2+} and ATPγS generates filamentous structures with a repeat unit of 2 recA monomers per 17 base pairs. As a consequence, the DNA is unwound by 11.5° per base pair, a value significantly different from that reported by the Koller group. The discrepancy may be related to differences in methodology. In fact, considerable structural variation can occur depending upon ionic strength and the presence or absence of nucleotide cofactors (78, 80, 81, 107). Williams & Spengler have found that the helical pitch of the recA protein filaments is 55 Å in the absence of nucleotide cofactors, and 93–100 Å when ATPγS is added. In these experiments, the pitch in the presence of DNA was 72–75 Å (80).

The stoichiometries reported for recA protein binding to duplex DNA have again varied somewhat. The electron microscopic observations of Koller and

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colleagues (79) and the studies of Dombroski et al (108) yielded a value of 1 recA monomer per 3 base pairs. A stoichiometry of 1 monomer per 4 base pairs was obtained using light scattering (101) or ATP hydrolysis (J. Lindsley, M. M. Cox, unpublished) as probes of binding.

The weak binding of recA protein to duplex DNA at pH 7.5 observed in the presence of ATP is the result of a slow step in the association pathway rather than an unfavorable binding equilibrium (101). If this step is bypassed (e.g. by a pH shift from 6 to 7.5), binding at pH 7.5 is quite stable (101). The rate-limiting step in binding appears to be initiation of DNA unwinding. Once nucleation occurs, propagation of a recA protein filament on the duplex DNA is rapid (101). The ATP hydrolysis that accompanies this process occurs only after the DNA is bound and unwound, i.e. ATP hydrolysis is not coupled to unwinding. Slow binding and unwinding of DNA is mirrored by a long lag in ATP hydrolysis, which can be on the order of hours in binding experiments carried out at pH 7.5. Binding to duplex DNA is therefore a complex process leading to a recA-nucleoprotein filament on extensively unwound duplex DNA, which is capable of ATP hydrolysis at a rate approaching the rate observed with ssDNA as cofactor (k_{cat} 20–22 min $^{-1}$) (101). The unwinding of duplex DNA is especially relevant to (and indeed, it is required by) the DNA strand exchange reactions promoted by recA protein described below. RecA protein effectively holds the DNA at or near the transition state for the exchange of strands.

HYDROLYSIS OF NUCLEOSIDE TRIPHOSPHATES BY REC A PROTEIN The first enzymatic activity to be associated with recA protein was its ssDNA-dependent ATPase (109, 110). In fact, both single- and double-stranded DNA stimulate ATP hydrolysis. The ssDNA-dependent reaction exhibits a broad pH optimum between 6.0 and 9.0 (92). The double-stranded DNA-dependent reaction, in contrast, exhibits a pH optimum near pH 6.0 (92, 111), paralleling double-stranded DNA binding (73). Values for the K_M for ATP range from 20 to 100 μM under various conditions (111, 112). The reaction proceeds with a turnover number of between 10 and 30 min^{-1}, again varying somewhat with conditions (84, 87, 101, 111, 112). The ssDNA-dependent ATPase activity has a Hill coefficient of 3.3 at pH 8 (as does the double-stranded DNA-dependent activity at pH 6.2), indicating that 3 or perhaps 4 recA monomers are required per hydrolytic cycle in a cooperative process in which binding of one ATP facilitates binding of further ATP molecules (111). It is therefore likely that the nucleoprotein filament described above is the active form of recA protein in the catalysis of ATP hydrolysis.

RecA protein–catalyzed hydrolysis of ATP is not restricted to the ends of these nucleoprotein complexes. Many or all recA monomers throughout the cooperatively assembled complex hydrolyze ATP. Thus, there is no correla-

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tion between the concentration of filament ends and either the initial rate or optimal turnover number for ATP hydrolysis (113). As described in a later section, this result also extends to the DNA strand exchange reaction.

In addition to ATP, hydrolysis of dATP, UTP, dUTP, and to a lesser extent CTP and dCTP is catalyzed by recA protein in the presence of single-stranded and duplex DNA (114, 115). In each instance hydrolysis stimulated by duplex DNA occurs only at acid pH (pH 5.5–6.5). In contrast to the *E. coli* recA protein, the recA protein from *Bacillus subtilis* has negligible ATPase activity. However, it can hydrolyze dATP at a rate comparable to that of the *E. coli* enzyme (116). Inasmuch as ATP can inhibit the dATPase activity of the *B. subtilis* recA protein analogue, it must be able to bind ATP. The significance of this stringent specificity for dATP is not known.

ADP and UTP are competitive inhibitors of the ATPase activity, indicating that there is only a single nucleoside triphosphate binding site per recA monomer (92, 115). Consistent with this idea is the finding that ATPγS, which is not hydrolyzed by the recA protein and is in fact a potent inhibitor of both the single- and double-stranded DNA-dependent ATPase (K_i = 0.6 μM), binds tightly to a single site per enzyme molecule (117). Modification of recA protein with the photoaffinity label 8-azido ATP both in the presence and absence of DNA results in covalent attachment of the azido ATP exclusively to Tyr-264 (118, 119). This residue is also the exclusive site of modification by another ATP analogue, 5'-P-fluorosulfonyl-benzoyladenosine (120). These and other findings suggest that Tyr-264 is located in the ATPase active site of recA protein and is positioned in close proximity to both the adenine ring and the triphosphate group of the ATP.

RecA protein does not catalyze any of the microscopic exchange reactions that are often associated with ATP hydrolysis. Experiments designed to detect [^{3}H]ADP⇌ATP, $HPO_4^- \rightleftharpoons H_2{}^{18}O_4$, and $HP^{18}O_4 \rightleftharpoons H_2O$ exchange were in each case negative, suggesting that ATP hydrolysis is irreversible (112).

RENATURATION OF COMPLEMENTARY SINGLE STRANDS The alignment of complementary DNA sequences is the simplest DNA pairing activity associated with the recA protein (114), and may underlie the much more complex strand exchange reactions to be considered below. The renaturation reaction catalyzed by recA protein differs in several important respects from DNA renaturation promoted by the single-stranded DNA-binding protein, SSB, of *E. coli* (121) and the T4 gene 32 protein (122). (*a*) RecA protein, in contrast to SSB, is required in subsaturating amounts [maximal rates of renaturation are observed at a stoichiometry of 1 recA protein per 30 nucleotides of ssDNA, which is approximately 10–15% of saturation (see above)] (123). In fact, saturating levels of recA protein are inhibitory. (*b*) Renaturation promoted by SSB follows second order reaction kinetics, as does nonenzymatic

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renaturation. These reactions proceed by a rate-limiting collision between homologous sequences, followed by a rapid "zippering up" of the strands to form a DNA duplex (124). Catalysis by SSB is thought to involve unfolding of regions of secondary structure, resulting in increased rates of nucleation (122), although other factors may also be important (121). In contrast, recA protein–promoted renaturation proceeds by a first order rather than a second order process, suggesting that there is rapid non–rate determining formation of an intermediate prior to complete renaturation (123). (*c*) RecA protein–catalyzed renaturation is stimulated by ATP (114, 123, 125). There is no effect of ATP or other nucleoside triphosphate on SSB-promoted renaturation. The role of ATP hydrolysis in recA protein–catalyzed renaturation is complex. At low Mg^{2+} concentrations (10 mM) maximal rates of renaturation are obtained in the presence of ATP; at high recA protein levels, ATP is absolutely required for catalysis of renaturation. A lesser stimulation is observed with ADP as cofactor, whereas ATPγS, which induces the irreversible binding of recA protein to ssDNA, permits only low levels of recA protein–promoted renaturation (123, 125).

When the Mg^{2+} concentration is increased from 10 mM (the optimal concentration in the presence of ATP) to 30 mM, the rate of ATP-independent renaturation increases 2- to 3-fold and proceeds at approximately the same rate as the ATP-stimulated reaction. Mg^{2+} concentrations in excess of 40 mM are inhibitory (123).

RecA protein can catalyze the renaturation of a (+) circular ϕX174 DNA strand with its (−) linear complement to generate RFII molecules at 10-mM Mg^{2+} in the absence of ATP (F. R. Bryant, I. R. Lehman, unpublished). This reaction, which may be related to the three-strand exchange reaction involving a circular single strand and a homologous linear duplex, described below, may proceed by either of two mechanisms. In one, the DNA strands are brought together directly by means of two DNA-binding sites on the recA protein molecule (DNA·recA·DNA). In the second, the DNA strands are brought together by interaction between recA protein molecules bound to different strands (DNA·recA·recA·DNA). The increase in the stoichiometry of binding of recA protein to ssDNA from 1 recA monomer per 4 to 1 recA monomer per 8 nucleotides in the presence of ATPγS suggests that a recA monomer contains two DNA-binding sites (83). Further support for the two binding site model for DNA renaturation has come from experiments in which stable recA protein (+) circular DNA complexes ($t_{1/2}$ of dissociation ~30 min) are mixed with the complementary (−) linear strand. Under these conditions RFII formation occurs at nearly the same rate as that observed upon addition of recA protein to a mixture of the (+) and (−) DNA strands (F. R. Bryant, I. R. Lehman, unpublished).

The notion of two DNA-binding sites is also consistent with studies of the

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mechanism of transfer of recA protein from one ssDNA molecule to another. Transfer is slow in the absence of nucleotide cofactors, but is greatly stimulated by ATP (83). In both cases transfer appears to be a cooperative process in which many recA monomers, possibly in the form of clusters, are transferred from one ssDNA molecule to another in a single event. Cooperative transfer appears to proceed by the intermediate formation of a complex between a recA protein–ssDNA complex, and a second ssDNA molecule, followed by transfer of the recA protein from the first to the second strand (83, 88, 89). The formation of a two-stranded structure of this kind provides an intermediate that would appear to be well suited to bringing complementary DNA strands together so that pairing can occur. Stimulation of the reaction by ATP may result from the increase in the rate of transfer of recA protein, which may then serve to increase the cycling rate of recA protein between different DNA strands in the search for homology.

DNA Strand Exchange

The activities of recA protein described above—binding to DNA, ATP hydrolysis, and the pairing of complementary DNA strands—converge in the DNA strand exchange reaction, which represents a more complete experimental model for the action of recA protein in vivo. Given the appropriate substrates, recA protein can transfer a DNA strand from one homologous partner to another (126, 127). In the course of this reaction (when four strands are involved), an intermediate identical to that predicted by Holliday can readily be detected (128). A thorough understanding of this process should provide a chemical basis for the central stages of general recombination.

The substrates that can be utilized by recA protein for strand exchange are limited only by topological constraints (126) and by the requirement for a short region of ssDNA to initiate the reaction (126–129). The reactions can then take a variety of forms and lead to a variety of products (Figure 2). The need for well-defined substrates and products for kinetic analysis, and the recognition that recA protein can produce heteroduplex DNA thousands of base pairs in length (126, 127), have led to the development of a system that has become standard in the analysis of recA protein–promoted DNA strand exchange, the exchange between linear duplex and circular single-stranded phage DNAs (127) (Figure 2*D*). The substrates and products of this reaction, which occurs over a convenient time period (10–30 min), are well-defined and readily distinguished by a variety of methods. Nearly a dozen different assays have been employed to measure various aspects of the reaction. Since the single-stranded circles isolated from virions are (+) strands, they cannot pair with each other; the reaction therefore avoids interference by recA protein–promoted DNA renaturation. The availability of a great variety of homologous, heterologous, and chimeric substrates provides a flexibility that

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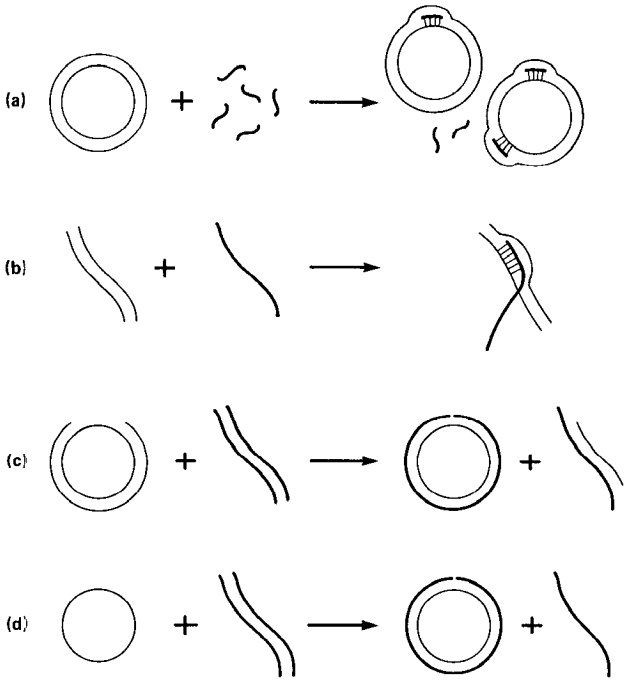


Figure 2. Strand exchange reactions promoted by recA protein.

has been exploited extensively. The reaction can be conveniently divided into three phases for study, each of which poses a different and interesting biochemical problem.

The first phase, complex formation or presynapsis, consists of the binding of stoichiometric amounts of recA protein to the available ssDNA (81, 130), resulting in the nucleoprotein filament described above that catalyzes ATP hydrolysis. The formation of this complex is related to the basic problem of how a protein binds nonspecifically and cooperatively to DNA. However, the ATP hydrolysis that occurs as a consequence of the binding complicates the analysis of this interaction. The product of the binding reaction is the active species in the subsequent phases of strand exchange. The structure and properties of the recA-ssDNA nucleoprotein filament are therefore central to any consideration of DNA strand exchange.

The second phase of the reaction involves alignment of the ssDNA within the nucleoprotein filament with complementary sequences in the duplex DNA (126, 131–133). The biochemical problem changes here. Binding to ssDNA effectively transforms recA protein into a duplex DNA-binding protein that now exhibits a sequence specificity dictated by the sequence of the ssDNA

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bound in the nucleoprotein filament. Several steps in this process have been identified.

Once homologous sequences are aligned, strand exchange begins. In the third phase of the reaction the (+) strand in the DNA duplex is displaced, and is replaced with the strand brought in by the nucleoprotein filament. This reaction is functionally equivalent to branch migration but differs from spontaneous branch migration (134) in that it requires ATP hydrolysis; in fact, it is the only phase of DNA strand exchange that requires ATP hydrolysis (127, 135), and it exhibits a unique polarity (136–138). Of special interest here is the coupling between a unidirectional reaction and chemical energy. The problem is a classical one in biochemistry, analogous in some respects to muscle contraction and ATP-driven ion pumps (139). Each of the three phases of the DNA strand exchange reaction will now be considered in detail.

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FORMATION OF REC A–SSDNA NUCLEOPROTEIN COMPLEX The central importance of ssDNA in the initiation of recA protein–promoted DNA strand exchange was recognized early (93, 97, 102, 114). As noted above, recA protein binds much more readily to ssDNA than to duplex DNA under conditions that are optimal for DNA strand exchange. Characterization of recA protein–ssDNA complex formation as a separate phase of DNA strand exchange has involved a variety of strategies. These include the use of DNA challenges (130), characterization of a lag in strand exchange (140), and isolation of the complex after its formation (81).

Formation of the complex requires the binding of stoichiometric amounts of recA protein to the ssDNA. As noted above, the values that have been reported for the binding site size for a recA monomer vary from 3 to 6 nucleotides. Secondary structure in the ssDNA impedes binding, imposing a barrier that can be circumvented by the addition of SSB (141, 84). Register & Griffith have recently found that the filaments assemble unidirectionally, 5'→3' along the ssDNA (142). This asymmetry may determine the polarity of the third phase of the reaction.

Formation of a complex active in strand exchange also requires ATP. Addition of ATP has several effects on the recA protein–ssDNA complex. These include a change in the range of nucleoside triphosphates that can be hydrolyzed by recA protein (F. R. Bryant, unpublished), as well as the change in the rate of transfer of recA protein between DNA molecules described above. These findings suggest that one or more ATP-induced changes in the state or conformation of recA monomers occurs in the complex. Structural evidence for such changes is presently limited to observations made by electron microscopy, and few details are available concerning changes at the molecular level. As already noted, characteristic differences can be observed by electron microscopy in recA protein–DNA complexes

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formed in the absence of nucleotide cofactors, in the presence of ATP, and in the presence of ATPγS. Leahy & Radding (143) have recently suggested that recA protein is in closest contact with the phosphate backbone of the DNA strand, leaving the bases free to participate in pairing reactions.

The rate of assembly of the active recA protein–ssDNA complex is affected by the nature of the ssDNA, the ionic conditions, and by the presence or absence of SSB. In the absence of SSB and with 10–15-mM $MgCl_2$, a discernible lag in strand exchange occurs, attributable to slow complex formation, which can be partially overcome by preincubation of recA protein with the ssDNA (140, 144). The lag is at least in part a function of secondary structure in the ssDNA. Thus, the rate of complex formation in the absence of SSB is enhanced by low Mg^{2+} concentrations, which eliminate most secondary structure in ssDNA (140, 141, 144), or by using polydT that lacks secondary structure (86, 87, 113, 145). When SSB is added, the rate of complex formation is greatly enhanced and the lag in strand exchange is abolished (81, 141). The role of SSB in the formation and maintenance of this complex is described in more detail below.

The recA-ssDNA nucleoprotein complex that results from this phase of the reaction is clearly the active species in the subsequent phases of strand exchange. Thus, there is a stoichiometric requirement for recA protein for the overall strand exchange reaction (146). Moreover, the rate and efficiency of strand exchange are functions of both the binding density of the recA protein in the complex and the stability of the complex (147). When SSB is added, the complexes have been shown to be kinetically competent as intermediates in strand exchange (130). Finally, when the complexes are separated from free recA protein and other components of the reaction, they are fully functional in strand exchange (81).

Once formed, the nucleoprotein complexes hydrolyze ATP with an apparent k_{cat} of about 30 min^{-1} at pH 7.5 and 37°C (84, 113). Hydrolysis occurs whether or not duplex DNA is added to initiate strand exchange, suggesting that much of the hydrolysis is irrelevant and not coupled to useful work. Alternatively, the ATP hydrolysis may reflect a system at idle, coupled to conformational changes that would result in strand exchange if homologous DNA were made available. The role of ATP hydrolysis will be considered in more detail below.

SEARCH FOR HOMOLOGY: SYNAPSIS The mechanism by which proteins that bind specific sequences locate their binding sites is determined to a large extent by interactions with nonspecific DNA (148). As described above, binding of recA protein to duplex DNA under conditions optimal for strand exchange is highly dependent on the presence of homologous ssDNA. To put it another way, the recA-ssDNA nucleoprotein complex is a sequence-specific

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duplex DNA-binding entity, with the specificity determined by the sequence of the ssDNA. The complex is much larger and more complex than such well characterized specific DNA-binding proteins as RNA polymerase, the EcoRI restriction enzyme, and the lambda and lactose repressors. Mechanistically, however, the problem of how this complex searches for homologous sequences in duplex DNA can be analyzed to a first approximation in terms of interactions with nonspecific (heterologous) and specific (homologous) DNA sequences using approaches similar to those employed in the simpler systems. The problem is rendered more tractable by the demonstration that ATP hydrolysis is not required; the search for homology can be completed in the presence of ATPγS, which is not hydrolyzed by recA protein (127, 135).

A number of studies have provided evidence that recA protein–ssDNA complexes can bind nonspecifically to heterologous duplex DNA. Such interactions have been detected in the presence of both ATPγS (93, 94, 149) and ATP (150–152). The rate of formation of these complexes is consistent with the idea that they are intermediates in the search for homology (152). Radding and colleagues have shown that these nonspecific interactions are manifested in vitro by large networks that link together many recA protein–ssDNA complexes and duplex DNA molecules early in the strand exchange reaction (151, 152). These networks effectively reduce the volume in which the search for homology must occur and can be harvested by brief centrifugation. Conflicting results have been obtained in experiments designed to investigate the mechanism by which homologous sequences are aligned. Gonda & Radding found that the search for homology is facilitated by the presence of lengths of heterologous DNA attached to the homologous duplex, with rates of synapsis increasing with the length of the heterologous tail (131), suggesting a processive mechanism. Julin et al, in a similar set of experiments, found that heterologous tails had no effect on the rate of the reaction (132). Wherease the experiments differ in the use of SSB in the latter study (resulting in higher rates of synapsis), the discrepancy has not been explained satisfactorily.

In studies initiated by the Radding group, two types of synaptic structures have been described. These differ depending on whether the paired strands of DNA are interwound or not. Paranemic joints, in which the strands are paired but not interwound, are characterized by a lack of a requirement for a free homologous end (99, 126, 153) and their greater sensitivity to protein denaturants (154). They have been observed directly by electron microscopy (155). Paranemic joints are formed fast enough to be considered intermediates in the synapsis pathway, and are very likely precursors of the plectonemic joints in which the DNA strands are interwound (99, 132, 154).

The interaction with duplex DNA triggered by binding of recA protein to ssDNA generally results in the unwinding of the duplex. With ATPγS, unwinding has been detected in the presence of both heterologous and

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homologous ssDNA (149). The apparent unwinding in the presence of heterologous ssDNA may reflect in part a significant ssDNA-independent binding of duplex DNA, which is detected in the presence of ATPγS (79, 101, 103, 108). When ATP is used, the unwinding reaction exhibits a considerable degree of dependence on homology in the ssDNA (96–99), implying that the unwinding occurs after homologous alignment of the two DNAs has occurred. The extent of unwinding has not been precisely determined. Several studies indicate that 100–300 base pairs of duplex DNA are instantly unwound upon formation of a paranemic joint (140, 156, 157), a necessary prelude to the formation of a plectonemic joint molecule in which the (−) strand of the linear duplex is interwound with (+) ssDNA. More extensive unwinding has also been observed. Since it is dependent on ATP and homologous ssDNA, it appears to be triggered by the formation of paranemic joints (or D-loops) (96, 98). When the unwinding is topologically trapped in a closed-circular DNA molecule, it is manifested by a superhelical density significantly greater than that of RFI DNA. Formation of this extensively unwound species is slow, requiring 20–40 minutes to reach completion (96, 98). The unwound species can therefore not be an intermediate in the synapsis pathway, since the formation of a plectonemic joint generally requires less than a minute. The slow unwinding may reflect a direct binding of excess recA protein to the duplex DNA, which can occur following D-loop formation (96, 157) or strand exchange (B. C. Schutte, M. M. Cox, unpublished). As described above, extensive unwinding is a characteristic of the binding of recA protein to duplex DNA.

A reaction scheme consistent with these results is presented in Figure 3. The first two steps have been detected in the presence of ATPγS and do not require ATP hydrolysis. The second step, (*b*), includes both the homologous alignment of the two DNA molecules and the subsequent unwinding of a short region of the DNA duplex. The third step, (*c*), in which the incoming circular single strand is interwound with its complement to form a plectonemic joint, does require ATP hydrolysis (154). This final step, (*d*), initiates and is probably indistinguishable from the branch migration of the third phase. For purposes of discussion, we will define the end of the second phase, i.e. synapsis, as the product of the second step in the pathway shown in Figure 3. The product of synapsis by this definition is therefore a ternary complex containing the ssDNA-recA nucleoprotein filament and duplex DNA, which has been bound, homologously aligned with ssDNA, and unwound. Elucidation of this very complex reaction in mechanistic detail awaits more extensive structural and kinetic analysis.

REC A PROTEIN–PROMOTED BRANCH MIGRATION Branch migration in solution under physiological conditions is a facile reaction. Rates measured in vitro were believed to account satisfactorily for the branch migration observed

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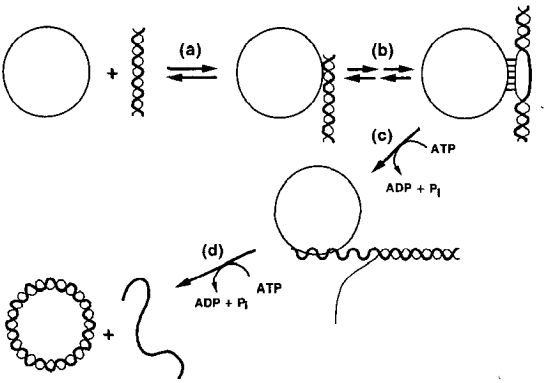


Figure 3. Reaction pathway for the transfer of a strand from a linear duplex to a circular ssDNA showing (*a*) nonhomologous interactions leading to (*b*) formation of a paranemic joint. Formation of a plectonemic joint (*c*) is coupled to ATP hydrolysis. Branch migration (*d*) is shown as a step separate from plectonemic joint formation, but is most likely an extension of the same process.

during recombination in fungi and other organisms (134). There was no reason, therefore, to expect this reaction to be catalyzed. The observations that branch migration in the presence of recA protein required ATP hydrolysis (127) and proceeded with a unique polarity (138, 156), however, clearly implicated recA protein as a catalyst in this reaction. The polarity of the reaction between circular single-stranded and linear duplex DNA proceeds in the 5'→3' direction relative to the invading single strand (136–138). This is identical to the direction in which recA filaments assemble on ssDNA (142). With another pair of DNA substrates, linear single-stranded and circular duplex DNA, it has recently been found that strand exchange may advance in the 3'→5' direction relative to the linear single strand (158). In this case, however, it is not clear whether the polarity observed is at the level of synapsis or branch migration.

RecA protein contributes two important properties to branch migration. First, by providing a unique direction, it ensures that heteroduplexes thousands of base pairs in length are created efficiently. The recA protein–catalyzed reaction is actually much slower than the spontaneous one [5–20 bp s^{-1} (146) vs 6000 bp s^{-1} (134)]. However, by limiting the direction of the reaction, efficiency is improved relative to the spontaneous but random process. Whereas the spontaneous process provides a 1 in 3 chance of migrating 950 base pairs in 10 min (134), the recA protein–catalyzed reaction has a nearly 100% chance of proceeding linearly for 3000 base pairs in the same time period. The second property is probably closely related to the first. Unlike the spontaneous process, recA protein–promoted branch migration can proceed efficiently past short DNA mismatches and pyrimidine dimers (159,

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160). It can even proceed past deletions or insertions hundreds of base pairs long (161). This property is probably of great utility to the cell (see ENERGETICS, below).

The active species in branch migration is the recA-ssDNA nucleoprotein filament formed in the first phase of the reaction. The mechanistic question then becomes: How is this filamentous structure employed to promote unidirectional branch migration coupled to ATP hydrolysis? Several basic characteristics of this reaction must be addressed in any consideration of mechanism. These include the location of recA protein at all stages of the reaction, the dynamic state of the filament, the location of the participating DNA strands relative to the filament, the specific role of ATP hydrolysis, and the finding by Bianchi et al that branch migration can proceed through extensive regions of nonhomology (161). Information about these basic features of DNA strand exchange is still very limited, as reflected in the variety of plausible models that can be proposed for the reaction (162–165).

The first question that can be asked about a filamentous system is whether the important events occur at ends or throughout the filament. An attractive model can be developed by drawing an analogy to two other filamentous systems, tubulin and actin. Hydrolysis of nucleoside triphosphates in these systems occurs at the ends of the filament and is coupled to a treadmilling reaction, which involves a net addition of subunits to one end of the filament at the expense of the other (166). Similarly, recA protein could promote unidirectional branch migration by coupling movement of the branch to association or dissociation of monomers at a filament end (162, 163). ATP hydrolysis by the recA nucleoprotein filament, however, is not restricted to the ends of the filament (113), and is not tightly coupled to complete association or dissociation of monomers (89, 90). Both observations provide evidence against treadmilling in recA filaments in the classical sense. An exchange of recA monomers between nucleoprotein complexes can be observed when the complexes are in transient contact (90). The role, if any, of this exchange of recA monomers in DNA strand exchange has yet to be determined.

A second and equally attractive model involves dissociation of recA protein at the branch point. RecA protein binds weakly to duplex DNA under conditions optimal for strand exchange. Inasmuch as the ssDNA in the recA nucleoprotein filament is converted to heteroduplex DNA as the branch point passes, dissociation of recA protein at the branch point might be expected. Direct observation of recA filaments during strand exchange by electron microscopy reveals that under some conditions dissociation might accompany movement of the branch (167). In fact, dissociation at the branch point has been incorporated into a model proposed by Howard-Flanders and colleagues (162). The electron microscopic observations, however, conflict with results

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obtained in solution and might reflect differences related to fixation of samples for microscopy. Results derived from patterns of ATP hydrolysis (113), DNase protection (168, 169), and the topology of the products of strand exchange (169) provide evidence that little net dissociation of recA protein occurs during the reaction. Instead they indicate that the recA protein is bound contiguously to the heteroduplex DNA well after strand exchange is complete. These findings do not conflict with the weak binding of recA protein to duplex DNA at neutral pH, since it has been demonstrated that the weak binding reflects a slow step in the association pathway rather than an unfavorable binding equilibrium (101). Strand exchange apparently bypasses this slow association step. These results taken together do not rigorously eliminate a role for association or dissociation in strand exchange, but they do indicate that the nucleoprotein filament remains relatively intact throughout the reaction.

A second question involves the location of the DNA strands undergoing exchange relative to the filament. One model has been suggested in which both DNA molecules are inside the spiral recA nucleoprotein filament (162). In another model, only one DNA molecule is inside the filament, with the other outside (164). The available information is again suggestive rather than definitive. The initiation of strand exchange results in an immediate decrease (up to 30%) in the rate of ATP hydrolysis that correlates precisely with the length of homology between the two DNA molecules involved (B. C. Schutte, M. M. Cox, unpublished). This finding suggests that some degree of homologous contact must occur almost immediately along the length of the homologous incoming DNA duplex. However, the duplex DNA acquires resistance to nuclease digestion with a time course that parallels the formation of heteroduplex DNA (169). Thus, the duplex DNA appears to be bound outside the filament until it is incorporated into heteroduplex. Much more detailed information is required about the structure of this complex and the space available within recA filaments for a more definitive answer to this question.

Fourier transform enhancements of electron micrographs of recA nucleoprotein filaments reveal a right-handed helical structure with a major groove (170). At least one DNA molecule lies within this complex, contacted primarily along the phosphate backbone (143). It is reasonable to suppose that the bases are exposed in the major groove of the filament, providing an active site for strand exchange. Inasmuch as DNA in its native form is a right-handed helix, rotation of the exchanging DNA molecules must occur to bring about unidirectional branch migration. This can occur, to a first approximation, in two ways, as illustrated in Figure 4. A model has in fact been proposed (164) in which the rotation shown in Figure 4*b* is coupled to strand exchange within the groove of the filament (Figure 5). Regardless of the

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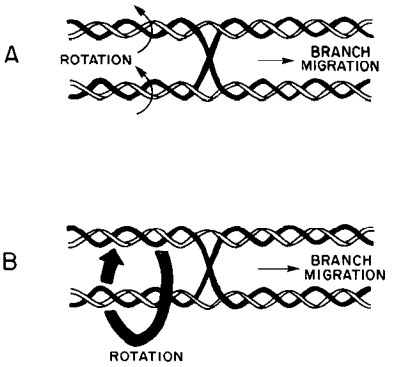


Figure 4. Rotation of right-handed DNA to produce branch migration. Rotation of (*A*) both DNA molecules or (*B*) one about the other produces branch migration in the direction indicated.

actual mechanism of strand exchange, the rotation required to bring it about ensures that a topoisomerase is required to resolve topological strain induced by the reaction.

ENERGETICS OF DNA STRAND EXCHANGE: TO WHAT IS ATP HYDROLYSIS COUPLED? RecA protein–promoted branch migration requires ATP hydrolysis. Indeed, a consideration of the principle of microscopic reversibility indicates that this reaction cannot be made unidirectional without an investment of chemical energy. ATP hydrolysis appears to be very inefficient, however, with over 100 ATPs hydrolyzed per base pair of heteroduplex formed under optimal conditions (112). All of the recA monomers in a nucleoprotein filament hydrolyze ATP in the absence of strand exchange. This is also true during strand exchange, with no correlation evident between rates of ATP hydrolysis and the number of migrating branch points (113). It is important to determine whether the apparent excess represents an idling mechanism, reflecting an activity that results in strand exchange when an appropriate substrate is available, or whether much of the ATP hydrolysis is not coupled to useful work.

The latter notion has in fact been suggested by Kowalczykowski and colleagues (112, 165). These workers noted that a favorable correlation can be drawn between ATP hydrolysis and strand exchange if it is assumed that most of the ATP hydrolysis is uncoupled from strand exchange and can therefore be subtracted from the total (165). Under most conditions, however, the ATP hydrolysis observed in the presence of ssDNA alone is significantly greater than the total ATP hydrolysis observed once strand exchange commences (B. C. Schutte, M. M. Cox, unpublished). In fact, the efficiency of the reaction can be improved to approximately 16 ATPs per base pair simply

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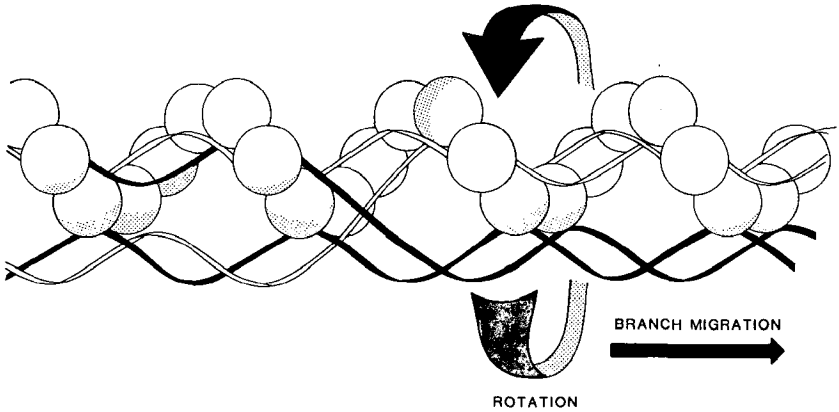


Figure 5. A model for recA protein–promoted branch migration. A recA nucleoprotein filament formed on one DNA molecule promotes branch migration by rotating a second DNA molecule (linked to the first via a crossover junction) around the outside of the filament. The resulting rotation is that illustrated in Figure 4*b*. See Ref. 163 for details.

by adding low concentrations of ADP (112). High levels of ADP cause dissociation of recA protein from the DNA, and the improved efficiency may be related to a shortening of the filaments under these conditions (112). Radding and colleagues have observed that branch migration is much less sensitive to ADP than the earlier phases of the reaction (98, 140), possibly indicating that relatively short recA filaments are fully competent to promote extensive branch migration. Nevertheless, these results indicate that not all of the ATP hydrolysis that occurs during strand exchange is required for branch migration.

On the other hand, it is possible that most of the ATP hydrolysis is involved in strand exchange and that the molecular events in the process occur throughout the filament rather than uniquely at the branch point. That is, an important advantage may be gained by the apparent waste of chemical energy. This notion involves a consideration of the strategy of employing a filament to perform strand exchange. Unidirectional branch migration could in principle be carried out efficiently by an enzyme or small complex possessing appropriate helicase and reannealing activities. However, *E. coli* may have evolved a complex filamentous system to carry out this reaction. The inefficiency that might result if all of the recA monomers in the complex hydrolyze ATP is significant. However, even this expense is trivial when compared to the energy required by the protein synthetic machinery to assemble the 352 amino acids of every recA monomer in the filament. This investment by the cell can only be rationalized in terms of an essential reaction. Extensive branch migration promoted by the filamentous complex of recA protein may therefore have a significant impact on cell survival: (*a*) it could provide sufficient

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energy to bypass DNA lesions, (*b*) it could provide protection for the DNA branch point, preventing premature resolution, and (*c*) it might exclude other DNA-binding proteins that could block branch migration. The excess ATP hydrolysis may not be required thermodynamically to promote branch movement, however, it may be a price that is paid to ensure that the branch is protected and that extensive branch migration can occur. Possible roles for branch migration in vivo are discussed below.

FOUR-STRAND EXCHANGES RecA protein promotes strand exchange between two duplex DNA molecules (82, 126, 128, 129, 171). To initiate the reaction, one of the molecules must have a single-stranded gap or tail and the second must have a free DNA end that can overlap the gap. Studies of these reactions have revealed the formation of classical Holliday intermediates by recA protein in vitro. These reactions are not as well characterized as the three-strand exchanges described above, reflecting in part the added difficulty in generating substrates and monitoring the reactions quantitatively. The recA nucleoprotein filament is again the active species in the reaction, and recA protein binding at the single-stranded region leads to a rapid and complete invasion of the adjacent DNA duplex (171; J. Lindsley, M. M. Cox, unpublished). The energetics of the four-stranded exchange reactions have not been investigated in detail, although efficiencies appear to be similar to those observed in the three-strand reaction (J. Lindsley, M. M. Cox, unpublished).

ROLE OF STRAND EXCHANGE IN VIVO Approximately 1200 monomers of recA protein are present in an *E. coli* cell under normal growth conditions (172, 173). Upon induction of the SOS system, this number can increase by as much as two orders of magnitude (172, 173). The recA protein is involved in recombination, postreplication repair, and numerous other processes. In its role as a recombinase, recA protein is probably responsible for the steps in recombination that it catalyzes in vitro: pairing and branch migration. Some in vivo evidence mirrors the findings in vitro. Yancy & Porter (174) have found that low concentrations of mutant recA protein interfere with the activity of wild-type recA protein in the cell. A likely interpretation of this finding is that the mutant monomers interfere with recA filament formation, underlining the importance of the recA nucleoprotein complex in vivo. The polarity observed in recombination in vivo is also consistent with the polarity observed in vitro (175).

The direct role of recA protein in postreplication repair suggests that it is essentially a recombinational process (176). RecA protein is uniquely suited to play a role in the repair of pyrimidine dimers or other lesions occurring in ssDNA. Whereas these lesions may represent a major barrier to DNA polymerase action, recA protein–promoted branch migration can move past

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them easily. The role of recA protein in this instance is very likely the conversion of the lesion-containing ssDNA into a duplex via branch migration (176), facilitating repair. The incoming strand would be derived from DNA on the opposite side of the replication fork. Inasmuch as a lesion in a single-stranded region is unrepairable, and therefore lethal, the importance of branch migration becomes apparent. The use of a recA nucleoprotein filament and the enormous investment in energy that this represents, may serve to ensure that this and other related functions occur in vivo with high efficiency.

Role of SSB in Strand Exchange

SSB plays an important role in homologous recombination in *E. coli* (177–179). A role in moderating the nuclease activity of the recBCD enzyme has been noted above. Important effects of SSB were noted in some of the earliest work on recA protein–promoted pairing reactions in vitro. In these early studies, SSB appeared to act simply in a sparing role, reducing the amount of recA protein required by binding to excess ssDNA (180, 181). Subsequently it was demonstrated that DNA strand exchange is stimulated by SSB (127, 130). RecA protein is required in stoichiometric amounts for optimal activity, whether or not SSB is present (146).

SSB binds to ssDNA as a tetramer of 18,873-dalton subunits (182). Binding is rapid and stoichiometric, with binding densities ranging from 1 tetramer per 33–65 nucleotides depending on conditions (183, 184). The monomer contains four tryptophans, and binding is manifested by a large quenching of the tryptophan fluorescence of the protein (183, 184). This property has proved to be useful in binding studies. It has recently been demonstrated that SSB exists in at least two DNA binding modes (183–185). The "low salt" binding mode is prevalent at NaCl or Mg^{2+} concentrations of less than 10 or 1 mM, respectively. This mode is characterized by a high degree of cooperativity and a binding site size of 33 nucleotides. A relatively smooth filament of SSB is often observed in the electron microscope under these conditions (185). The "high salt" binding mode is prevalent above 200 mM NaCl or at 10 mM $MgCl_2$, and is characterized by a very low degree of cooperativity and a binding site size of approximately 65 nucleotides. This form is characterized by a "beads on a string" appearance when bound to ssDNA (185). At intermediate salt concentrations, the two forms can coexist (184). These findings explain a variety of observations regarding SSB obtained under different conditions (186–192).

When SSB exists in its "low salt" binding mode, binding of ssDNA by SSB and recA protein appears to be strictly competitive. SSB will displace recA protein almost entirely (147). Stimulation of recA protein–promoted DNA strand exchange is observed, however, only under conditions in which SSB exists predominantly in its "high salt," low-cooperativity binding mode. These conditions (10-mM Mg^{2+}) are optimal for the strand exchange reac-

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tion. Even here, SSB will rapidly displace recA protein from ssDNA in the absence of ATP (84). Upon addition of ATP, the steady-state binding equilibrium between the two proteins is displaced in favor of recA protein (84). If recA protein is added prior to SSB, the SSB does not displace the recA protein. Instead it plays a significant role in establishing a stable, stoichiometric, and highly active recA protein filament on the ssDNA (84, 146, 147). On circular ssDNA, the resulting complex is uniform and unbroken as viewed in the electron microscope (76, 80, 81). When SSB is added first, there is a long lag in the binding of recA protein (84). However, given sufficient time, recA protein can displace the SSB and the resulting complex is the same as that formed when SSB is added last (130, 146). This order of addition effect explains the finding that SSB inhibits recA protein–promoted ATP hydrolysis (180, 192). When SSB is added last, no inhibition occurs, and in fact an apparent enhancement of ATP hydrolysis is observed (84, 147, 193).

Stimulation of DNA strand exchange by SSB can be traced directly to an effect on the formation of recA-ssDNA complexes in the first phase of the reaction (146). Stimulatory effects of SSB on the formation of recA nucleoprotein filaments have been noted in a number of studies (76, 81, 185). Radding and colleagues have demonstrated that recA protein binding to ssDNA is impeded by DNA secondary structure (81, 141); this barrier to binding is removed upon addition of SSB (81, 84, 141).

Several explanations for the role of SSB in establishing a uniform recA-nucleoprotein filament on ssDNA have been suggested. The first and most straightforward is that SSB serves to denature secondary structure in the DNA (81, 141). The SSB is then displaced by recA protein to form the contiguous recA nucleoprotein filament. In support of the idea that SSB has only a transient action, Radding and coworkers have shown that when recA protein was bound to ssDNA under conditions that did not favor the formation of secondary structure (low Mg^{2+}), recA protein bound uniformly to the DNA. After further addition of Mg^{2+} up to concentrations optimal for strand exchange, the reactivity of the complexes was significantly improved relative to complexes that had not been subjected to Mg^{2+}-shift (81, 141).

Other studies, however, have demonstrated that SSB is not displaced from the recA protein–ssDNA complex (84). As measured by quenching of the intrinsic SSB fluorescence, an interaction of SSB with recA protein–ssDNA complexes was demonstrated. This interaction, which is continuous for periods greater than an hour, is ATP-dependent, and requires sufficient SSB to saturate the ssDNA. The association has the effect of increasing the binding density of recA protein and enhancing the apparent k_{cat} for recA protein–promoted ATP hydrolysis (84). The association is required to maintain the stability of recA protein–ssDNA complexes. In contrast, the stability and uniform binding of complexes formed via the Mg^{2+} shift do not persist.

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Within a period of 20 min after the shift to high Mg^{2+} concentration, the complexes revert to a form equivalent in all respects to recA protein–ssDNA complexes formed at 10 mM Mg^{2+} in the absence of SSB (147). This deterioration does not occur in the presence of SSB (147).

Two models have been proposed to accommodate these results. In one, proposed by Kowalczykowski and coworkers (193, 193a), SSB prevents deterioration of the recA protein–ssDNA complexes by continually melting-out secondary structure and allowing rebinding of recA protein. Thus, SSB is used reiteratively to maintain the stability of the recA complex. In the second model, there is a direct interaction of SSB with the recA protein complexes (84). In this case it is the joint recA protein–SSB complex that denatures and binds to regions of secondary structure.

Data presently available suggest that SSB plays a role that may be more complicated than the transient or reiterative denaturation of secondary structure in DNA. Under conditions optimal for DNA strand exchange, the interaction between SSB and recA protein is not competitive. In fact, the amount of each protein in the complex is sufficient, separately, to saturate the ssDNA (84, 90). Maintaining high levels of SSB increases rather than decreases the stability of the recA complexes in dilution experiments (147). Moreover, the level of fluorescence quenching of SSB observed after complex formation is unaffected by the addition of excess recA protein or SSB (84). Exchange of recA protein between free and bound forms, which might be expected if it were cycling in and out of regions of secondary structure, is not observed in the presence of SSB (90). It has also been demonstrated that SSB that has participated in the formation of recA-ssDNA complexes is effectively sequestered and unable to participate immediately in further complex formation (D. A. Soltis, B. Stockman, M. M. Cox, I. R. Lehman, unpublished).

The "high salt" binding mode of SSB is characterized by a cooperativity parameter (ω) of approximately 50, which is low enough to suggest that little or no interaction occurs between SSB tetramers (183, 184). Lohman and coworkers have noted that the binding site size for SSB in this binding mode increases from 65 to 77 nucleotides when M13 ssDNA is substituted for polydT (184). They further suggested that this change might reflect blocking of SSB from regions of secondary structure. Under similar conditions, only 80% of a sample of ssDNA is protected from DNase digestion in the presence of excess SSB (169). These results suggest that neither recA protein nor SSB can bind to regions of significant secondary structure under these conditions. If this interpretation is correct, removal of secondary structure should involve a joint complex of the two proteins. However, attempts to isolate such complexes have been unsuccessful (194), nor have they been detected by electron microscopy. Some alterations in the structure of recA-ssDNA complexes formed in the presence of SSB have been noted, leading to the

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suggestion that mixed complexes may exist in which SSB and recA protein are not separated into domains (80). Finally, it should be noted that the stimulatory effect of single-stranded DNA-binding protein is not unique to *E. coli* SSB; the T4 gene 32 protein and the phage λ β protein can also stimulate recA protein–promoted DNA strand exchange (195). In sum, physical evidence for a direct interaction between recA protein and SSB is still lacking and is clearly required before the notion of such a complex can be accepted.

Whatever the mechanism of SSB action, these studies underline the importance of the recA nucleoprotein filament. The activity of recA protein in every instance correlates positively with an increase in the binding density of recA protein and the formation of unbroken filaments; interference with filament formation in every instance inhibits recA protein action. The active species in recA protein–promoted DNA strand exchange is clearly a uniform stoichiometric recA nucleoprotein filament.

RESOLUTION OF HOLLIDAY STRUCTURES

The final step in general recombination is the resolution of the Holliday intermediate. As described above, the recBCD enzyme could, by virtue of its nuclease activity, play a role in this reaction. Recently, however, an enzyme has been isolated from bacteriophage T4–infected cells, endonuclease VII, the product of T4 gene 49, that exhibits a demonstrable specificity for the cleavage of Holliday junctions (196–199). Cells infected with gene 49 mutants accumulate a highly branched multimeric form of T4 DNA called very-fast-sedimenting (VFS) DNA (196), and the infections are abortive. In vitro, the enzyme cleaves DNA specifically at the base of extruded cruciforms, which are used as analogues of the Holliday intermediate (198, 199). The cuts are symmetrically placed on both strands of the DNA, 2 or 3 nucleotides 5' to the end of the cruciform in each case (198). The resulting DNA molecule is linear with hairpin ends and single ligatable nicks at positions corresponding to the stem base of the cruciform (198, 199). A similar activity has recently been detected in yeast extracts (200, 201).

RECOMBINASES IN OTHER CELLS

Proteins closely related to recA protein of *E. Coli* appear to be widely distributed among bacteria. RecA-like proteins have been isolated from *B. subtilis* (116), *Salmonella typhimurium* (202), and *Proteus mirabilis* (203). Another recA-like protein, the product of the *uvsX* gene of bacteriophage T4, has been isolated from T4-infected cells (204–206). Although catalyzing many of the DNA pairing reactions catalyzed by recA protein, the uvs x protein hydrolyzes ATP at an approximately 20-fold greater rate than recA protein. Surprisingly, it produces AMP and PPi as well as ADP and Pi (206).

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Only ssDNA functions as a cofactor for ATP hydrolysis. Notably, the T4 gene 32 protein strongly stimulates the activity of uvs x protein, possibly in a manner analogous to the effect of SSB on recA protein–promoted reactions (204–206). These enzymes are all very similar to recA protein, with the most interesting distinction being the hydrolysis of dATP but not ATP by the *B. subtilis* enzyme noted above.

Only one recA-like enzyme has been purified from eukaryotic cells, the rec1 protein of *Ustilago maydis* (207–209). This protein promotes many of the pairing and strand transfer activities associated with the *E. coli* recA protein. However, rec1 protein–promoted strand exchange exhibits the interesting difference that the polarity of branch migration is the opposite of recA protein (207). The rec1 protein also exhibits a pronounced Z-DNA binding activity, which may be related to the mechanism by which pairing and synapsis reactions are promoted by this enzyme (209).

A variety of recA-like activities have been detected in mammalian cell extracts (210–212), but to date none of these has been purified. In several instances the activity differs from recA protein in a basic property, in particular, the absence of a requirement for ATP (212). Nonetheless these studies suggest that studies of homologous recombination with purified enzymes from *E. coli* may be generally applicable to recombination in higher cells. On the other hand, the apparent absence of a gene with the pleiotropic effects of the *recA* gene in many organisms including yeast suggests that interesting alternative mechanisms remain to be discovered.

Many gaps remain in our understanding of the molecular mechanism of general recombination. The ready accessibility of the recA-recBCD-SSB system from *E. coli,* coupled to the importance of the biological and biochemical questions that remain to be addressed, ensures that this prototype will occupy a central place in studies of general recombination for years to come.

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Volume 56, 1987

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CONTENTS

SUMMING UP, *Konrad Bloch* 1

FRACTIONATION AND STRUCTURAL ASSESSMENT OF OLIGOSACCHARIDES AND GLYCOPEPTIDES BY USE OF IMMOBILIZED LECTINS, *T. Osawa and T. Tsuji* 21

DYNAMICS OF MEMBRANE LIPID METABOLISM AND TURNOVER, *E. A. Dawidowicz* 43

TOPOGRAPHY OF GLYCOSYLATION IN THE ROUGH ENDOPLASMIC RETICULUM AND GOLGI APPARATUS, *Carlos B. Hirschberg and Martin D. Snider* 63

COMPLEXES OF SEQUENTIAL METABOLIC ENZYMES, *Paul A. Srere* 89

AMINOACYL tRNA SYNTHETASES: GENERAL SCHEME OF STRUCTURE-FUNCTION RELATIONSHIPS IN THE POLYPEPTIDES AND RECOGNITION OF TRANSFER RNAs, *Paul Schimmel* 125

INOSITOL TRISPHOSPHATE AND DIACYLGLYCEROL: TWO INTERACTING SECOND MESSENGERS, *Michael J. Berridge* 159

COMPLEX LOCI OF *DROSOPHILA*, *Matthew P. Scott* 195

ENZYMES OF GENERAL RECOMBINATION, *Michael M. Cox and I. R. Lehman* 229

TRANSFER RNA MODIFICATION, *Glenn R. Björk, Johanna U. Ericson, Claes E. D. Gustafsson, Tord G. Hagervall, Yvonne H. Jönsson, and P. Mikael Wikström* 263

FERRITIN: STRUCTURE, GENE REGULATION, AND CELLULAR FUNCTION IN ANIMALS, PLANTS, AND MICROORGANISMS, *Elizabeth C. Theil* 289

IMPACT OF VIRUS INFECTION ON HOST CELL PROTEIN SYNTHESIS, *Robert J. Schneider and Thomas Shenk* 317

INTRACELLULAR PROTEASES, *Judith S. Bond and P. Elaine Butler* 333

THE STRUCTURE AND FUNCTION OF THE HEMAGGLUTININ MEMBRANE GLYCOPROTEIN OF INFLUENZA VIRUS, *Don C. Wiley and John J. Skehel* 365

(continued)

INTRACELLULAR CALCIUM HOMEOSTASIS, *Ernesto Carafoli* 395

DNA MISMATCH CORRECTION, *Paul Modrich* 435

ALTERNATIVE SPLICING: A UBIQUITOUS MECHANISM FOR THE GENERATION OF MULTIPLE PROTEIN ISOFORMS FROM SINGLE GENES, *Roger E. Breitbart, Athena Andreadis, and Bernardo Nadal-Ginard* 467

INHIBITORS OF THE BIOSYNTHESIS AND PROCESSING OF N-LINKED OLIGOSACCHARIDE CHAINS, *Alan D. Elbein* 497

THE NUCLEUS: STRUCTURE, FUNCTION, AND DYNAMICS, *John W. Newport and Douglass J. Forbes* 535

PROTEIN SERINE/THREONINE KINASES, *Arthur M. Edelman, Donald K. Blumenthal, and Edwin G. Krebs* 567

G PROTEINS: TRANSDUCERS OF RECEPTOR-GENERATED SIGNALS, *Alfred G. Gilman* 615

THE MOLECULAR BIOLOGY OF THE HEPATITIS B VIRUSES, *Don Ganem and Harold E. Varmus* 651

MOLECULAR GENETICS OF MYOSIN, *Charles P. Emerson, Jr. and Sanford I. Bernstein* 695

INTERFERONS AND THEIR ACTIONS, *Sidney Pestka, Jerome A. Langer, Kathryn C. Zoon, and Charles E. Samuel* 727

ras GENES, *Mariano Barbacid* 779

BIOSYNTHETIC PROTEIN TRANSPORT AND SORTING BY THE ENDOPLASMIC RETICULUM AND GOLGI, *Suzanne R. Pfeffer and James E. Rothman* 829

CYCLIC AMP AND OTHER SIGNALS CONTROLLING CELL DEVELOPMENT AND DIFFERENTIATION IN *DICTYOSTELIUM*, *Günther Gerisch* 853

RECEPTORS FOR EPIDERMAL GROWTH FACTOR AND OTHER POLYPEPTIDE MITOGENS, *Graham Carpenter* 881

PROTEIN GLYCOSYLATION IN YEAST, *M. A. Kukuruzinska, M. L. E. Bergh, and B. J. Jackson* 915

P450 GENES: STRUCTURE, EVOLUTION, AND REGULATION, *Daniel W. Nebert and Frank J. Gonzalez* 945

INDEXES

Author Index 995

Subject Index 1059

Cumulative Index of Contributing Authors, Volumes 52–56 1079

Cumulative Index of Chapter Titles, Volumes 52–56 1082

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