The RecA Protein: Structure and Function

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I. INTRODUCTION

The year 1990 marks the 25th anniversary of the discovery and naming of the recA gene of *Escherichia coli* by Clark and Margulies.[1] The recA protein is a central component in the related processes of recombinational DNA repair and homologous genetic recombination. This same protein is also involved in the cellular S.O.S. response to DNA damage. The recA gene was cloned in 1976 by McEntee[2] and the recA protein was subsequently purified by several groups.[3-7] *In vitro* recA protein mediates a set of DNA strand exchange reactions (Figure 1) that provide an experimental window on the central steps in homologous genetic recombination. RecA protein also acts as a coprotease in the cleavage of lexA repressor, several related repressors, and the umuD protein,[4,8-11] providing a mechanistic explanation for its function in the regulation of S.O.S. More recently, evidence has appeared for a direct cellular role of recA protein in mutagenesis.[12,13]

This review focuses on recA protein-mediated DNA strand exchange, and the role of this reaction in DNA recombination and repair. The substrates illustrated in Figure 1 are typical of those used in *in vitro* studies. RecA protein exhibits a number of subsidiary activities that are related to its function in promoting DNA strand exchange. These include binding to single- and double-stranded DNA, DNA-dependent ATP hydrolysis, and self-assembly into filaments in the presence or absence of DNA. Correspondingly, the active recA species in DNA strand exchange is a nucleoprotein filament that covers a ssDNA or a gapped duplex DNA as the first step in the reaction. ATP is required for strand exchange, and the exchange proceeds unidirectionally, 5′ → 3′, relative to the ssDNA or the single strand gap.

Despite over a decade of intensive study, many of the central issues concerning the mechanism of recA-mediated DNA strand exchange remain unresolved. In particular, the manner in which the two DNAs are aligned, the relative movement of DNA strands, the function of ATP hydrolysis, and the molecular role of the recA filament remain open questions.

RecA-like strand exchange proteins are widely distributed among prokaryotes (Table 1). Those that have been studied *in vitro*, such as the recA proteins of *Proteus mirabilis*[14] and *Bacillus subtilis*,[15] and the uvsX protein of bacteriophage T4,[16] exhibit an obvious structural and functional relationship to the recA protein of *E. coli*.

Proteins that promote strand exchange have also been purified or partially purified from eukaryotes ranging from yeast to humans.[17-21a] Aside from an immunological relationship demonstrated in a few cases,[22] however, these proteins have little or no evident mechanistic relationship to recA protein. In particular, most do not require ATP and do not appear to form filaments. These proteins will not be covered here, except to note the important implication their lack of an ATP requirement has for any discussion of the energetics of DNA strand exchange.

An exhaustive coverage of the literature relevant to recA-mediated strand exchange alone would be prohibitive. This review is intended as an update of Cox and Lehman[23] and will concentrate on the past 4 years. An attempt will be made to rationalize the overall system in terms of a biological perspective that encompasses the unique and sometimes perplexing biochemical characteristics of recA protein. One central theme is that recA protein is almost certainly a repair system first and a recombination system second. This is not a novel insight, but it is featured here because the perception of what is mechanistically reasonable changes when the structural and energetic features of the system are viewed as evolutionary adaptations to the exigencies of cellular DNA damage.

Every effort is made to present all relevant results and ideas. Many of the key issues remain controversial, however, and our efforts to provide an adequate synthesis of a vast and rapidly growing literature make it inevitable that some things will be inadequately covered. The reader is encouraged to consult several other recent summaries for somewhat different perspectives.[24-27]

II. ON ENERGY, STRUCTURE, AND BIOLOGICAL FUNCTION

Strand exchange can be thought of as a combination of three essential molecular events: the *homologous alignment* of two DNA molecules, a *strand switch* that exchanges individual bases between two thermodynamically equivalent pairing partners, and *DNA rotation*. The rotation is a requirement imposed by the helical structure of DNA. In principle, any, all, or none of these processes could be coupled to ATP hydrolysis in the recA system.

The event that triggered the past decade's intensive research in recA-mediated DNA strand exchange was the 1978 reports

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by the Ogawa and Roberts laboratories[3,4] that recA protein is a DNA-dependent ATPase. The role of chemical energy in this system remains arguably the most perplexing and controversial issue concerning the mechanism of the reaction. The simple problem is that recA protein uses a great deal of ATP to promote a reaction that apparently should require little or none. In a typical three-strand exchange carried out *in vitro*, approximately 100 ATPs are hydrolyzed per base pair of heteroduplex DNA formed. In addition, the active form of recA protein in this reaction is a nucleoprotein filament that can involve thousands of recA monomers.

Table 1
Eubacterial Analogs of *E. coli* K-12 RecA Protein

Organism	Recomb. def.	Gene			Protein				Ref.
		Cloned	Hybrd.	Seq.	Recomb.	Coprot.	Cross-reacts	Purif.	
Gamma purple — enteric									
Aeromonas caviae	+	+	+		+				268
Citrobacter intermedius			+						269
Erwinia carotovora	+	+	+[a]	+	+	+[b]	+	+	149,270–272
E. chrysanthemi	+	+			+	+[b]			273
Escherichia adecarboxylata			+						269
E. alkalescens			+						269
E. aurescens			+						269
E. blattae			+						269
E. coli B/r		+	+	+	+	+	+	+	149,270,271
E. dispar			+						269
Klebsiella aerogenes			+						269
K. pneumoniae			+						271
Proteus mirabilis	+	+	±	+	+	+	+	+	14,154,269,274–276
P. rettgeri			−						269
P. vulgaris		+	+[a]	+	+	+	+	+	149,270,271
Salmonella typhimurium	+		+			+	+	+	269,271,277,278
Serratia marcescens	+	+	+	+	+	+			262,279
Shigella flexnari		+	+	+	+	+	+	+	149,270,271
Vibrio anguillarum		+			+	+			280
V. cholerae	+	+	−		+	+	+		281–283
Yersinia pestis		+			+	+			284
Gamma purple — nonenteric									
Haemophilus influenzae	+	+		+[c]	+	−			285,286
Legionella pneumophila		+	−		+	+[b]	+		287
Pseudomonas aeruginosa	+	+	+(−[a])	+	+	+	+		146,271,288–292
P. putida	+	+			+				157,293
P. syringae	+	+	−		+				294
Beta purple bacteria									
Acinetobacter calcoaceticus	+	+							157
Methylophilus methylotrophus		+	+		+	+			295
Neisseria gonorrhoeae	+	+			+				296
Thiobacillus ferrooxidans		+		+	+	+	+		147,297
Alpha purple bacteria									
Agrobacterium tumefaciens	+	+	+	+[c]	+				148,263,298,299
Aquaspirillum magnetotacticum		+	+	+	+	+			264,300
Rhizobium japonicum			+						298
R. leguminosarum			+						298
R. meliloti	+	+	+	+	+				148,298
Rhodobacter capsulatus	+								301
Cyanobacteria									
Anabaena variabilis		+		+	+	−			265,302
Gloeocapsa alpicola		+			+	+			303
Synechococcus sp		+	+	+	+	+	+		148,304

Table 1 (continued)
Eubacterial Analogs of *E. coli* K-12 RecA Protein

Organism	Recomb. def.	Gene: Cloned	Gene: Hybrd.	Gene: Seq.	Protein: Recomb.	Protein: Coprot.	Protein: Cross-reacts	Protein: Purif.	Ref.
Gram-positive bacteria									
Bacillus subtilis	+	+	−[a]	+		+[b,d]	+	+	15,271, 305–308
Micrococcus radiodurans	+								309
Staphylococcus aureaus	+	+		+[c]					310,311
Streptococcus faecalis	+								312
S. pyogenes	+								313
Bacteroides									
Bacteroides fragilis		+	−		+	+	+		314

Note: Bacterial species for which a recA-like function has been reported are grouped according to phylogenetic divisions,[315] arranged in the order of decreasing relatedness to *E. coli* K-12 (γ purple-enteric). The first column indicates whether a recombination-deficient mutant has been isolated. The next three columns indicate whether the recA-like genes have been either cloned, detected by hybridization with the *E. coli* K-12 recA gene, or sequenced. No distinction has been made between weak and strong homology in the hybridization experiments tabulated. The next two columns indicate whether the recA protein analogs have been shown to functionally complement *E. coli* K-12 recA mutants for recombinase or coprotease activity. The next column indicates if cross-reaction of the recA protein analog with polyclonal antibodies against *E. coli* K-12 recA protein has been detected. The final column indicates whether the protein has been purified.

[a] Proposed ATP binding site coding region as described by Knight, *et al.*[271]
[b] Does not promote cleavage of bacteriophage λ cI repressor.
[c] partial sequence.
[d] *in vitro*.

The thermodynamic and kinetic barriers to reaction are small enough that the complexity of the recA system seems like biological overkill. The strand exchange reactions of Figure 1, involving homologous DNA substrates, should have an equilibrium constant at or near 1.0. In most cases the number of base pairs is equivalent in both product and substrates. Once a branched intermediate (such as a Holliday junction) is formed, spontaneous branch migration is rapid in the absence of proteins.[28]

There are several ways to rationalize this apparent paradox: (1) the *in vitro* system may lack a factor that renders it more efficient *in vivo;* (2) energy may simply be wasted; and (3) the "wasted" energy may address a biological problem, such as DNA repair, that is not adequately modeled in the *in vitro* system.

In the absence of the discovery of a suitable factor, the first possibility is virtually impossible to test rigorously. It can be argued that such a factor should be abundant in the cell, since it must presumably bind to and affect the activity of thousands of recA monomers. No *E. coli* protein with the required properties has been reported. A small molecule that binds and regulates recA protein activity may be a better hypothesis. The only real candidate uncovered to date is ADP, the effects of which are described below.

The idea that energy is wasted in the *in vitro* systems now in common use is supported by two compelling experimental

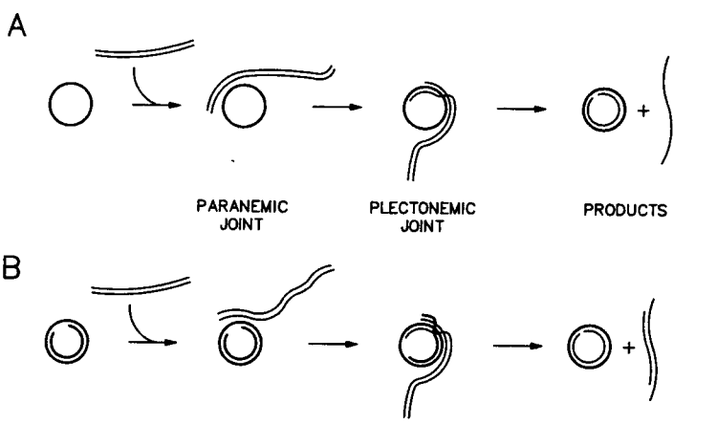


FIGURE 1. RecA protein-mediated DNA strand exchange reactions. The substrates shown are generally derived from bacteriophage DNAs. (A) A three-strand exchange; (B) a four-strand exchange.

facts. The first is the discovery of eukaryotic proteins that promote DNA strand exchange in the absence of ATP (cited above). The second is the recent observation of Menetski et al.[29] that recA protein itself can promote significant strand exchange in the presence of ATPγS (which is hydrolyzed minimally) under certain conditions. While none of these reactions matches the recA and ATP-mediated process in terms of prod-

uct formation, it is very clear that DNA strand exchange requires little energy and can occur with little energy input.

This discussion is incomplete, however, without considering the cellular function of the recA system. As Campbell has noted,[30] much more thought has been given to the mechanism of recombination than to its function. Biological form generally reflects function, and the view that ATP hydrolysis serves a productive purpose in recA-mediated strand exchange can be developed by considering the biological problem addressed by the recA system. It has been argued[30-33] that little selective advantage exists for the maintenance of a system whose only function is homologous genetic recombination. Genetic variation resulting from a recombination event can be deleterious as well as advantageous. Genetic recombination, however, is essential for the repair of certain classes of DNA damage where an intact complementary strand does not exist and the information required for repair must come from a separate DNA molecule. This includes double-strand breaks and DNA lesions left in single-stranded gaps as a result of a bypass by the cellular replication apparatus.[34-39] Not surprisingly, DNA lesions of these types are highly recombinogenic *in vivo*.[39-44a]

The types of damage that require recombinational repair occur at significant levels. Daily background levels of oxidative damage in human cells are one event per 130,000 bases in the nucleus and 1/8000 bases in the mitochondria. This translates into about 10^4 events in the nucleus of a human cell each day,[45] some of which result in double-strand breaks. In higher eukaryotes, efficient repair of lesions of this kind is essential to maintain the integrity of the genome of germ line cells, and it has been argued that this is the primary function of recombination during meiosis.[31-33] It has also been suggested that the repair of DNA lesions may account for all of the recombination occurring in mitosis.[36] The link between recombination and repair is perhaps most apparent in yeast. When homologous chromosomes are available to guide recombinational repair, 50% of yeast cells survive levels of ionizing radiation sufficient to produce 10 double-strand breaks per cell.[46] However, when one copy of chromosome III in *Saccharomyces cerevisiae* diploids is replaced by a chromosome from *S. carlsbergensis* that is functionally equivalent to chromosome III but lacks precise homology over 50% of its length, the rate of chromosome loss in response to ionizing radiation increases by 20- to 50-fold.[47] DNA repair can therefore be viewed as the primary function of homologous genetic recombination systems in terms of impact on cell viability. The recA system is clearly involved in the repair of double-strand breaks and in postreplication repair.[38,39,48-50] Repair may indeed be the primary mission of the recA system, with recombination itself simply a byproduct.

Unambiguously demonstrating a direct role for recA protein in repair is not straightforward, because virtually any effect of recA mutants observed *in vivo* can be attributed to a loss of some S.O.S. function. Nevertheless, the evidence for a direct role is extensive and the relevant literature is expanding rapidly. For example, the molecular basis for the inviability of dam$^-$ recA$^-$ cells appears to be their inability to repair double-strand breaks produced by mismatch repair.[51] A more recent result consistent with a direct repair function is that the UV sensitivity of strains with the recF143 and uvrA6 mutations is relieved without induction of S.O.S. by a valine to methionine change at residue 37 of recA protein.[52] This recA mutant protein, recA803, produces joint molecules more efficiently *in vitro* than the wild-type protein. Other evidence has been reviewed elsewhere.[34]

To illustrate the role of recombination in repair, one model for postreplication repair is outlined in Figure 2. In this model, based on the work of Howard-Flanders and co-workers, an efficient and extensive strand exchange reaction is central to the process. Other models for postreplication repair have been outlined elsewhere.[53] A directed strand exchange as depicted in Figures 1 and 2, however, is difficult to envision in the cell without an input of chemical energy. The rotation that must occur by means of rotary diffusion in recA-mediated strand exchange in the presence of ATPγS[29] is almost certainly pre-

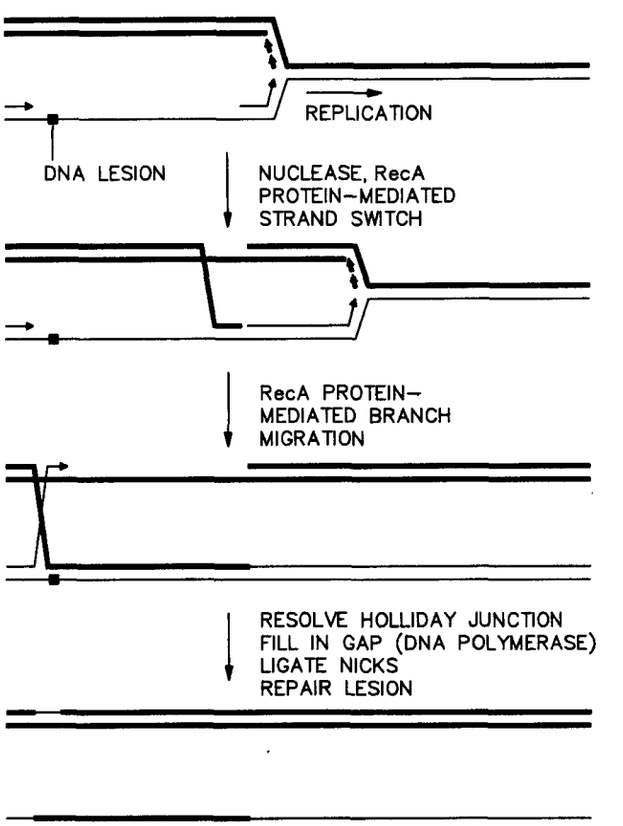


FIGURE 2. A postulated role for recA protein in postreplication repair. Repair of a DNA lesion left behind in single-stranded DNA after the replication fork has passed requires that this DNA be made duplex. This is accomplished by recruiting a complementary strand from the opposite side of the replication fork and pairing it with the lesion-containing strand via recA-mediated strand exchange. (Adapted from a model proposed by Howard-Flanders and co-workers.[38])

cluded when the substrate becomes the *E. coli* chromosome, tightly constricted and packaged by histone-like proteins in the cell. The problem is especially apparent when one or both DNAs contains a DNA lesion that must be bypassed. The required reaction must efficiently proceed through thousands of base pairs, and do so in a cellular environment filled with a wide variety of structural barriers that are generally lacking in *in vitro* model systems.

As a DNA repair system, the molecular design of the recA system is easily rationalized. The function becomes the promotion of an efficient strand exchange reaction under conditions where the reaction is essential for cell survival. The filament effectively excludes other DNA binding proteins (which would block any form of spontaneous branch migration) and serves to protect the branchpoint from premature nuclease attack. ATP hydrolysis would greatly enhance the probability for a successful recombinational repair event by rendering the reaction unidirectional and allowing strand exchange to proceed past any structural barrier likely to be encountered. RecA protein-mediated strand exchange *in vitro* (with ATP) proceeds efficiently past pyrimidine dimers,[54] other lesions,[55] and short heterologies.[56]

As a repair process, DNA strand exchange is not simply a problem of breaking one set of base pairs and forming another. To move branch migration past these structural barriers, the function of ATP hydrolysis can be envisioned as mechanical rather than catalytic. The properties of the system outlined below, including the characteristics of the ATPγS-mediated reaction now being elucidated, are consistent with this view.

III. RECA FUNDAMENTALS

A. The Protein

RecA protein contains 352 amino acids with a combined molecular weight of 37,842.[57,58] The amino acid content is unremarkable except for relatively low numbers of tyrosines (seven) and tryptophans (two). In solution recA protein takes up a variety of forms, with the equilibrium between species determined by buffer and salt conditions. In the most comprehensive study to date, Brenner et al.[59] defined four distinct aggregation states present in the absence of DNA: monomers, rings 12 nm in diameter containing 5 to 8 (probably 6) monomers, rods 10 nm in diameter and 50 to 200 nm in length, and, finally, large bundles of rods. Formation of bundles is facilitated by millimolar levels of $MgCl_2$ and inhibited by monovalent salt and nucleotides. The bundles give rise to a large light-scattering signal and this signal had been interpreted previously to represent formation of rods by Morrical and Cox.[60] The bundle formation process competes with DNA binding, indicating that bundles do not bind DNA.[60] The predominant forms under conditions optimal for DNA strand exchange are the 12 nm rings and short rods.[59] It is not clear which species represents the fundamental unit from which filaments are assembled on DNA, but the 12 nm rings represent an attractive candidate.

In a complementary study using equilibrium ultracentrifugation, Brenner et al.[60a] detected recA monomers, trimers, hexamers, dodecamers, 24-mers, and higher oligomers in reversible equilibrium, with the distribution determined by solution conditions. Assembly of oligomeric forms of recA protein is entropically driven.[60a,60b] Wilson and Benight[60b] have found that the formation of filaments (or rods) involves uptake of one proton and one $MgCl_2$, and the release of five to six NaCls. Assembly is driven by the release of about 70 water molecules.[60b]

B. Binding to DNA and Filament Formation

It has been recognized for over a decade that the active species in recA-mediated reactions *in vitro* is a nucleoprotein filament with a fixed recA:DNA stoichiometry. Subunit mixing experiments *in vivo*, employing inactive mutant or truncated recA proteins in addition to the wild-type protein,[61-63] provide complementary evidence that recA nucleoprotein filaments are the physiologically relevant species in recA-mediated cellular processes. The filament has a regular structure and a prominent feature is a large helical groove. For a thorough illustration and description of filament structure the reader is referred to a summary of the computer-enhanced images derived from electron microscopy contributed by Stasiak and Egelman.[64]

Any discussion of recA protein binding to DNA is complicated by the fact that recA must bind to two DNA molecules, up to four strands total, to carry out DNA strand exchange. The interaction with each DNA strand is distinct, necessitating an appropriately descriptive system of nomenclature. The DNA on which the nucleoprotein filament initially forms is generally ssDNA or gapped duplex. We refer to this DNA simply as DNA1, and to nucleoprotein filament formation on this DNA as primary DNA binding. The ssDNA or the strand that spans the gap in gapped duplex is referred to as the initiating strand or as one of two + strands in the reaction. Its complement is referred to as such or as a − strand. Binding of this nucleoprotein filament to another DNA (DNA2) is referred to as secondary DNA binding. DNA2 is virtually always duplex DNA, and it may be either heterologous or homologous to DNA1.

1. Primary Binding to ssDNA

The importance of ssDNA in nucleoprotein filament formation was apparent in the earliest studies on purified recA protein.[23,26] At pH 7 and above, binding to ssDNA is much faster than binding to dsDNA, and nucleoprotein filaments tend to form on DNA that is entirely or partially single-stranded. Primary binding to ssDNA occurs in the presence or absence of ATP. In the absence of ATP a "collapsed" filament is observed in the electron microscope that exhibits a helical pitch of 64 Å and an axial rise per nucleotide of 2.1 Å.[64,65] Addition

of ATP or ATPγS gives rise to an extended filament with a helical pitch of 95 Å and an axial rise per nucleotide of 5.1 Å. The DNA in this complex is extended by 50 to 60% relative to B form duplex DNA.[23,26,64,65] In the presence of ATPγS, there are approximately 6 recA monomers and 18 to 19 nucleotides per helical turn.[66-69] RecA protein is bound to the DNA along the phosphate backbone, with the bases accessible in the major helical groove of the filament.[68,70,71] The binding site for ssDNA (the initiating strand) is located deep in the helical filament groove near the filament axis.[72] Binding is largely sequence-independent, although preferential binding is observed to some oligonucleotide homopolymers such as poly dT.[73,74] Binding is also characterized by a high degree of cooperativity.[23,59,75,76] Optimal binding to poly dT requires a polymer >50 nucleotides in length.[77] Since recA protein binds tightly to poly dT, optimal binding would require significantly longer random sequence ssDNA polymers. Secondary structure in ssDNA acts as a barrier to complete binding. This barrier can be circumvented by the addition of *E. coli* single-strand DNA Binding Protein (SSB) (see Radding, Reference 26).

RecA filament assembly occurs primarily in the 5′ → 3′ direction on ssDNA,[78] and this polarity of assembly is reflected in a clear structural polarity in the overall filament.[79] The polarity of the assembly process ensures that on a linear ssDNA, more recA protein will be found near the 3′ end than near the 5′ end. This is a likely explanation for the observation of Konforti and Davis[80,81] that joint molecules are preferentially formed at the 3′ end of linear ssDNAs. A recA nucleoprotein filament formed on circular DNA is illustrated in Figure 3.

2. Primary Binding to Gapped DNA

On gapped duplex DNA, binding initiates in the gap and filament assembly proceeds rapidly to incorporate the flanking duplex.[82-84] Assembly again proceeds 5′ → 3′ relative to the strand in the gap (initiating strand). Like ssDNA, duplex DNA is bound along the minor groove and the phosphate backbone.[68,70,71,85] The major groove of the DNA is arrayed in the helical groove of the filament. The binding involves ionic interactions (perhaps as few as two per monomer[76]) and does not involve intercalation of tryptophan or tyrosine residues.[85,86] The bases of the DNA are oriented perpendicular to the filament axis.[86-86b]

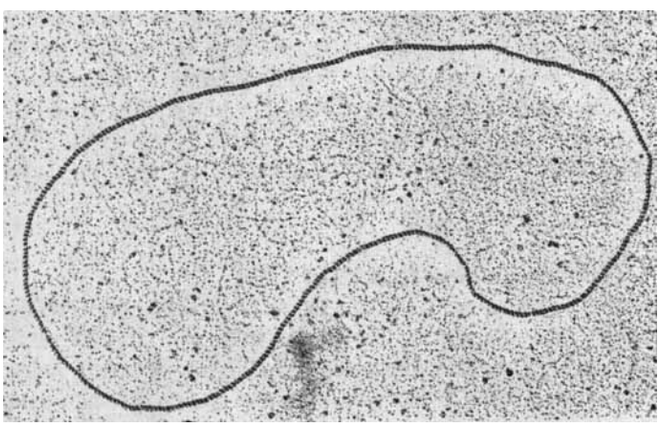


FIGURE 3. A recA nucleoprotein filament formed on a circular duplex DNA (M13 mp8) in the presence of ATPγS. Note the regular helical striations evident. (Courtesy of Ross B. Inman.)

In addition to the longitudinal polarity of the filament structure described above, there is a distinct asymmetry in the binding of the two strands of a duplex DNA. This is made apparent in DNAse protection experiments employing gapped duplex DNA substrates. When recA protein is bound, the initiating strand is protected from DNAse cleavage 2- to 3-fold better than its complement.[84,87] This implies distinct binding sites within the filament with different characteristics for the two strands of a duplex DNA. This in turn reflects an important functional distinction between the two strands. During strand exchange, the initiating strand remains bound within the filament throughout the reaction. In a 4-strand exchange (Figure 1B), its complement is moved out of the filament and replaced with a strand from DNA2.

3. Primary Binding to Duplex DNA

RecA protein will also bind directly to completely duplex DNA in a reaction that is very pH sensitive. At pH 7.5, the binding to B form DNA is negligible on the 30 to 60 min timescale of most strand exchange experiments, and for several years this was taken as evidence that recA protein cannot bind to duplex DNA under these conditions. This perception has had important effects on mechanistic proposals for the strand exchange reaction. For example, as the recA-bound single strand in Figure 1A becomes duplex during strand exchange, one expectation would be that recA protein must dissociate and this has been incorporated into many models for strand exchange.[64,88-90]

Recent studies have demonstrated that the barrier to duplex DNA binding is kinetic rather than thermodynamic, however, and recA can bind stably to duplex DNA even at pH 7.5.[91-93] The slow step is the nucleation of binding, with propagation of the filament to form a saturated complex occurring rapidly once nucleation takes place. This slow nucleation is manifested by a lag in binding that becomes longer as pH is increased. The minimal binding pathway that accounts for the kinetic properties of the system observed to date consists of a rapid pre-equilibrium followed by nucleation as outlined in Figure 4. Not surprisingly, it is the nucleation step that is adversely affected by pH.[92] The binding process up to and including the nucleation step involves an uptake of approximately two protons per recA monomer.[92] The nature of the rapid pre-equilibrium step is not clear, although a current working hypothesis is that it is a weak association with the DNA that does not involve any structural changes in the DNA.[92] Although the

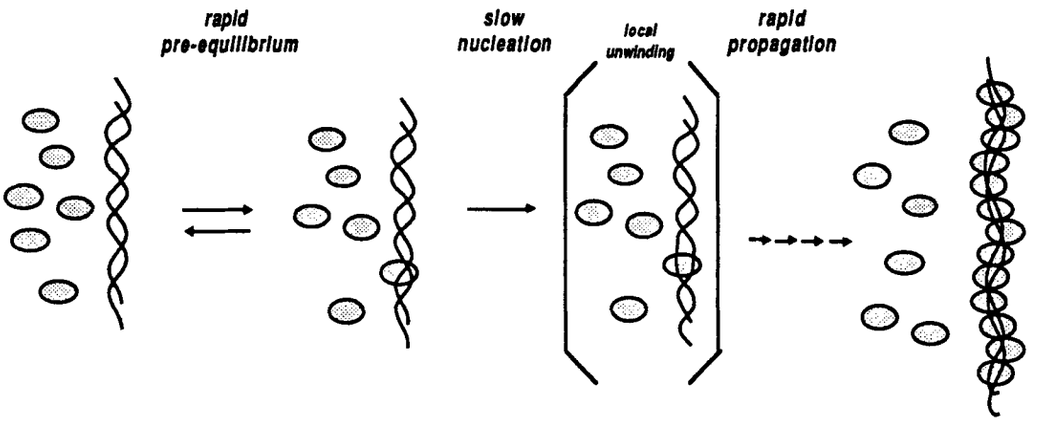


FIGURE 4. Pathway for recA protein binding to duplex DNA. The second step (nucleation) is generally rate-limiting. ATP is hydrolyzed only after the complex is formed. (See text for details).

intrinsic affinity of recA protein for duplex DNA is not as great as for ssDNA,[94] filaments formed on nicked circular duplex DNA are stable for an hour or more at pH 7.5.[91] It is now clear that recA protein remains bound to heteroduplex DNA after strand exchange is complete under at least some conditions.[94-96]

Unlike binding to ssDNA, recA protein binding to duplex DNA exhibits an absolute requirement for ATP or an ATP analog such as ATPγS.[91,92] Stable binding in the presence of ATP requires an ATP-regenerating system to prevent ADP accumulation.

Binding is also coupled to an important structural change in the DNA. When bound by recA protein, duplex DNA is significantly underwound and extended. In the presence of ATPγS, the DNA is extended by a factor of 1.5 to 1.6, with an axial rise per base pair of 5.1 Å and a helical pitch of 95 Å,[64,65,69,97-99] the same as for ssDNA. This suggested that the DNA was also underwound, and Stasiak and Di Capua[100] determined that the DNA was underwound by 43%. This corresponds to 18.6 bp per turn and a rotation per base pair of 19.4° (compared with 34.3° in B form DNA). This result has been confirmed by Dombroski et al.[68] in a study using different techniques. When ATPγS is replaced by ATP, the underwinding is reduced slightly to 39.6%.[101] This corresponds to 17.4 base pairs per turn. Nordén et al.[86b] have found that this underwinding does not reflect strand separation. Interestingly, the degree of underwinding observed in the presence of ATP varies very little from one recA-DNA complex to the next, implying a broad structural homogeneity in these complexes even while ATP is being hydrolyzed.[101] ATP hydrolysis is not coupled to binding or DNA underwinding, but rather is a consequence of the formation of the complex.[91] DNA extension and the underwinding of duplex DNA in recA nucleoprotein filaments has functional significance in that it is likely to represent a DNA structural state that is activated for strand exchange.

The slow nucleation step in duplex DNA binding is inseparably coupled to DNA underwinding, and this slow step can be facilitated by a variety of structural or physical perturbations that tend to make the DNA "unwindable." The most effective is a single-strand gap, as already discussed. Optimal binding rates (negligible binding lags) are observed with gaps (or 5′ tails) of 50 nucleotides or more.[102] Other perturbations that facilitate binding are negative DNA superhelicity,[91] regions rich in A-T base pairs,[93] DNA lesions,[103,103a] increased temperature,[93] and regions that take up a left-handed Z form structure.[104,105]

4. Z DNA

The Z DNA interaction, in particular, has received attention because of the demonstrated recombinogenic properties of Z DNA forming sequences *in vivo*.[106] RecA protein binds faster to Z DNA than to B DNA,[104] but again the effect is kinetic rather than thermodynamic and involves the nucleation step.[105] The more rapid binding to Z DNA can be traced to more rapid nucleation. The final recA complex formed on Z DNA is a right-handed helical filament that is structurally indistinguishable from that formed on B DNA.[105,106] The complexes formed on Z DNA are actually less stable than those formed on B DNA, as demonstrated in competition experiments.[105] There appears to be only a single pathway for recA protein binding to duplex DNA, and the effects observed to date involving an apparent preferred binding to various structural variants of DNA can be accounted for by effects on nucleation.

5. Stoichiometry

The question of DNA binding site size has become surprisingly complex and controversial. In most studies using random-sequence DNA (ss or ds), a binding site size of 3 to 4 nucleotides or base pairs[23,105,107-110] is observed. The most direct and unambiguous measurements tend to support a site size of

3.[66,68,108,109] In at least three cases, however, a site size approximately twice as large (6 to 8 nucleotides or base pairs) has been observed. This is seen when poly dA or a chloroacetaldehyde-modified ssDNA called ε DNA* is used as substrate,[111-113] or when ATPγS is used in experiments measuring the binding of ssDNA.[114] Interestingly, the higher site size occurs only when the measurement involves a DNA-based signal, such as the fluorescence quenching used to monitor recA protein binding to ε DNA. A site size of ~3 is seen on ε DNA when a protein-based signal (ATP hydrolysis) is used.[113] This could represent a change in conformation under some conditions that includes a change in site size, but Takahashi et al.[86] have rejected this possibility on the basis of the physical properties of the different complexes, and no corresponding structural change has been noted in the extensive studies on recA protein using electron microscopy. Two other possibilities remain: (1) either the instrinsic site size is 3 to 4 and the higher apparent site size reflects binding of two DNAs to the same recA filament, or (2) the intrinsic site size is 6 to 8 and the apparent lower site size reflects the formation of a dimeric filament.[113] These possibilities are virtually impossible to distinguish kinetically, but other evidence tends to argue against a dimeric filament (2). The higher site size is observed only with certain DNA cofactors, and is never observed when dsDNA is used as a substrate.[86] If recA protein is separately bound to both DNA molecules in a strand exchange reaction before they are mixed, strand exchange is blocked.** In addition, no dimeric filaments bound to a single DNA molecule have been observed by E.M. and the mass measurements of Di Capua et al.[66] on standard filaments, coupled to measurements that indicate there are 17 to 18 bp of duplex DNA per turn in the filament,[66,68,99,101,115] strongly support an intrinsic site size of 3. More recent studies by Zlotnick et al.,[115a] employing a sedimentation assay to study recA-poly dT interactions, also indicate that the inherent site size is 3 to 3.5, and that conditions in which a site size of 6 to 7 is observed reflect binding of two DNAs to the same filament. In the absence of structural data supporting the dimeric filament hypothesis, the best explanation for the larger site sizes seen in some experiments is a binding of the recA nucleoprotein filament to a second heterologous DNA strand. Possible modes of binding are described under Section IV.B below.

6. Nucleoprotein Filament Assembly and Disassembly

As indicated above, filament assembly on ssDNA occurs 5′ → 3′.[78] Binding to ssDNA substrates of 6 to 12,000 nucleotides in length can be completed in less than 2 min, suggesting optimal assembly rates (assuming 1 recA/3 nucleotides) of as much as 30 to 40 monomers s^{-1}. There is no evidence that nucleation is strictly rate limiting in the ssDNA binding reaction, however. On duplex DNA, nucleation is facilitated by ssDNA tails or gaps. A 5′ tail is much more effective in this respect than a 3′ tail.[82-84,110] This is consistent with a predominant filament growth in the 5′ to 3′ direction along the initiating strand, in this case defined by the tail. The mechanism by which 3′ tails facilitate assembly is unclear; the strands in the resulting complexes are equally sensitive to DNAse, suggesting that the filaments are oriented randomly.[84] Beginning with a 5′ tail or gap that provides a unique nucleation site, assembly on a 7229 bp flanking duplex is again complete in less than 2 min under optimal conditions, indicating the rate of assembly at pH 6.0 or 7.5 is at least 20 s^{-1} [84] and may be several times higher.

Disassembly of the filaments on duplex DNA can also be observed at pHs >6.5. This is an end-dependent process. It is not observed with recA filaments formed on nicked circular DNA[84,91] as long as an adequate ATP regeneration system is present. The time required for disassembly increases linearly with DNA length, indicating that each DNA molecule has a single filament that dissociates from an end at a uniform rate. In addition, only one of the two ends appears to be involved.[84] Filament disassembly is inhibited by 5′ single-strand tails, providing evidence that this process primarily occurs at the 5′ end and proceeds 5′ → 3′. This means that assembly and disassembly occur primarily at opposite ends and proceed in the same 5′ → 3′ direction relative to the initiating strand.[110] The rate of disassembly increases with pH over the range 6.5 → 8.0, with a maximum rate of 3 monomers s^{-1} observed above pH 8.0.[84] At pH 7.5, the rate of assembly at the 3′ end is typically at least an order of magnitude greater than the observed rate of disassembly at the 5′ end.

Assembly and disassembly at opposite ends suggest that under some conditions recA protein filaments treadmill, as observed for cytoskeletal filaments such as tubulin or actin.[116] A steady-state treadmilling reaction, in which assembly and disassembly are balanced, has not yet been observed in the recA system, although such a reaction on duplex DNA might well occur under appropriate conditions. Possible functions for the assembly, disassembly, and putative treadmilling reactions are discussed in Section IV.C (below).

Since assembly at one filament end and disassembly at the other can readily be observed in the same test tube,[110] the dissociation constants for recA protein are dissimilar at the two ends. This implies that these processes must be coupled in some manner to ATP binding or hydrolysis.[116] The recA system is not typical of other filaments that treadmill in that recA-mediated ATP hydrolysis occurs uniformly throughout the filament.[77,96,117] In the filament interior, ATP hydrolysis is not coupled to association or dissociation.[84,110,118] It can be argued, therefore, that neither assembly nor disassembly represents the

*The chloroacetaldehyde reaction produces fluorescent derivatives of cytidylate and adenylate as detailed in Reference 113A.

**This experiment has been done by several students in this lab and the result has been independently communicated to me by several other workers. To our knowledge, it has never been published.

raison d'etre for ATP hydrolysis in this system. One hypothesis is that hydrolysis leads to a series of conformation changes that may have more than one function, but result in dissociation only when the monomer in question is located at the 5′ end of the initiating strand and the pH is sufficiently high. Evidence that ATP hydrolysis has a role in this disassembly process is provided by the observation that low levels of ATPγS can prevent dissociation altogether by binding to only a small subset of monomers within the filament.[84,110,119] ATPγS is a tight binding competitive inhibitor of ATP hydrolysis,[120] and amounts of ATPγS that lower the rate of ATP hydrolysis by only 10% have a remarkable stabilizing effect on the entire filament.[84,110,119] A monomer on the 5′ filament end that cannot hydrolyze ATP by virtue of binding to ATPγS evidently cannot dissociate and effectively caps the filament even though ATP hydrolysis proceeds in other monomers.

7. A Final Note on Filament Stability

In the electron microscope, filaments formed on duplex DNA in the presence of ATP generally appear either fragmented[82,121] or absent.[64,78,89,90] The former observation suggests that multiple partial filaments are formed on long duplex DNAs. These could be oriented randomly on the DNA or there could be several shorter filaments that load at the 5′ tail and migrate 5′ → 3′ by means of a treadmilling-like process. The idea of fragmented or multiple short filaments is generally supported by the incomplete protection from DNAse digestion conferred on duplex DNA by these filaments, and by the observation (described above) that the DNA is underwound somewhat less in the presence of ATP than with ATPγS.

The observed kinetics of assembly and disassembly on duplex DNA in the presence of ATP, however, are inconsistent with multiple short filaments on duplex DNA and instead indicate that each DNA is bound by a single uniform filament when sufficient recA protein is present to saturate the DNA. On 5′ tailed duplexes, assembly occurs rapidly from the unique nucleation site provided by the tail,[84,110] and disassembly from that end, which would permit migration (by treadmilling) of a short filament into the duplex, is inhibited.[110] As noted above, assembly is generally much faster than disassembly even when substrates are employed that optimize disassembly rates. This makes a steady-state treadmilling process unlikely under conditions typically employed in these experiments. Individual recA monomers (or filaments) do not move or slide along the DNA.[84] The rate of nucleation at sites external to the single-strand tail is so low that independent nucleation events outside the tail can be assumed to be negligible on the rapid timescale of filament assembly on 5′ tailed substrates. Since nucleation is strictly rate-limiting in the formation of recA-dsDNA nucleoprotein filaments, the potential for multiple short filaments generated by simultaneous independent nucleation events on the same DNA molecule is negligible. Rates of disassembly are also consistent with a reaction with single uniform filaments. The linear dependence of dissociation time on DNA length is observed for DNAs from a few hundred to 12,000 bp in length.[84] If dissociation were occurring from multiple short and randomly oriented filaments, this length dependence could not exist for DNAs longer than the average filament length, and stable filaments on circular duplex DNAs would not be observed. The kinetic arguments in general support the idea that single uniform filaments coat the DNAs, and that any observed fragmentation of these filaments might occur during spreading for electron microscopy. However, the possibility that multiple shorter filaments may exist under some conditions, oriented and treadmilling in the same direction, is still viable and intriguing.

More work will be needed to resolve this issue completely, but it serves to highlight a persistent experimental problem in the recA system. E.M. observations often indicate no binding of recA protein to duplex DNA in the presence of ATP under conditions in which binding can be demonstrated unambiguously by other methods. Shaner et al.[82] observed fragmented filaments only after optimizing fixation protocols to preserve the complexes, and this work is virtually unique in its detailed effort to reconcile the discrepancies between E.M. and solution measurements. This work and that of Register and Griffith[121] have documented the problems involved in preserving recA filaments on duplex DNA in the presence of ATP for electron microscopy. Indeed, in those cases where fixation protocols were reported, all E.M. studies that have indicated a general dissociation of recA protein from heteroduplex DNA[64,89,90] were carried out using fixation protocols now recognized, from the work cited above, not to preserve these complexes. In the absence of a crystal structure for recA protein, electron microscopy has been the most important source of structural information, and there has been good agreement between E.M. and other methods in the measurement of all recA complexes with ssDNA and with duplex DNA in the presence of ATPγS. The lower affinity of recA protein for duplex DNA when ATP is present apparently renders preservation of these complexes during spreading particularly difficult, however, and care must be taken in the interpretation of unbound duplex DNA in recA reactions observed by E.M. in the presence of ATP.

C. ATP Hydrolysis

1. General

RecA protein is a DNA-dependent ATPase, and the protein has a single active site for binding and hydrolyzing ATP and other nucleoside triphosphates.[122-124] ATP hydrolysis plays a role in DNA strand exchange, although its function has not yet been rigorously defined at the molecular level. The turnover number for ATP is relatively low for an ATPase. The optimal k_{cat} on ssDNA approaches 30 min^{-1}.[23,77,96] On dsDNA the optimal k_{cat} is about 30% lower, 21 to 25 min^{-1}.[84,91,105] Reported K_Ms for ATP generally range from 20 to 150 μM.[23,105]

Under conditions used for strand exchange, ATP hydrolysis is nearly completely DNA-dependent. At pH 7.5, the rate of ATP hydrolysis is reduced 2000-fold if DNA is absent. The active species in ATP hydrolysis is a recA filament, with the result that DNA oligonucleotides shorter than 60 nucleotides are poor cofactors.[77] As indicated above, ATP is hydrolyzed uniformly throughout the filament, with no detectable enhancement at filament ends.[77,96,117] The k_{cat} for ATP hydrolysis is relatively constant on both ssDNA and dsDNA between pH 6 and 9. The low levels of ATP hydrolysis generally observed with dsDNA at pHs above 6.5 reflect slow binding to dsDNA.[91-93] Once bound, recA-dsDNA complexes can be shifted from pH 6 to pH 8 with no detectable change in ATP hydrolysis. ATP hydrolysis, in fact, has proven to be a reliable assay for DNA binding and can be the assay of choice because of the simple and accurate spectrophotometric methods available for its measurement.[84,91,94,105,110] A notable and unexplained anomaly is a 4-fold reduction in k_{cat} observed when a left-handed Z DNA substrate is used as DNA cofactor.[105]

RecA protein does not promote exchange of $[^3H]ADP \rightleftharpoons ATP$, $HPO_4^- \rightleftharpoons H_2{}^{18}O$, or $HP^{18}O_4 \rightleftharpoons H_2O$ at detectable levels, indicating that ATP hydrolysis in this system is both macroscopically and microscopically irreversible.[125]

In addition to ATP, most naturally occurring rNTPs and dNTPs are hydrolyzed at some rate by recA protein.[126,127] In most cases, hydrolysis of another nucleotide occurs at a lower rate and does not support activities such as DNA strand exchange. An exception is dATP, which is hydrolyzed as well as rATP and actually enhances DNA strand exchange under some conditions.[113] Notably, the recA protein from *B. subtilis* is specific for dATP, and hydrolysis of rATP by this protein is negligible.[15] The cellular logic of this arrangement is unclear.

Hydrolysis of ATP in the absence of DNA is generally low but detectable. DNA-independent ATP hydrolysis have a k_{cat} of 0.1 min^{-1} at pH 6.0, its pH optimum. This decreases to 0.015 min^{-1} at pH 7.5 under solution conditions typically used for DNA strand exchange. Surprisingly, salt at higher concentrations has a dramatic stimulatory effect on DNA-independent ATP hydrolysis.[128] At concentrations of 1.5 to 2.0 *M*, many salts stimulate recA-mediated ATP hydrolysis at rates at or even slightly above the rates observed with DNA at low salt.[128] The rates exhibit a 3rd to 4th order dependence on salt concentration. A recA aggregate or small filament is required for ATP hydrolysis in high salt, just as it is in the DNA-dependent reaction.

Many nucleotides act as competitive inhibitors of recA-mediated ATP hydrolysis; ADP and ATPγS are the best characterized. Both ADP and ATPγS act as competitive inhibitors of ATP hydrolysis, and the inhibition kinetics in both cases are complicated by cooperativity between adjacent monomers in recA filaments. The detailed inhibition mechanisms of ADP and ATPγS are nevertheless complex and distinct. In addition to competitive inhibition, ADP mediates a cooperative dissociation of filaments from DNA. This effect is a function of the ADP/ATP ratio and is the reason DNA-dependent ATP hydrolysis proceeds only to about 60% of completion under most conditions.[119,125] In contrast, ATPγS (at subsaturating levels) actually enhances ATP hydrolysis under some conditions (notably at ATP concentrations below K_M).[119] This effect is seen in both the DNA-dependent and high salt reactions. The effects of ATPγS and ADP are not additive. Instead they are antagonistic inhibitors, and low amounts of ATPγS (enough to inhibit ATP hydrolysis ~10%) are sufficient to prevent ADP-mediated dissociation of recA filaments from DNA.[119] These results provide support for the observations of Kowalczykowski and co-workers[129,130] described below that ATP and ADP stabilize different recA protein conformations.

The dissociative effects of ADP have caused some confusion. Under most conditions, measurable dissociation of recA protein from a nucleoprotein filament requires the presence of a significant amount of ADP. In experiments carried out with an ATP regeneration system, this effect is minimized except as noted above in the discussion of end-dependent filament disassembly. There is no strict coupling between dissociation and the ATP hydrolytic cycle. When ADP levels are allowed to rise, net dissociation is detected at ADP/ATP ratios greater than 0.05. As the ADP/ATP ratio increases, the degree of dissociation (net) increases gradually.[119] This is observed as a gradual decrease in the amount of recA protein on all DNAs in the population rather than a complete dissociation of some filaments and none in others.[119] The effect becomes complete at ADP/ATP ratios ≥1.0. Small amounts of ADP have been observed to enhance the efficiency of DNA strand exchange,[125] and this increase in efficiency correlates with a shortening of the filaments in the reaction.[119] Possible explanations for these effects are discussed in Section IV.C.

Another experimental fact of note is that the dissociative effect of ADP exhibits hysteresis, i.e., if ADP is added to an ongoing reaction, competitive inhibition is observed immediately but the dissociation characteristic of that particular ADP level is not completed for several minutes.[131] This must be kept in mind when designing experiments with ADP, since the full effect of this inhibitor is often not observed immediately after its addition.

2. Conformation Changes

A growing number of reports provide evidence that ATP hydrolysis results in one or more recA protein conformation changes. Kowalczykowski and co-workers[129,130] have shown that ATP and ADP result in "high DNA affinity" and "low DNA affinity" states of recA protein, respectively. That ATP and ADP stabilize different protein conformations is reinforced by the inhibition studies cited above. McEntee and co-workers[132] have used limited trypsin digestion to provide evidence for a conformation change when free recA protein binds to ATP, dATP, dTTP, or ATPγS. McEntee[132a] has also found that

substitution of phe or ser for tyrosine 264 (a residue that is involved in ATP binding to recA protein — see Section III.D.3) results in a protein that is proficient in cleaving the lexA repressor in the presence of ATPγS and DNA. The mutant does not promote strand exchange, however, suggesting either that the conformations required for lexA cleavage and strand exchange are distinct or that one or more conformation changes are required for strand exchange, while one suffices for lexA cleavage. Egelman and Stasiak[133] have also observed local conformation changes in the recA filament resulting from the (very slow) hydrolysis of ATPγS. It is not clear whether the conformations observed in these studies are comparable, or if the observed changes represent the only changes that occur. It does seem clear that one or more changes in conformation take place in conjunction with the ATP hydrolytic cycle. Elucidation of the nature and sequence of these changes will become more important once the molecular function of ATP hydrolysis in this system is unambiguously defined.

3. Cooperativity

ATP hydrolysis on any given recA monomer in a recA filament may occur independently or it may be coordinated in some manner with the hydrolytic cycles of adjacent monomers. The latter possibility evokes the hypothesis that ATP hydrolysis may be propagated longitudinally through the filament in the form of coordinated waves (Figure 5).

The simplest argument for some form of coordination can be found in the cooperativity evident in the ATP hydrolytic reaction. Both the ssDNA and dsDNA-dependent reactions exhibit Hill coefficients near 3.0.[119,126] Cooperativity with a similar Hill coefficient is also evident in the inhibition patterns observed with ADP.[119]

An effect of the conformational state of one monomer on the activity of its neighbors is also seen in the observation that low levels of ATPγS activate ATP hydrolysis under some conditions.[119] Given that there is only a single nucleotide binding site on recA protein, this effect must be mediated by protein-protein interactions. The very interesting observation of Menge and Bryant[134] that GTP hydrolysis by recA protein is activated by low concentrations of ATP may represent a closely related phenomenon. GTP by itself is hydrolyzed poorly by recA protein.[126] All of these results are ambiguous, however, with respect to the "coordinated wave" hypothesis. The cooperativity with respect to ATP observed in these studies could reflect a general activation of the filament to a state competent for ATP hydrolysis, rather than the higher level of coordination suggested above.

An interesting clue suggesting that coordination may exist, however, can be found in the end-dependent filament disassembly reaction from linear duplex DNA. The maximum observed rate of filament disassembly is 200 recA monomers min^{-1} $filament^{-1}$,[84] or one recA monomer every 0.3 s. It can be argued that an ATP hydrolytic event is required for monomer dissociation, since the reaction is blocked by ATPγS. If ATP hydrolysis is a prerequisite for dissociation, then hydrolysis must occur in the 5′ end monomer no more than 0.3 s after it is exposed by dissociation of its neighbor. The highest reported k_{cat} for ATP hydrolysis on

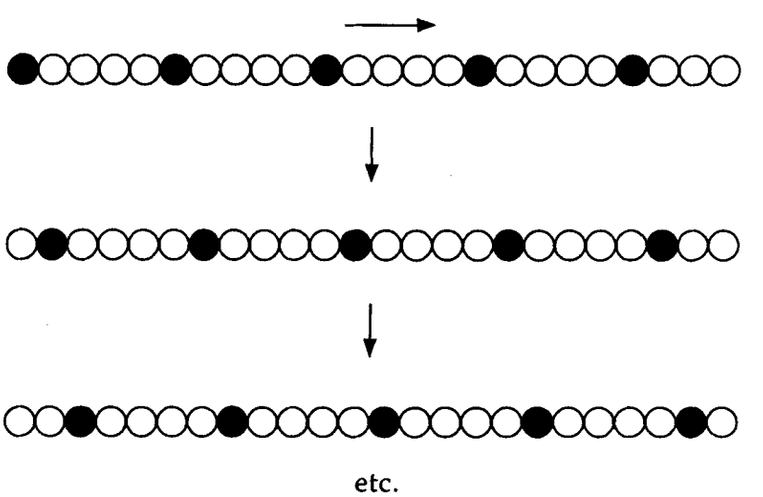


FIGURE 5. Hypothetical organization of ATP hydrolysis in recA filaments in coordinated waves. Dark circles indicate recA monomers where an ATP hydrolytic event is occurring. The progression from top to bottom indicates that each hydrolytic event in one monomer stimulates a new event in the adjacent monomer to its right as drawn. Waves are indicated at every sixth monomer in the filament in this depiction, but the separation could be smaller or greater.

duplex DNA is 25 min^{-1}, or one hydrolytic event every 2.4 s. If ATP hydrolysis were entirely random or uncoordinated, an ATP hydrolytic event would occur in the end monomer 1.2 s (on average) after the previous dissociation event exposed it. This is too slow by a factor of four to account for the maximum observed disassembly rate. This suggests that the hydrolytic cycles of adjacent monomers are coordinated and offset by about 0.3 s. If coordinated waves of ATP hydrolysis were separated by about 8 monomers, each monomer in the interior would hydrolyze an ATP every 2.4 s, giving the observed k_{cat}. If the assumptions described above are varied somewhat, this same calculation yields a separation between waves of anywhere between 6 to 12 monomers. This is an admittedly speculative exercise, and the idea clearly requires further investigation.

4. On Filament States and the "Accordion" Hypothesis

A hypothesis has been advanced that ATP hydrolysis occurs simultaneously in all monomers throughout the filament. The idea is based on the E.M. observation that recA filaments in the presence of ATP exhibit the extended structure described above, while in the presence of ADP they are collapsed. Simultaneous ATP hydrolysis throughout the filament might therefore be expected to bring about an accordion-like cyclic extension and retraction of the filament.[64,65]

Several sets of results, however, provide a compelling experimental case against this hypothesis. The ADP state is not observed in the course of normal ATP hydrolysis, even though the rate of ATP hydrolysis is relatively slow and a significant fraction of filaments might be expected to be in this state at any given time. The conformation changes observed by Egelman and Stasiak[133] in the course of the very slow hydrolysis of ATPγS by recA filaments do not include a collapse to anything approaching the ADP state. The degree of underwinding of duplex DNA in recA filaments hydrolyzing ATP is remarkably constant from one filament to the next,[101] indicating that the filaments are maintained in a homogeneous (extended) structure during ATP hydrolysis. Finally, the ADP state is observed only when ADP is the only nucleotide present. Kinetic studies[119,131] indicate that the ATP and ADP states are mutually exclusive rather than interconvertible. When ATP is present, ATP-state filaments are formed on DNA; when ADP is present, ADP-state filaments are formed on DNA. But when ATP and ADP are present in about equal amounts, the incompatibility of the two forms leads to filament disruption.[119,131] When one nucleotide is replaced by or added to the other, a change in state appears to require complete dissociation of the pre-existing filament.[131] These results indicate that ATP hydrolysis is not simultaneous throughout the filament and that collapse to an ADP state is prevented during normal ATP hydrolysis by the presence of a subset of recA monomers that have bound ATP at any given moment.

D. Structure of RecA Protein

For the last 12 years, work on recA protein function has been pursued without the benefit of detailed information about the three-dimensional structure of this protein. Nevertheless, this period has produced a wealth of data that provides some information on recA protein structure and its relationship to function. The structure itself should be known by the time this review appears. The crystal structure of the recA protein has been solved to 2.4 Å resolution, although as of this writing, analysis of the structure has not proceeded far enough to include it here.[135] The prospect of knowing the three-dimensional structure of recA protein makes a summary of the related literature especially appropriate.

Structural information and structure/function correlations in this system have been derived from primary sequence analysis, chemical modification, and the analysis of mutant or truncated proteins. Some of the correlations deduced from this work are summarized in Figure 6, and the work itself is reviewed below. One caveat worth noting is that Figure 6 is of necessity an oversimplification, and that not all results are in agreement with the indicated functional assignments. Much of the data on which these assignments are based are indirect. In some instances an effect of a mutation or other alteration that appears to implicate a given region in a particular function may instead reflect a longer-range effect on the structure of the protein.

The literature also includes three predictions of the structure[136-139] based on the analysis of primary structure by means of several algorithms[140-142] and a measurement of the α-helical content of the protein by circular dichroism.[136] The crystal structure represents a definitive test of this work and no additional comment will be offered here.

1. Primary Structure

The amino acid sequence of the recA protein is presented in Figure 7. The recA gene from 16 bacterial species has been sequenced in total or in part, and these are also included in Figure 7 for comparison. All references to the primary structure use a residue numbering system that begins at alanine — the N terminal amino acid found in the mature *E. coli* K-12 protein.

As indicated in Table 1, a recA-like activity is widely distributed in eubacteria and has evidently remained a part of bacterial physiology since pre-Cambrian times. It is estimated that the cyanobacteria and the gamma purples diverged approximately 1.5 billion years ago.[143] The tertiary structure of this protein has remained conserved throughout the bacterial kingdom as measured by cross-reactivity with polyclonal antibodies raised against *E. coli* K-12 recA protein.

Available sequence information is sufficient to identify invariant residues that should highlight regions of functional significance. The recA sequences in Figure 7 were multiply-aligned using a pattern-induced alignment method.[144,145] Homology with the *E. coli* K-12 recA protein ranged from 56% (*A. variabilis* and *Synechococcus* sp.) to 100% (*E. coli* B/r and *S.*

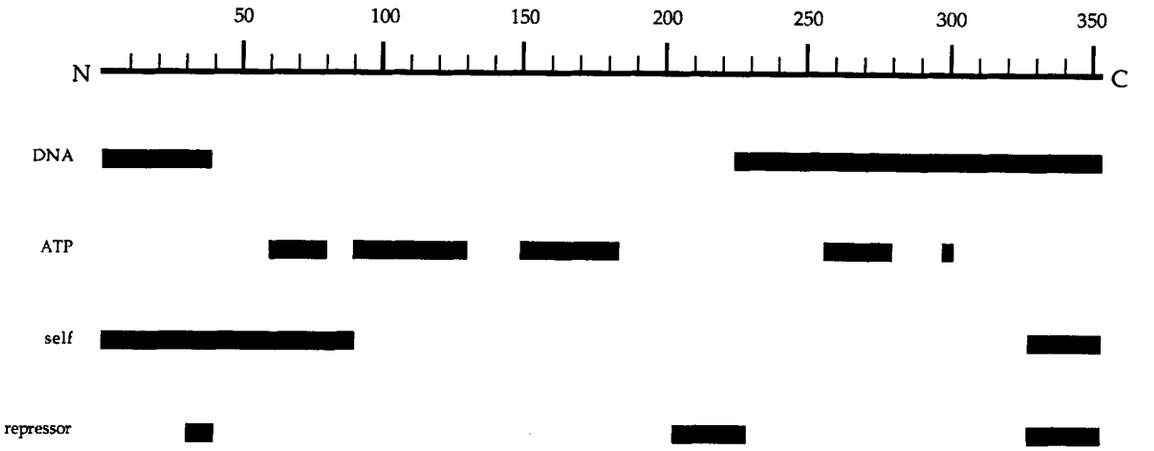


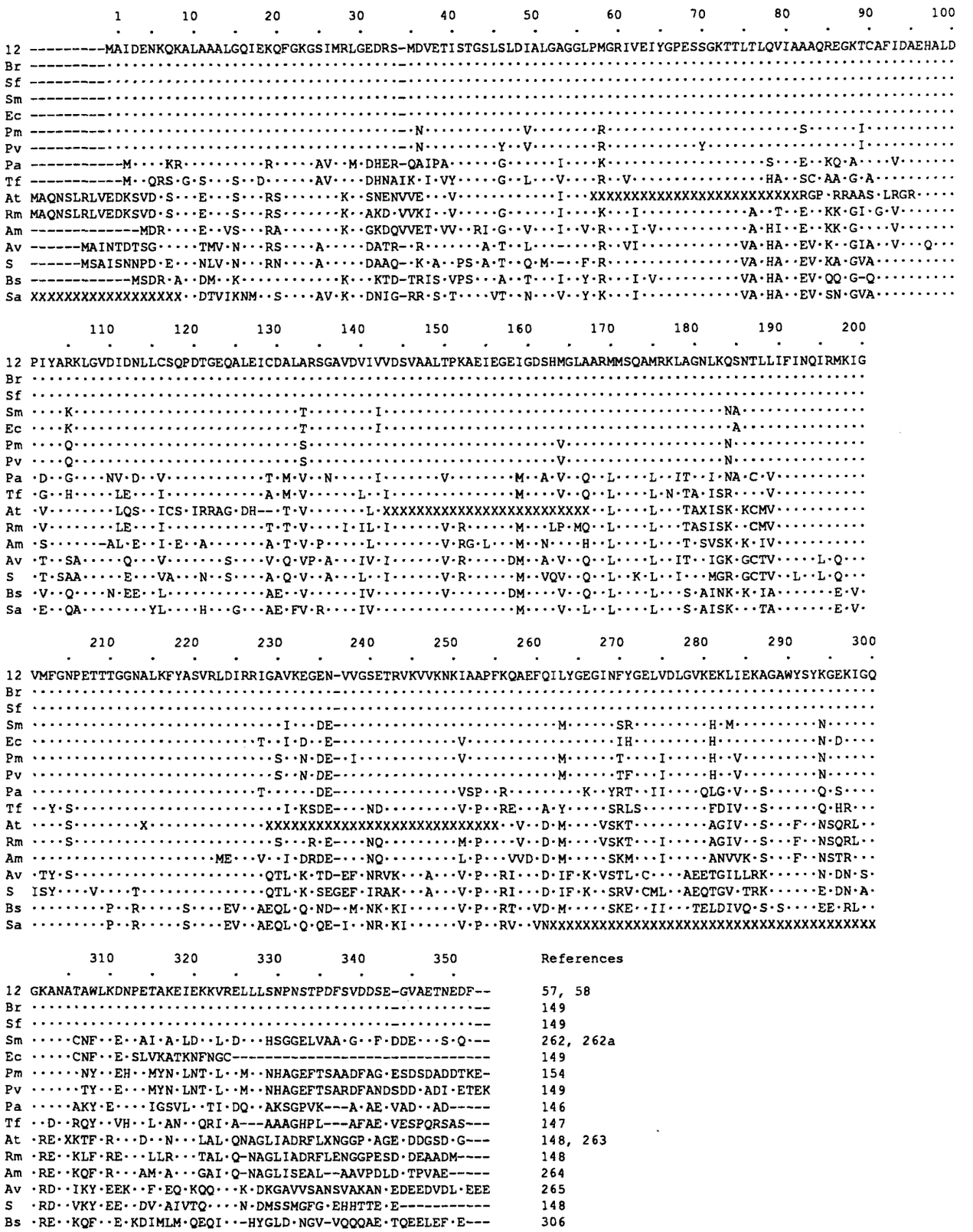
FIGURE 6. Postulated functional domains within the primary structure of recA protein. Assignments are based on data derived from interspecies sequences comparisons, chemical modification, and mutant analysis. The assignments are subject to a variety of caveats related to data interpretation, many of which are described in the text.

flexnari). A total of 105 residues are invariant in the samples now available. A schematic summarizing interspecies homologies is presented in Figure 8.

A number of conclusions can be drawn from this information and complementary data that are expanded upon below. First, there are two regions with a low degree of homology that may have functional significance. The C terminus (approximately the last 40 residues) of all the analogs is highly variable but still rich in acidic residues.[146-149] It has been postulated that this area is involved in ATP binding,[150] binding to dsDNA,[151] and/or the modulation of the coprotease activity.[138] A second area in which the sequences diverge is from residues 31 to 40.[146,147] It has been suggested that this area, in conjunction with the C terminus, modulates the coprotease activity.[146] Second, there are two conserved regions that are likely to be involved in nucleotide binding. Residues 60 to 80 contain a consensus sequence for a nucleotide binding fold.[146,148,152-154] In particular, the more narrowly defined consensus sequence described by Gill et al.[155] is nearly perfectly conserved among all analogs shown. Zhao and McEntee[155a] have found that substitution of gln, glu, or met for lysine 72 produces a protein that is essentially inactive, reinforcing the idea that this region is critical for recA protein activity. A second region important in nucleotide binding (residues 257 to 204) was proposed by Knight and McEntee.[123] Tyrosine-264 was identified as the sight of attachment of a photoactivable nucleotide analog.[156] This residue is conserved among a majority of the analogs shown. Finally, on the basis of the analysis of certain recA protein mutants, it has been proposed that residues 204 to 229 are important in repressor recognition.[137,138] This area is generally well conserved. These functional assignments for segments of the recA protein are also included in Figure 6. For an overview of the evolutionary relationship of bacterial recA analogs and additional discussion of the functional significance of conserved sequences, see Miller and Kokjohn.[157]

A number of studies have attempted to define a consensus sequence for a nucleotide binding fold among ATP-requiring enzymes. Walker et al.[152] examined five enzymes (bovine ATPase β, *E. coli* ATPase β, *E. coli* ATPase α, adenylate kinase, and recA protein) and found the conserved sequence of G-X-X-X-X-G-K-(T)-X-X-X-X-X-X-I/V preceded by a basic amino acid. In adenylate kinase, the residues corresponding to residues 67 to 74 in the recA protein form a flexible loop with the sequence G-X-G-X-G. In adenylate kinase, AMP may interact with this loop. A lysine following the loop may interact with the α-phosphate of nucleotides in adenylate kinase.[158] In a similar study, Higgins et al.[153] found the conserved feature of G-X-X-G-S/T-G-K-S/T among the recA protein and several nucleotide binding bacterial membrane transport proteins. Finally, a comprehensive study of 50 enzymes, including those mentioned above, yielded the nucleotide binding fold consensus sequence of G/A-X-X-G-X-G-K-S/T flanked by hydrophobic residues.[155] By any of these criteria, the recA protein sequence between residues 60 and 80 represents a nucleotide binding fold. In particular, the consensus sequence of Gill[155] appears between residues 66 and 73. The sequence in this region is invariant among all bacterial recA proteins for which sequences are available, except for a ser to tyr change at residue 70 seen in *Proteus vulgaris*.

Sequence alignment of the recA protein with heterologous DNA binding proteins has also been carried out in an attempt to identify those regions involved in DNA binding. Kawashima et al.[137] compared the primary structure of several ssDNA binding proteins, including SSB, the recA protein, and the bacteriophage T4 gene 32 protein. The presence of residues at



the N-terminus of recA similar to those present in the DNA binding sites of the other proteins led them to conclude that this region was involved in DNA binding. On the other hand, Prasad and Chiu[159] compared similar proteins with the constraint of maximizing the alignment of aromatic and basic residues for stacking and electrostatic interactions, respectively. They concluded that the C-terminus was involved in DNA binding. It is likely that more than two regions of the recA protein are involved in DNA binding, although, as already indicated, the aromatic residues are not involved in stacking interactions.[85,86] A protein that appears to be largely functionally equivalent to the recA protein is the uvsX protein of bacteriophage T4. Sequence alignment between recA and uvsX (Figure 9) reveals considerably less homology than between the bacterial recAs seen in Figure 7. About 20% of the residues are identical.

FIGURE 7. Multisequence alignment of eubacterial recA protein analogs. The deduced primary sequences were aligned using a pattern-induced alignment method.[144,145] The numbering of residues begins at alanine — the N terminal residue found in the mature *E. coli* K-12 recA protein. A dash (—) represents an insertion in the primary sequence. A dot (.) below the sequence of *E. coli* K-12 recA protein indicates an invariant residue. Symbols at left denote bacterial species as follows: 12 — *E. coli* K-12, Br — *E. coli* B/r, Sf — *S. flexnari*, Sm — *S. marcescens*, Ec — *E. carotovora*, Pm — *P. mirabilis*, Pv — *P. vulgaris*, Pa — *P. aeruginosa*, Tf — *T. ferrooxidans*, At — *A. tumefaciens*, Rm — *R. meliloti*, Am — *A. magnetotacticum*. Av — *A. variabilis*, S — *Synechococcus*, Bs — *B. subtilis*, Sa — *S. aureaus*. The alignment is consistent with several published alignments involving subsets of these sequences.[146-149,154,262-266]

2. RecA Missense Mutants

Approximately 200 missense mutants of the recA protein have been identified. Of these, approximately 30 have been sequenced.[160] Table 2 summarizes the properties of the latter that have been studied *in vitro*. Table 3 indicates the genotype of these mutants along with other mutants that have not been characterized *in vitro*. Figure 8 locates each of the mutants on the recA protein's primary sequence.

a. RecA1

This mutation is a gly to asp change at position 160.[137] A number of studies have characterized its *in vitro* properties. At

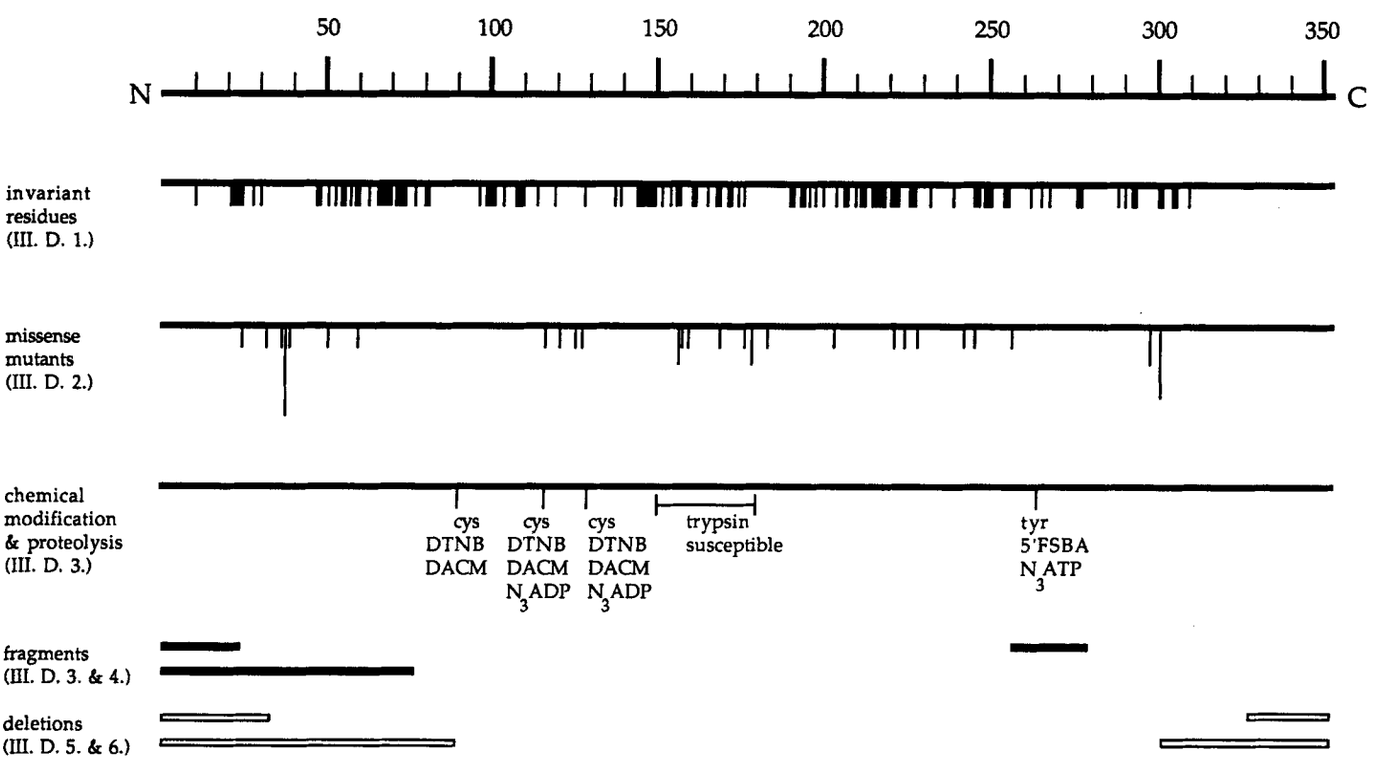


FIGURE 8. Schematic summaries of data and structural variants described in Section III.D. The bar at top represents the primary structure of recA protein. On subsequent lines are schematics showing: invariant residues in the comparison of the sequences of 16 eubacterial analogs (Figure 7); the location of known missense mutations in the recA gene described in Section III.D.2; the location of residues identified in chemical modification and proteolysis experiments (Section III.D.3); the recA protein fragments described in Sections III.D.3 and 4; and the truncated recA proteins described in Sections III.D.5 and 6. The open bars in the bottom schematic denote deleted sequences. For missense mutants, the length of the lines is proportional to the number of known mutants involving a given residue.

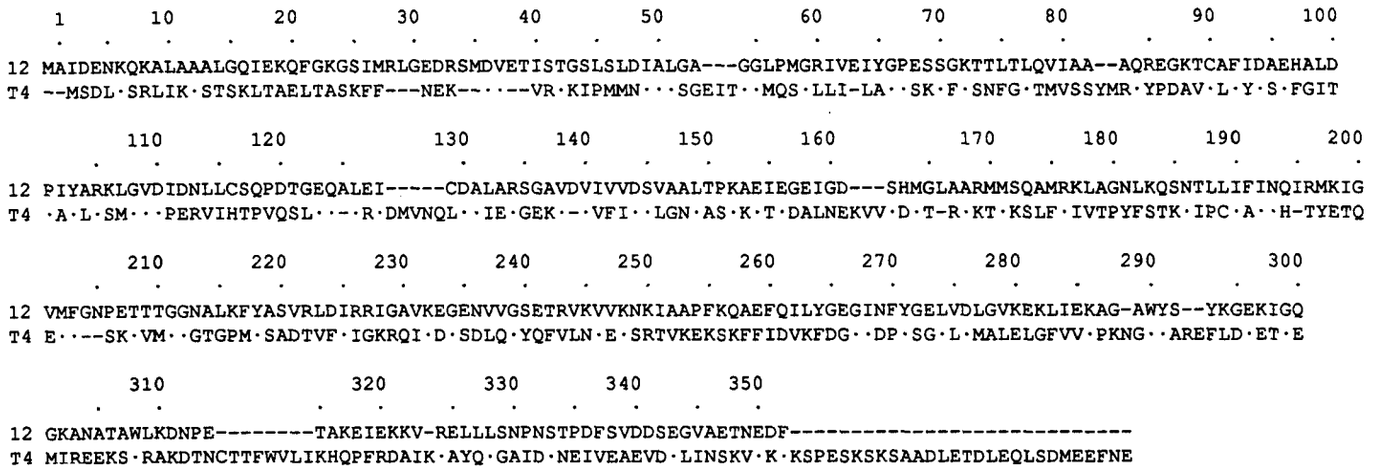


FIGURE 9. Sequence alignment of *E. coli* K-12 recA and phage T4 UsvX proteins. The notation used is the same as in Figure 7. The following sequences were used: 12 — *E. coli* K-12, T4 — T4 UvsX. In general, the alignment did not agree with that generated by Fujisawa et al.[267]

pH 7.5 and in the presence of ATP, it has a reduced affinity for ssDNA[7,161] and can be displaced by SSB.[162] In the absence of ATP, it has wild-type affinity for ssDNA[161,163,164] with an apparent stoichiometry of 3.5 nucleotides/monomer.[164] Under similar conditions, it does not bind dsDNA.[163] However, at approximately pH 6, its ssDNA affinity in the presence of ATP returns to wild-type levels.[137] At pH 7.5, recA1 protein binds ATP[163] without any induced conformational change.[3] It has no ssDNA-dependent ATPase activity.[3,4,7,137,162,163] Decreasing the pH once again restores activity.[137,162] As is expected, this mutant cannot carry out strand exchange,[162,163] although it will promote the renaturation of ssDNA in the absence of ATP[164] but not in its presence.[161,164] DNA coaggregates (networks) do not form with this mutant.[163,164] Finally, there is no coprotease activity for the λ repressor[4] except when the pH is lowered to 6 or when ATPγS is used as a cofactor.[137] *In vivo*, this mutant is recombination deficient[1] and will negatively complement a recA$^+$ host for recombinase activity.[61]

Kawashima et al.[137] offered the explanation that the amino acid change caused a decrease in the affinity for ATP due to the introduction of a negative charge that would be neutralized at a lower pH. Rusche et al.[163] proposed that the mutation resulted in a nonfunctional second DNA binding site. In support of the latter interpretation, Bryant and Lehman[164] found that in the presence of ATPγS, the stoichiometry of the binding of DNA did not increase by a factor of two, a phenomenon observed with the wild-type protein that has been explained by the binding of two DNA molecules to the nucleoprotein filament (as discussed in Section III.B.5). Bryant[162] tested the effect of the introduced charge by constructing an asn-160 mutant that would mimic the asp of the recA1 mutant in structure and H bonding properties. He found that the ATPase activity returned to wild-type levels; however, (asn-160)recA could not carry out strand exchange nor resist SSB displacement. Electrostatic repulsion alone did not rationalize the recA1 mutant's phenotype. With the assumption that glycines are well conserved in proteins and often provide for flexibility in the α carbon backbone,[165] they proposed that residue 160 was located in a loop region. The recA1 mutant's properties would be the result of a decrease in the flexibility of this postulated loop region. This proposal is supported by the fact that gly-160 is conserved among the eubacterial analogs reviewed above. Also, it is located within a trypsin susceptible region[132] (to be described below) that may be a linker between two domains of the recA protein.

b. RecA44

This mutant is a change from valine-246 to methionine.[137] *In vivo*, the mutant is defective in both recombinase and coprotease activities.[137,166] Its thermosensitive nature may reflect an instability in the higher order structure of this region of the protein.[137] This residue is conserved in recA analogs among the bacterial species listed in Figure 7.

c. RecA91

Glycine-229 is changed to serine in this mutant.[138] Ogawa and Ogawa[138] found that recA91 permits the induction of prophage λ *in vivo* but not prophage φ80, suggesting this region may be involved in the recognition of repressors.

d. RecA142

Isoleucine-225 is changed to valine in the recA142 mutant.[12] Kowalczykowski et al.[167] showed that *in vitro* this mutant can bind ssDNA and hydrolyze ATP in a ssDNA-dependent manner. However, both activities are more sensitive to ionic strength than with the wild-type protein. Furthermore, SSB inhibits this ssDNA-

Table 2
***In vitro* Properties of RecA Mutants**

Allele no.	DNA binding: ATP	DNA binding: ss +	DNA binding: ss −	DNA binding: ds +	DNA binding: ds −	DNA binding: DNA	ATP: Bind	ATP: Hydrolysis ss	ATP: Hydrolysis ds	ATP: Coagg.	Joint molec.	Strand exch.	Renatur.: ATP	Renatur.: +	Renatur.: −	Self-assoc.	DNA	Coprot.: ss	Coprot.: ds	Ref.
1		−	+		−		+	−				−		−	+					3,4,7,137, 161,163,164
142		+[a]						+[a] −[b]	−	−	−	−				+		+[c] −		167,168
430		+[a]	+	−[c,d]				+	+[a]					+[a]		+[a]		+[c]	−[c,d]	137,161,168, 173,174
441		+		↑				+	+		+	+						+	↑	4,8,137,173,
																		↑[e]		174, 184–190,316
629								+[f]	+[f]	+[f]				+[f]				+		5,184,187
803											↑									52
1202		↑					↑											↑		196,197,205, 206
1211		↑					↑											↑		197,205,206

Note: Summary of the known *in vitro* properties of recA missense mutants. The symbols +, ↑, ↓, − denote normal, increased, decreased, or absent activities. Where no symbol is present, no information about that activity was available. Abbreviations used are ss, single-stranded DNA; ds, double-stranded DNA; coagg., coaggregation; joint molec., joint molecule formation; strand exch., strand exchange activity; renatur., renaturation of complementary single strands; self-assoc., self-association; coprot., coprotease activity. DNA binding and renaturation activities are broken down according to the presence or absence of ATP (+, −), and DNA cofactors (ss or ds) are specified for ATP hydrolytic and coprotease activities. The symbols, of course, are qualitative and in many cases represent an oversimplification of effects observed. (See text for details.)

[a] Sensitive to ionic strength.
[b] In the presence of SSB.
[c] In the presence of ATPγS.
[d] With UV irradiated DNA.
[e] More pronounced at 42°C.
[f] Not at 28°C.

dependent ATPase activity. The dsDNA-dependent ATPase activity is also reduced. This mutant cannot coaggregate single- and double-stranded DNA. It is defective in joint molecule formation. Subsequently, it cannot carry out a strand exchange reaction. RecA142 is proficient in its self-assembly properties. Roberts and Roberts[168] showed that there is coprotease activity only in the presence of ATPγS. *In vivo*, this mutant is recombination deficient[169,170] and defective in the induction of prophage λ by UV or mitomycin C treatment.[170-172] However, the mutant is proficient for spontaneous (non-S.O.S.) induction of λ.[169,171,172] Early reports postulated that this mutant was defective in its interactions with DNA[171,172] and/or ATP.[172] This leads to a susceptibility to displacement by SSB,[167] providing a rationale for the recombination deficient phenotype *in vivo*. The binding competition between recA142 and SSB may also explain its inability to induce prophage under DNA-damaging conditions. At a more fundamental level, the recA142 mutant displays a different binding affinity toward ssDNA. It is the mutant's inability to form the ATP binding-induced high-affinity ssDNA-binding state that differentiated it from the wild-type protein. This residue is nearly invariant among the analogs in Figure 7. Interestingly, however, the *B. subtilis* and *S. aureaus* recA analogs have the same ile→val amino acid substitution at this position. This suggests that other structural differences in the recA proteins of these two bacterial species compensate for the presence of valine at this position and indicate that it should be possible to isolate intragenic suppressors of the recA142 mutation.

e. RecA430

The recA430 mutant (formerly named LexB30) has a glycine to serine change at residue 204.[137,173] *In vitro*, it can bind ssDNA in the absence and presence of ATP, although the latter reaction is very sensitive to ionic strength.[161] The ssDNA-dependent ATPase activity is also more sensitive to ionic strength than the activity of the wild-type protein.[168] In the presence of ATP, purified recA430 protein can reanneal two homologous single-stranded DNA molecules.[161,168]

In vivo, this mutant is recombination proficient[175-180] but

Table 3
Genotypes of RecA Mutants

Allele no.	Residue no.	Amino acid: Wild type	Amino acid: Mutant	Ref.
1	160	Glycine	Aspartic acid	137
13	51	Leucine	Phenylalanine	160
44	246	Valine	Methionine	137
56	60	Arginine	Cysteine	160
91	229	Glycine	Serine	138
142	225	Isoleucine	Valine	12
430	204	Glycine	Serine	137,173
441	38	Glutamic acid	Lysine	137,173,184
	298	Isoleucine	Valine	
629	32	Aspartic acid	Glycine	184
	38	Glutamic acid	Lysine	
	298	Isoleucine	Valine	
718	28	Glutamic acid	Lysine	198
	126	Leucine	Valine	
730	38	Glutamic acid	Lysine	160
801	257	Glutamine	Proline	160
803	37	Valine	Methionine	52
1201	157	Glycine	Aspartic acid	196
	301	Glycine	Serine	
1202	184	Glutamine	Lysine	196
1203	128	Isoleucine	Isoleucine	196
	169	Arginine	Cysteine	
1206	177	Lysine	Lysine	196
	301	Glycine	Aspartic acid	
1207	301	Glycine	Aspartic acid	196
1211	38	Glutamic acid	Lysine	197
1212	179	Alanine	Valine	196
1213	179	Alanine	Valine	196
1219	158	Glutamic acid	Lysine	196
1222	25	Serine	Phenylalanine	196
1235	39	Threonine	Isoleucine	196
1601	301	Glycine	Serine	196
1602	157	Glycine	Aspartic acid	196
1730	117	Serine	Phenylalanine	12
1734	243	Arginine	Leucine	12
1735	222	Arginine	Cysteine	160
2020	121	Threonine	Isoleucine	160

Note: The amino acid changes of various recA missense mutants.

deficient in the induction of prophage λ.[168,175-178,181,182] Induction is generally deficient for other lambdoid phage[181,182] but not for bacteriophages ϕ80 and P21.[181] *In vivo*, this mutant exhibits a low level of coprotease activity toward the LexA repressor.[181,182] *In vitro* this mutant does not promote the cleavage of the cI repressor in the presence of ssDNA and ATPγS.[168] Somewhat surprisingly, however, Lu and Echols[174] showed that the coprotease activity toward the LexA repressor in the presence of ssDNA (but not dsDNA) is efficient. RecA430 is nevertheless deficient in lexA protein binding relative to wild-type recA when compared side by side in an assay involving retention and cleavage of lexA on recA protein-agarose columns.[182a] The recA or recA430 proteins in these columns were activated for lexA cleavage by binding to short dT oligomers and ATPγS.

Other S.O.S. functions for which this mutant is defective are UV resistance[178] and radiation mutagenesis.[183] This could be explained by the defect in the promotion of LexA cleavage.[137,168,174] A salt-dependent conformation change may explain the ionic strength effects.[137] Gly204 is invariant among the bacterial recAs listed in Figure 7.

f. RecA441

RecA441 (formerly *tif*-1) is a double mutant — glutamic acid 38 to lysine[137,173,184] and isoleucine 298 to valine.[184] *In vitro*, the mutant forms ternary complexes with ssDNA and ATP more efficiently than wild type.[185,186] The same is true for its interactions with dsDNA.[174] An enhanced ATPase activity of recA441 relative to wild-type recA has been observed with both ssDNA and dsDNA cofactors.[174,185-187] This is generally a kinetic effect, however, and reflects more rapid or complete binding of recA441 to these DNAs. The intrinsic k_{cat} for ATP hydrolysis is the same for recA441 and wild-type recA protein.[188] Finally, the ssDNA-dependent coprotease activity toward the cI repressor[4,8,185,187,189,190] is increased relative to wild type.[186] In the presence of SSB, the activity is enhanced even more.[186] The dsDNA-dependent coprotease activity is also more efficient than wild type.[174] The aforementioned experiments were performed at 37°C. At 42°C, the increase in ATPase and coprotease activities is even more pronounced.[186]

This mutant was described as a thermosensitive inducer of prophage λ where induction occurred in the absence of DNA damage at 42°C.[191-194] RecA441 is recombination proficient.[193]

Lavery and Kowalczykowski's comprehensive biochemical study[186] offered the following model. As mentioned, the most pronounced differences between recA441 and wild-type protein occur at elevated temperature and in the presence of SSB. Under these conditions *in vivo*, the recA441 protein can compete efficiently with SSB for the extant regions of ssDNA in the cell. For instance, recA441 can bind at replication forks and become activated for coprotease activity without the need for DNA damage.

It has been proposed that the mutation affects recA interactions with ssDNA and/or ATP.[174,187,194,195] In part, the glutamic acid 38 to lysine change would improve the interactions with DNA by the introduction of a positive charge.[137,138,196] Knight et al. also proposed that the change of ile298 → val may affect nucleotide binding due to its proximity to tyrosine 264.[184] In comparing recA441 to recA1211 (glutamic acid 38 to lysine), Wang and Tessman[197] proposed that residue 38 did yield a constitutive protease phenotype. Furthermore, since recA1211 was not temperature-sensitive, they proposed that residue 298 was a temperature-sensitive suppressor of the first mutation in recA441.

g. RecA629

This mutant is an intragenic suppressor of recA441. The first two amino acid changes are the same as recA441—glutamic acid 38 to lysine and isoleucine 298 to valine, and it includes an additional change with aspartic acid 32 changed to glycine.[184] At 42°C, this mutation cannot induce prophage λ. This mutant is cold labile, i.e., the mutant exhibits normal activity for ssDNA dependent ATPase,[5,187] aggregation of ds- and ssDNA in the presence of ATP,[5] and the renaturation of ssDNA in the presence of ATP at 37°C.[5] However, at 28°C, all activities are defective.[5] At 37°C it has wild-type coprotease activity toward the cI repressor.[187] *In vivo*, the mutant causes cold sensitivity to recombination and repair.[5] McEntee and Weinstock[187] proposed that the change at residue 298 was in a DNA binding domain. The more recent work of Knight et al.[184] suggested that this change modulated ATP binding since it was located near tyrosine 264. The observed difference in phenotype between recA441 and recA629 suggests that there is an ionic interaction occurring between lysine 38 and aspartic acid 32 in recA441 that lends stability to an active conformation.

h. RecA718

RecA718 is a derivative of recA441 that combines the glutamic acid 38 to lysine change with a unique leucine 126 to valine change.[198] Work performed *in vivo* by Witkin et al.[199] shows that a recA718 cell requires DNA damage to induce the S.O.S. response, i.e., there is no recA441-like response to increased temperature. Furthermore, such a cell is slightly UV sensitive and displays increased levels of UV mutagenesis. They proposed that the recA718 mutant is defective in DNA repair. The recA protein has a role in induced replisome reactivation (IRR).[103,200,201] Witkin et al.[202] have proposed that the recA718 mutant is either defective for a direct role in IRR or for its interactions with the UmuD protein. McCall et al.[198] drew the conclusion that the first amino acid change endows the protein with constitutive coprotease activity possibly in the same manner as recA441 protein. The second change suppresses that activity in some temperature-independent way, such as reducing cofactor affinity. Amplification of the protein overcomes this reduced affinity.

i. RecA803

The recA803 mutant (originally *srf*-803) represents a substitution of methionine for val-37.[52] Madiraj et al.[52] established that under suboptimal conditions (i.e., 10 m*M* Mg^{2+} and no SSB) more joint molecules were formed at a higher rate with recA803 protein than with wild-type recA protein. When the Mg^{2+} was removed, this observed disparity between the two disappeared, suggesting that ssDNA secondary structure was the differentiating factor. When SSB was introduced, recA803 protein was much less susceptible to inhibition than wild-type recA protein. They proposed that the recA803 protein effectively competes with SSB due to an increased affinity for ssDNA, suggesting that the N-terminus is involved in ssDNA binding.

j. RecA1200s and recA1600s

Tessman and Peterson[203] developed a method that permits efficient isolation of coprotease constitutive recA mutants. These mutants were roughly divided into two classes based on whether they were recombination proficient or deficient. Tessman and Peterson[204] showed that the protease constitutive, recombinase proficient mutants were capable of promoting the autodigestion of the LexA, phage 434, and phage 21 repressors. The protease constitutive, recombinase deficient mutants had constitutive activity for the LexA repressor only, although they did display inducible coprotease activity for the other two repressors. A number of the mutations have been sequenced,[196] and generally map in three clusters that may define different structural or functional domains. They proposed that the first domain from residues 25 to 39 (recA1211, recA1222, and recA1235) affected DNA interactions since the mutations introduced basic or aromatic amino acids. The second domain from residues 157 to 184 (recAs 1201, 1202, 1203, 1212, 1213, 1219, 1602) affected nucleotide binding since these mutant's protease activities were modulated by the introduction of cytosine and guanidine into the medium that possibly inhibited ATP binding competitively.[194] Finally, residues 298 to 301 (recAs 441, 1206, 1207, 1601) affected both ssDNA and nucleotide binding since this region interacts with both of the former regions.

Both recA1202 (glutamine-184 to lysine) and recA1211 (glutamic acid 38 to lysine) were studied *in vitro*.[205,206] Both bind ATP better than wild type and, in the presence of ATP, they bind ssDNA better.[206] The coprotease activity with the LexA repressor in the presence of ATP was also stronger than wild type.[206] These mutants could also be activated for coprotease activity by rRNA and tRNA, whereas wild type could not.[205] They concluded that the constitutive protease activity *in vivo* could be explained by the larger range of positive effectors available to them. Finally, four of these mutants have changes at residues that are invariant in the bacterial recA analogs described in Figure 7 — recA1203, 1206, 1207, 1601. These mutants are protease constitutive, recombinase defective *in vivo*. The gln 184 to lys change in recA1202 is found in the wild-type sequence of six of the recA analogs in Figure 7.

k. RecA1700s

Dutreix et al.[12] isolated some mutants that showed differential coprotease activity. RecA1730 (serine 117 to phenylalanine) could not promote the cleavage of the LexA repressor but did promote the cleavage of the φ80 and λ repressors. RecA1734 (arginine 243 to leucine) promoted the cleavage of the LexA and λ repressors, but not the φ80 repressor. Since both could cleave the λ cI repressor, they assumed that these mutants were not deficient in ternary complex formation with ssDNA and ATP. These residues could then be involved in

repressor recognition either through specific contacts or affecting changes in the local conformation of the recA protein. RecA1734 is recombination proficient yet recA1730 is not. Since this mutation is localized near cysteine 116, which is important in ATP hydrolysis,[207] this mutant could be defective in its ATPase activity.

I. RecA (Am)

Weisemann and Weinstock[208] screened a large number of mutations at the three cysteine residues (90, 116, and 129) of the recA protein. For each mutant they tested the ability to survive UV-irradiation, to carry out homologous recombination, and to induce S.O.S. They found that position 90 was probably involved in ternary complex formation, i.e., binding ATP and/or ssDNA, since some amino acid substitutions at this position had affected all three functions. Position 116 is probably involved in coprotease activity since a change to serine conferred a constitutive coprotease phenotype. Position 129 is probably in a hydrophobic region since only hydrophobic substitutions resulted in a functional protein. Their results also extended the chemical modification studies discussed below. Kuramitsu et al.[207] showed that cysteine 116 was important in ATPase activity. Weisemann and Weinstock showed that a change to serine resulted in near-normal ATPase activity. This residue then does not have a direct role in catalysis. Instead, its effect in the chemical modification studies must be either a steric one or an effect on local conformational structure.

3. Chemical Modification

To date, the structure of the recA protein has been probed with photoreactive ATP analogs, with reagents that modify cysteine residues, and with monoclonal antibodies.

The photoaffinity ATP analog, 8-azidoadenosine 5′-triphosphate (N_3-ATP), has been used to locate the region of the recA protein responsible for ATP binding/hydrolysis. The binding of N_3-ATP depends on the native conformation of the protein, since heat denaturation prevents crosslinking.[123,150] Attachment of N_3-ATP is inhibited by ATP as well as the same competitive inhibitors of ATPase activity, namely, ADP and ATPγS.[123,127,150] Last, N_3-ATP is efficiently hydrolyzed to N_3-ADP in a ssDNA-dependent reaction.[123,127] Tyrosine-264 is the unique site of N_3-ATP attachment.[156] The results with another ATP analog, 5′-*p*-fluorosulfonyl-benzoyladenosine (5′-FSBA), mirror those of N_3-ATP.[124] These treatments terminate recA protein's ATPase activity.

Given the fact that both analogs reacted with the same residue, Knight and McEntee were able to outline some constraints on the possible orientations of the substrate and active site. They generated a model of the substrate-active site complex with the following features.[156] First, the adenine ring of ATP is in the anti-conformation about the glycosyl bond when it binds to recA protein. Second, tyrosine-264 is positioned such that its benzylic carbons and atoms of the polypeptide backbone are in close proximity to the C-8 of adenine, where the azido moiety of N_3-ATP is located. Finally, the sulfonyl fluoride moiety of 5′-FSBA is positioned next to the hydroxyl of tyrosine-264, meaning 5′-FSBA is in an extended conformation when it binds to recA protein.

Knight and McEntee carried out the same chemical modification studies on T31, a 31-residue trypic peptide[209] that included tyr-264. The results were the same except that UTP and TTP did not inhibit photolabeling of T31, whereas they did for the protein. This suggests that T31 contains residues necessary for establishing purine contacts, but residues responsible for nonspecific ionic contacts with ribose or phosphate were missing.[158,210] Furthermore, the phosphates of ATP appear unnecessary for interaction with T31 since adenine can also serve as a competitive inhibitor of the labeling reaction.[209]

In complementary work focused on tyrosine-264, this residue was replaced by either serine or phenylalanine by Freitag and McEntee.[132a] The resulting proteins produce a general recA$^-$ phenotype *in vivo*. *In vitro*, both proteins bind DNA and are proficient in lexA repressor cleavage when ATPγS is used in place of ATP. Neither mutant protein will promote strand exchange, however, and rates of ATP hydrolysis are reduced substantially.

Ogawa et al.[138] performed photoaffinity labeling experiments using N_3-ADP, and showed that both cysteine-116 and cysteine-129 were modified. ADP is a competitive inhibitor of ATP binding and there is no evidence for more than one nucleotide binding site. The modification of different amino acid residues with N_3-ATP and N_3-ADP may reflect the different conformations of the recA protein that are stabilized by ATP and ADP.

Kuramitsu et al. showed that all three cysteine residues (90, 116, and 129) can be covalently bound to 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB).[136] In the presence of ATP or ADP, two cysteines were protected (90 and 129) and modification of cys-116 was accelerated. The same results were found when cysteines were reacted with *N*-[7-(dimethylamino)-4-methylcoumarinyl]maleimide (DACM). They concluded that the three cysteines were in the vincinity of the ATP binding site and that cys-90 and cys-129 were concealed by bound ATP.[137,207] However, the results of Knight and McEntee[156] provide evidence that the differences in chemical modification data obtained in the presence and absence of a ligand may reflect protein conformational changes at regions removed from the site of ligand binding.

Ikeda et al.[210a] have used a number of monoclonal antibodies to probe recA protein structure-function relationships. One of these, IgG ARM321, recognizes a region of the recA protein between residues 89 and 127 and inhibits the ATP hydrolysis and homologous pairing activities. This is the same region defined by the cysteine residues described above. Another an-

tibody, IgG ARM414, with similar effects on recA protein activities, binds to a region between residues 233 and 256.[210a] This is near the region defined as part of the ATP binding site by McEntee and co-workers.[156] Two additional antibodies, IgG ARM191 and ARM193, both bind to recA protein near the carboxyl-terminus and inhibit dsDNA binding and protein-protein interactions, respectively.[210a] These results are generally consistent with observations derived from studies of recA proteins that have been truncated at the C-terminal end as outlined below.

4. Protein Fragments

Kobayashi et al.[132] analyzed recA protein-NTP complexes to determine their sensitivity to proteases such as trypsin. They found that tryptic digests produced four bands on SDS-PAGE gels that were either absent or at much lower levels in digests of uncomplexed protein. Their model proposes that the binding of NTPs causes a region near the middle of the primary structure (residues 150 to 180) to become more susceptible to trypsin cutting. This result sheds light on another phenomenon. RecA1 protein, whose mutation lies within this putative linker region, is apparently defective because it cannot undergo a necessary nucleotide-induced conformational change.[3] In addition, many of the recA1200 protein mutants, described by Wang and Tessman,[203,204] are located in this linker region. These results, then, support the idea that this region is a linker (between two domains) that is affected by an ATP-induced conformational change. The induced conformational change would protect the cysteine residues from alkylating agents.

Yu and Egelman[211] have generated a three-dimensional reconstruction of the structure of the nucleoprotein filament from electron micrographs. In support of the 2-domain model, this image analysis has allowed them to visualize two domains within the recA monomer in the presence of a nucleotide analog, ADP-A1F_4^-.[211] Moreau and Carlier[211a] have shown that ADP-A1F_4^- binds to recA protein and functions as a nonhydrolyzable analog of ATP.

5. C-Terminus

As noted above, the C-terminal region is the least well-conserved part of the *E. coli* recA gene sequence. Benedict and Kowalczykowski[151] studied the properties of a recA protein fragment that was missing approximately 50 amino acids from its C-terminus (≈15%). This fragment has wild-type ATPase activity. In the absence of SSB, this fragment has increased strand assimilation activity (i.e., assimilation of ssDNA into homologous duplex DNA to form heteroduplex DNA molecules). In the presence of SSB, it behaves as the wild-type protein. It has identical activity to wild-type protein with respect to ssDNA affinity and ssDNA-dependent ATPase activity. Important differences follow. First, the fragment showed no lag (presumably the slow, rate-limiting nucleation step) and a higher rate of ATP hydrolysis in the dsDNA-dependent ATPase activity. Second, the fragment has a higher affinity for duplex DNA. Third, the fragment had a greater tendency to aggregate in the presence of dsDNA. Alterations in the recA-dsDNA interactions brought about by deleting these residues would explain these differences. The function of the C-terminal region could be to modulate recA protein's interactions with dsDNA. The C-terminal region is rich in acidic amino acids and consequently is charged. Perhaps it interferes electrostatically or sterically with binding to one or both strands of the DNA duplex. Also, the C-terminus may be involved in modulating a conformational change that affects recA protein's interactions with adjacent monomers in the nucleoprotein filament or with the dsDNA.

Ogawa et al.[138] generated the truncated mutant recA5327 protein that lacked 25 amino acids at the C-terminus. Its aggregation properties were slightly different than wild type, suggesting that a region near the C-terminus may be involved in protein-protein interactions. Furthermore, when this mutant protein is present on a plasmid within a host bearing a wild-type chromosomal copy of the recA protein, the cells become sick and filamentous, suggesting that the C-terminal region may be involved in coprotease activity.

Finally, Larminat and Defais[212] studied the truncated mutant recA335, which is missing 17 amino acids from the carboxy-terminus. They concluded that those residues were not essential for homologous recombination, UV resistance, and the induction of the S.O.S. response.

6. N-Terminus

Sedgwick and Yarranton[62] found that a plasmid encoding 22% of the N-terminal portion of the recA structural gene decreases recombinase and postreplication repair activity in $recA^+$ cells. Plasmids that carried less than 10% of the structural gene had little effect. They concluded that the fragment and full-length proteins were forming inactive multimers. Therefore, the N-terminal region is involved in the protein self-assembly process.[63] In agreement with this, trypsin digestion of the first 33 residues of the recA protein inhibited protein aggregate formation.[137,138] Finally, *in vitro* affinity chromatography of truncated recA proteins has shown that a region within the first 90 residues of the N-terminus is important in self-assembly.[213] In addition, this work has demonstrated that a hydrophobic surface of the recA protein[214] plays a major role in monomer recognition in solution.[213]

Zlotnick and Brenner[215] studied the interactions of a 24 residue peptide (N-terminal fragment of the recA protein) with DNA. They found that in the presence of DNA, the peptide formed an α-helix. A positive face of the helix could bind DNA by electrostatic interactions. The affinities for various oligonucleotides were the same as that found with full-length recA protein (poly(dT) > εDNA > poly(dA) > dsDNA).[73]

IV. RECA PROTEIN-MEDIATED DNA STRAND EXCHANGE

A. Overview

The properties and activities of recA protein described above come together in this reaction, which lies at the heart of recombination or recombinational repair. As already indicated, recA-mediated strand exchange reactions are unidirectional, require ATP, and can involve either three or four DNA strands (see Figure 1).

Strand exchange requires that the recA nucleoprotein filament bind to a second DNA (DNA2) and align homologous sequences between DNA1 and DNA2. This is followed by a strand switch to create a crossover junction (a Holliday structure when four strands are involved). Extensive regions of heteroduplex DNA are then produced by means of a unidirectional strand exchange (a facilitated branch migration process). This final process requires rotation of the right-handed helical DNA substrates as shown in Figure 10.

As is evident in Figure 10, strand exchange between two helical DNA molecules involves both longitudinal (branch movement parallel to the helix axis) and rotational components. These are coupled so that unidirectional application of energy along the axis of the filament will result in rotation in a predictable direction and an application of energy into directional rotation around the circumference of a filament cross-section will result in longitudinal displacement of the branch. Any chemical energy involved in strand exchange must be coupled to one or both of these motions.

Based on current evidence, the remainder of this review will focus on a likely strand exchange reaction pathway that can be summarized as follows:

1. The nucleoprotein filament already described is formed on DNA1, then binds and aligns a homologous DNA2 to form a novel right-handed triple helical structure as the key pairing intermediate. The triple helix is quite unstable in the absence of recA protein. This intermediate was originally proposed by Howard-Flanders and coworkers,[88] but its detailed structure may deviate significantly from the original formulation.
2. Within the triple helix, the initiating strand (ssDNA, DNA1) is paired in a Watson-Crick mode with a complementary strand derived from DNA2, at least over short regions, i.e., at least one form of the triple helix is one in which a strand switch has already occurred. The displaced strand of DNA2 may be present in a Hoogsteen or other related hydrogen-bonding mode. This is consistent with the view that the strand switch required for strand exchange does not require ATP hydrolysis, but is instead facilitated simply by the binding energy available in a recA nucleoprotein filament. A reaction of this kind is sufficient to explain the three-stranded exchange reaction of Figure 1A involving entirely homologous DNA molecules, as demonstrated by the extensive recA-mediated strand exchange observed by Menetski et al.[29] in the presence of ATPγS.

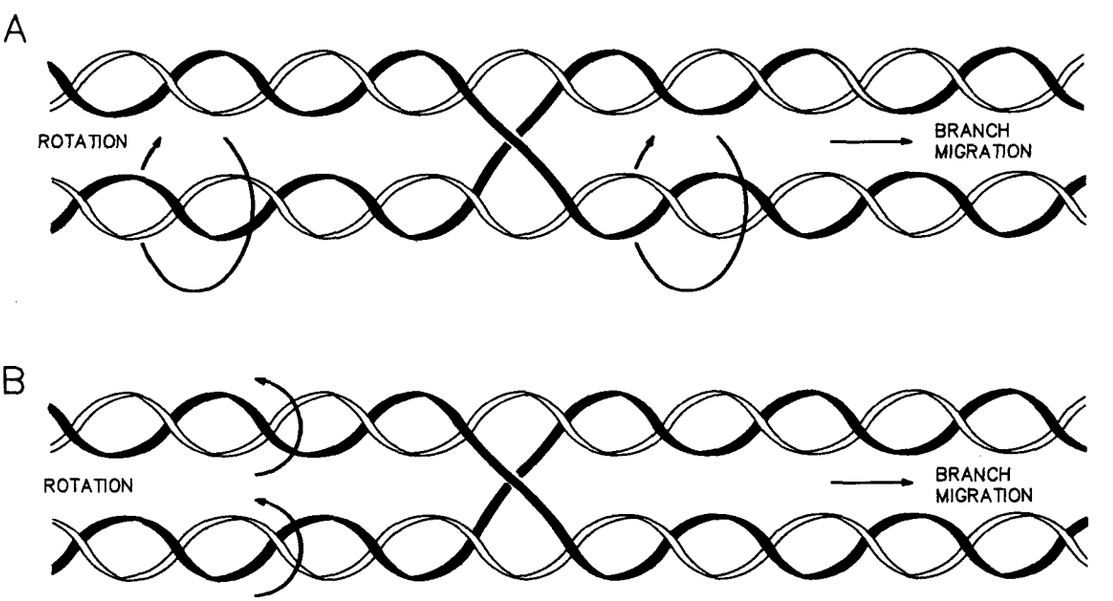


FIGURE 10. The right-handed helix of DNA must be rotated as shown to bring about strand exchange. The two DNAs could be rotated one about the other (panel A) or together about their longitudinal axes (panel B).

3. ATP *hydrolysis* in this system is not coupled to strand exchange per se, but instead represents an augmentation of the process that permits strand exchange to bypass structural barriers in the DNA as required during DNA repair. An alternative view, that ATP hydrolysis is required only for recA protein dissociation after strand exchange is complete, will also be considered. These two proposals for the use of chemical energy in this system are not necessarily incompatible and models can be developed that include both. Several current models for strand exchange will be discussed, as well as prominent alternative proposals regarding the themes described above.

B. Binding to DNA2

RecA protein binds to DNA1 as a sequence-independent DNA binding protein. Once this nucleoprotein complex is formed, however, it becomes a sequence-specific DNA binding entity. The sequence specificity with respect to DNA2 is determined entirely by the nucleotide sequence of DNA1. The ultimate purpose, of course, is the homologous alignment of the two DNA molecules.

1. The Search for Homology

The initial binding involves a heterologous interaction that must logically precede homologous alignment. Nonspecific binding of the nucleoprotein complex to a second DNA molecule has been characterized in several studies, most of them from the Radding laboratory.[216-221] Binding to heterologous DNAs is observed with both ATP and ATPγS, and it occurs with kinetics consistent with the idea that the resulting complexes are intermediates in the process of homologous alignment.[221] This binding implies the existence of a second DNA binding site for a DNA molecule in the nucleoprotein filament, one that is distinct from the first binding site that includes the positions occupied by the initiating strand and (when DNA1 is a duplex) its complement (Figure 11). This second DNA binding site must bind to heterologous duplex DNAs. Evidence for a second DNA binding site that can accomodate heterologous DNA includes the coaggregation of heterologous duplex DNAs promoted by recA nucleoprotein filaments[220,221] and binding studies carried out with several different methods.[86,222]

A second DNA binding site is almost certainly located in the large helical groove of the filament, as suggested by Howard-Flanders and co-workers.[88,223] Assuming this is the site where heterologous DNAs are bound, there must be sufficient room in this site to prevent steric interference with DNA1. The filament groove must in any case have space for at least three DNA strands because they clearly coincide in the first paired recombination intermediates as described below. When DNAs 1 and 2 are homologous, there is clear evidence that at least three DNA strands can be accomodated in the filament groove.[86,222] When DNA1 is single-stranded, and DNA2 is a heterologous duplex, binding of DNA2 occurs but is weak,[222,224]

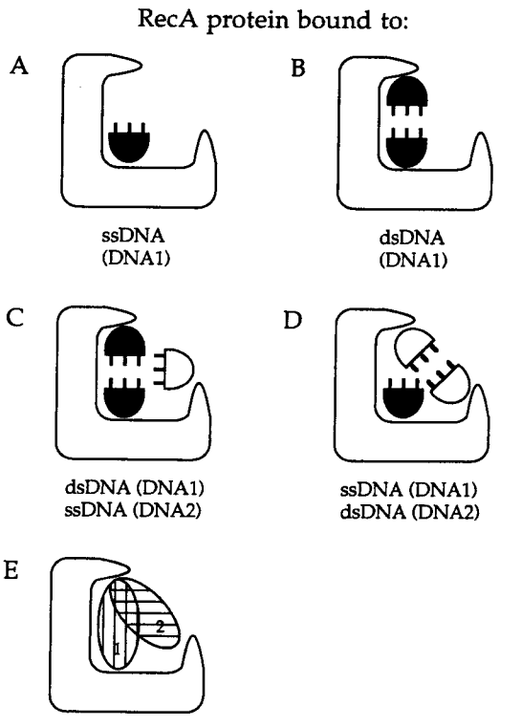


FIGURE 11. Postulated DNA binding sites in the recA nucleoprotein groove. Closed and open symbols represent strands derived from DNA1 and DNA2, respectively. The position occupied by the lone ssDNA at top, left, is intended to represent the binding site for the initiating strand. G-C base pairs are represented, although hydrogen bonding patterns are entirely speculative. Other patterns are possible, especially in the case of arrangements shown in panels C and D (see Figure 17). In panel E the hypothetical binding sites 1 and 2 are indicated by shading. The overlap is indicated because (as described in the text) either DNA1 or DNA2, but not both, may be duplex.

indicating that some steric interference may exist in this situation. An apparent exception to this rule appears to occur when DNA1 is the fluorescent derivative of ssDNA called εDNA.[86] Strong binding of both DNA1 and DNA2 when they are heterologous is observed only when both are single strands. If DNA1 is duplex, little binding of a duplex DNA2 is observed even if it is homologous to DNA1, indicating that strong binding in the groove is limited to three strands.[86,222] The fact that either DNA1 or DNA2, but not both, may be duplex suggests that DNA binding sites 1 and 2 overlap to some extent (see Figure 11). It is worth noting that the capacity to bind three but not four strands of DNA, evident in a growing number of studies, provides another interesting functional link to DNA repair processes.

Another possible location for a heterologous DNA binding site is external to the groove.[225] Any DNA bound to such a site would be separated from DNA1 (Figure 12). Such a site

could facilitate pairing by holding DNA2 in close proximity to DNA1, in much the same manner as a heterologous site in the groove. If all monomers in the filament possessed this site, six grooves would be formed longitudinally along the outside of each filament parallel to the filament axis (Figure 12). A DNA2 bound in this extended site would be positioned parallel to DNA1, and it would span the major filament groove once each helical turn of the filament (Figure 12). This arrangement would facilitate a sampling interaction between the two DNAs while minimizing steric interference and any need for an energetically costly interwinding of the DNAs prior to the alignment of homologous sequences. This putative site will be referred to as binding site 3 below. If it exists it must be in addition to, rather than in lieu of, a second site in the groove, because at least three strands clearly coincide in the groove once homologous alignment occurs as described below. A third site of this kind would not only facilitate homologous alignment, it is also a requirement for one model for DNA strand exchange (see Section IV.C below). At this time the existence of site 3 must be regarded as strictly hypothetical. Evidence that suggests a third DNA binding site has been provided by Takahashi and Schnarr,[226] who used lexA repressor cleavage kinetics as an indirect measure of recA-polynucleotide interactions. Kubista et al.[86a] have also found evidence for three DNA binding sites, two internal and one external to the filament, in a recent study using flow linear dichroism and nuclease digestion methods.

2. *Homologous Alignment and Paranemic Joints*

The first paired intermediate in strand exchange has been called a paranemic joint, and it was initially defined by Radding and co-workers[56,227,228] with substrates in which strand exchange following the formation of joints is precluded. With these substrates (Figure 13), the joints are highly unstable when recA protein is removed.[56,227-229] Paranemic joints were determined to be kinetically competent as intermediates in strand exchange by Riddles and Lehman.[229]

Several types of solution measurements and direct observation by electron microscopy demonstrates that homologous alignment in paranemic joints can extend over thousands of base pairs.[90,96,230] As described above, DNA1 is extended and (if duplex) underwound. In the paired complex, DNA2 is also significantly underwound,[230] as it must be for alignment with DNA1. The structure of this pairing intermediate represents

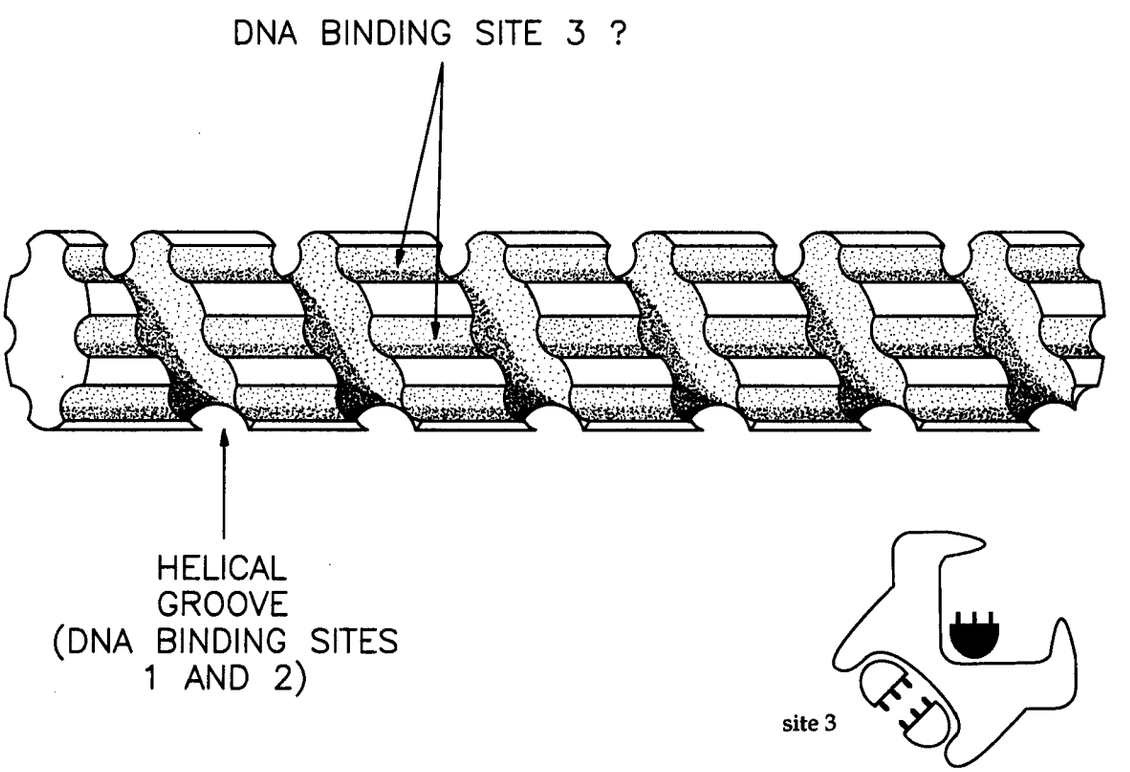


FIGURE 12. A postulated third DNA binding site on the exterior of the filament. Panel A illustrates the six "site 3s" that might exist as linear crevices along the outside of the filament and parallel to the filament axis. There are six recA monomers per filament turn, and each monomer would contribute part of one of these six site 3s, as shown in a schematic monomer cross-section in panel B.

one of the key unresolved issues in the recA system, and it is this question to which we now turn.

3. A Triple-helical Recombination Intermediate: The RecA Triplex

Homologous alignment of two DNAs is presumed to require formation of base pair-specific-intermolecular hydrogen bonds. The most probable interaction points are the hydrogen bond donor and acceptor groups available in the major groove that are not involved in Watson-Crick base pairing. Alignment over a significant region would require interwinding of the DNAs to form a three- to four-stranded helix, as proposed for the paired intermediate in recA-mediated strand exchange by Howard-Flanders and co-workers in 1984.[88] Proposals for the detailed structure of this intermediate have been strongly influenced by the four-stranded DNA model described by McGavin[231] and Wilson[232] (Figure 14). Interduplex hydrogen-bonding in this structure is optimal for like base pairs. A three-stranded version of this model can be generated simply by removing one strand.[88,223]

RecA protein promotes both three- and four-stranded exchange reactions, leading to the expectation that both three- and four-stranded pairing intermediates must exist. Three-stranded paranemic joints have been detected by a wide variety of methods.[56,90,96,227-230] The first clear demonstration of duplex-duplex pairing has been reported recently by Conley and West.[233]

Much evidence, however, indicates that a functional and structural distinction exists between three- and four-stranded joints, and suggests that a three-stranded joint is of fundamental importance in virtually all recA-mediated strand exchange reactions. As already noted, recA filaments readily bind up to three strands of DNA, but not four. Correspondingly, in a side-by-side comparison, three-stranded paired joints are formed much more readily than four-stranded joints.[234] The homology-dependent underwinding of DNA2 that represents the most reliable manifestation of joint formation is weak in the four-stranded case and is not observed at all unless DNA2 is initially negatively supercoiled.[233,234] Furthermore, four-strand exchange reactions are almost always initiated in a three-strand region.[38,235-237] The reactions are stimulated by at least two orders of magnitude when the homologous end of DNA2 overlaps the single-strand gap of DNA1.[234,238] Optimal reaction efficiency requires either ~40 or ~100 base pairs of overlap depending on whether the assay used measures formation of early pairing intermediates[238] or strand exchange products,[234] respectively. All of this is in general accord with other properties of the system. Recent studies of DNA binding indicate that three, but not four, DNA strands are readily accomodated in the helical filament groove.[86,222] The strong kinetic predisposition of recA protein to bind ssDNA tends to direct it to regions where three-stranded structures can be formed. Single-stranded DNA is also highly recombinogenic *in vivo*.[39] These results indicate that three-stranded joints may be functionally distinct from four-stranded joints and that three-stranded structures that have no four-stranded analog need to be considered.

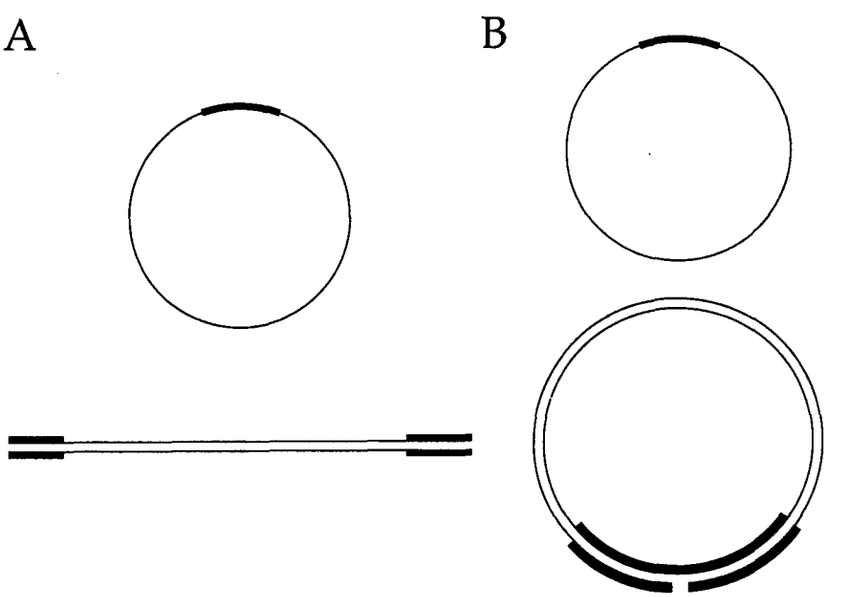


FIGURE 13. Substrates used to study paranemic joint formation. Strand exchange between homologous sequences (thin lines) in the substrate pairs is precluded by flanking heterologous sequences (thick lines). The substrates in panel A may form a paired structure with net interwinding; those in panel B may not.

The function of four-stranded intermediates remains to be determined. For reasons cited above, most work and thinking to date has focused on three-stranded structures. Models proposed to date for three-stranded pairing are summarized in Figure 15. Only the topological arrangement of strands is presented and hydrogen-bonding interactions are unspecified. The variety of models reflects the important secondary issue of topological balance. All of the structures except one incorporate triple helices. If long DNA molecules, i.e., chromosomes, are to be aligned over any distance, any right-handed interwinding of the two DNAs must generate an equivalent number of compensatory left-handed turns. This problem could be addressed by the recA protein itself or by a mechanism external to the system.

Models A, E, and F represent proposals for structures in which the topological problem is solved by compensatory DNA binding modes inherent to the recA filament. The side-by-side model (A) was proposed by us several years ago.[239] It has proven to be unworkable and will not be considered further here as a structure for homologous alignment. In structure F, right-handed triple helices are balanced with interspersed left-handed triple helices, similar to arrangements suggested by Fishel and Rich[240] and Christiansen and Griffith.[241] In structure E, the right-handed triple helix is balanced by left-handed toroidal turns of DNA2 about DNA1 without homologous contact. This structure represents a solution to the topological problem originally proposed by Wilson.[232] These turns could be stabilized by the nucleoprotein filament. In structure B to D, no topological compensation is present, and in these cases it must be assumed that any topological problems created by formation of a right-handed (B) or left-handed (C) triple helix are resolved in the cell by means external to the recA system (i.e., topoisomerases). Structure B is essentially that proposed by Howard-Flanders et al.[88] Structure D is a variant of B in which right-handed triple-helices are interspersed with regions in which DNAs 1 and 2 are side by side and not in contact. In these regions DNA2 could be either unbound or bound in the hypothetical site 3 illustrated in Figure 12.

Observation by electron microscopy of putative three-stranded DNA structures has been reported several times.[89,90] The DNAs appear to be duplex and the presence of three strands is inferred on the basis of DNA length measurements.

Obtaining structural information on these complexes is complicated by the fact that the joints formed with the substrates in Figure 13 are unstable in the absence of protein. This problem can be overcome in part by crosslinking the joints with psoralen prior to removal of protein. Studies of this kind have provided evidence consistent with structure D in Figure 15.[242] When the circular single-strand is paired with a linear duplex with heterologous ends (Figure 13A), long crosslinked joints are produced, averaging nearly 2500 base pairs in length.[242] When the same single strand is paired with a circular duplex (Figure 13B) the average joint length was reduced to less than 200 base pairs.[242] This indicates that formation of long joints requires interwinding of the DNAs, providing a topological argument consistent with the formation of a triple helix. Since recA nucleoprotein helical filaments are right-handed even when recA binds initially to Z form DNA, a triple helical pairing intermediate would almost certainly be right-handed. Since the lengths of the joints formed with the substrates of Figure 13A and B are so different, recA protein evidently does not facilitate the formation of a topologically balanced structure. This suggests that the pairing function of recA nucleoprotein filaments is focused on the formation of right-handed triple-helices with the resulting topological consequences resolved by other systems in the cell.

The detailed structure of this triplex is not known, but it is important to note that it is almost certainly a novel structure. Stable triple-helical DNA structures have been described, but they generally are limited to sequences with a polypyrimidine sequence bias and like strands are antiparallel. The known structural parameters that must apply to any recA-stabilized triple helix make it unique. In particular, like strands must be

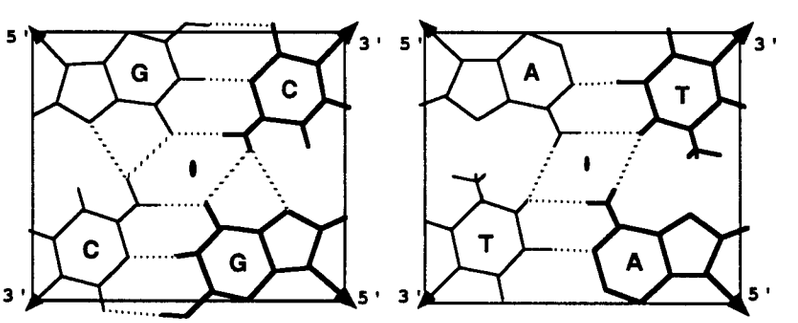


FIGURE 14. Hydrogen-bonding interactions in the four-stranded DNA recombination intermediate postulated by McGavin[231] and Wilson.[232]

parallel, any sequence must be accomodated, and the DNA must be extended and underwound so that the axial base separation is 5.1 Å and each helical turn contains 18 base pairs (triplets).

The observed crosslinking patterns in these joints are complex[242] and suggest that shorter multiple triple-helical regions encompassing a few hundred base pairs are interspersed with regions where DNAs 1 and 2 are side by side. These multiple contacts are not necessarily required for strand exchange and may be formed only when further steps in strand exchange are topologically precluded. They suggest, however, that a range of structures may be formed in this system. One of the observed crosslinking patterns is especially intriguing and is described in the next section. A caveat here is that the multiple crosslinking patterns may reflect incomplete crosslinking of a longer uniform three-stranded structure.

Late in the strand exchange reaction, a three-stranded DNA species has been detected that lingers in many of the substrate molecules and exhibits a remarkable degree of heat stability.[243] The nature of this structure and its relationship to the unstable paranemic joints formed when strand exchange is topologically precluded remains to be determined, although one possibility is outlined below.

4. Formation of Paranemic Joints May Be Coincident with the Strand Switch in DNA Strand Exchange

There are two psoralen crosslinking patterns derived from paranemic joints that appear to reflect the presence of triple helical structures. In the most straightforward one, all three strands are crosslinked. In the other, the circular single-strand is crosslinked to one of the strands of the duplex (Figure 16). The strand derived from the duplex is probably the complementary strand, because psoralen efficiently links DNA strands that are Watson-Crick base paired.[244] Although other conformations are not excluded, this result suggests that at least one form of the triple helix formed by recA nucleoprotein filaments is one in which the two strands of DNA2 have been separated and the ssDNA initiating strand is paired with its complement. In this scenario, the structure stabilized by the recA nucleoprotein filament is one that resembles products rather than substrates or a transition state, and, in effect, the strand switch has already occurred (Figure 17). This "strand-switched" triple helix may be related to the stable three-stranded structures observed by Rao et al.[243] and may also explain the observation of significant strand exchange in the presence of ATPγS.[29]

Note that when DNA1 is single-stranded, the DNA strand binding site that would bind its complement (when recA is bound to duplex) is unoccupied (Figure 11A vs. B). Binding energy pro-

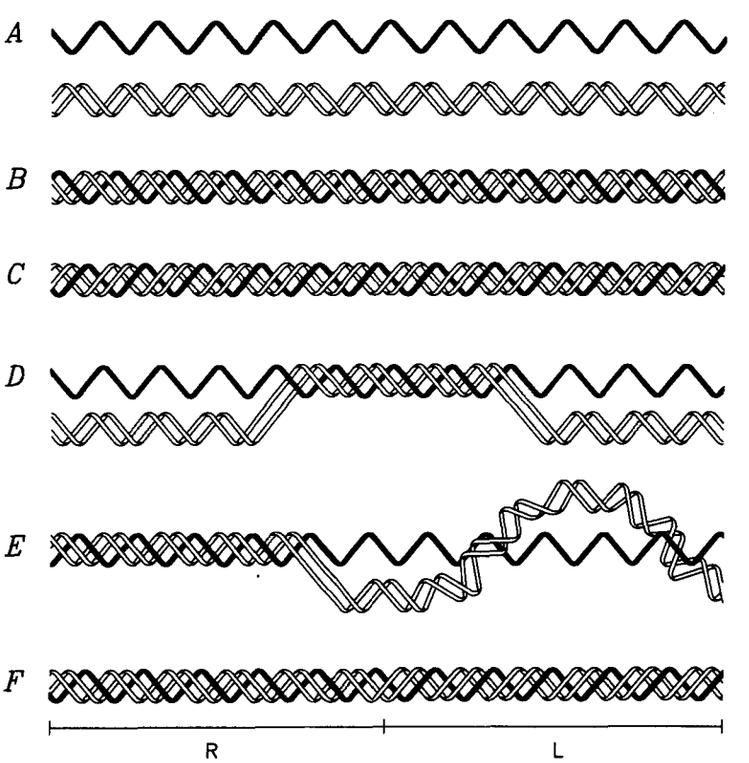


FIGURE 15. Hypothetical structures for three-stranded paranemic joints. (See text for details.)

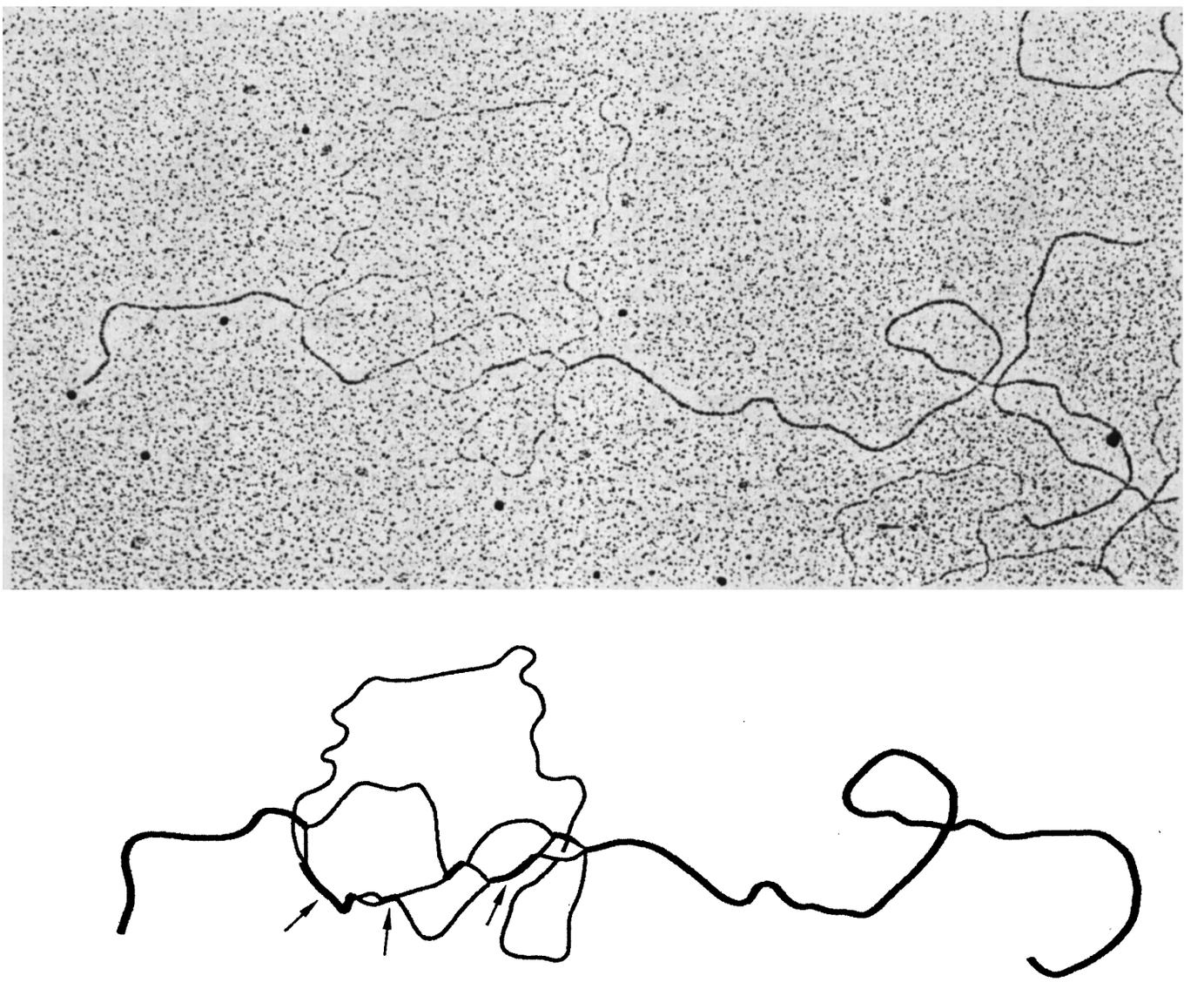


FIGURE 16. Crosslinking of DNA strands in a paranemic joint by the psoralen derivative AMT. In the interpretive drawing, thin and thick lines are single-strand and crosslinked double-strands, respectively. Arrows indicate segments where the single-stranded circle (see Figure 13A) is crosslinked to one of the two strands of the linear duplex. Segments in which all three strands are crosslinked are not seen in this example but were common in other joints in this experiment.[242] The linear duplex has heterologous sequences at either end (Figure 13A) that preclude a complete strand exchange reaction.

vided by both the recA protein and the ssDNA bound in the initiating strand site could facilitate this strand switch. A reaction of this kind need not require ATP hydrolysis. This represents a variation of a mechanism proposed by Menetski et al.[29] to explain ATPγS-mediated DNA strand exchange. The ATPγS reaction represents compelling evidence that this process or something very similar must occur. A similar process may account for recA protein-mediated renaturation of complementary ssDNAs.[5,164,245-247]

C. The Mechanism of RecA-Mediated DNA Strand Exchange

1. Vital Statistics

After formation of the pairing intermediate described above, a strand exchange occurs in the presence of ATP that may be thought of as a facilitated branch migration reaction. Strand exchange is unidirectional, proceeding 5′ → 3′ relative to the

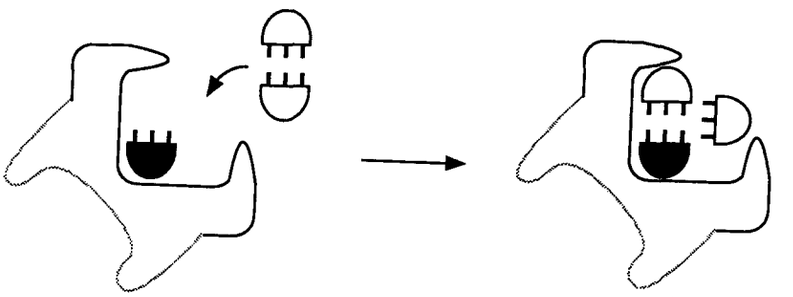


FIGURE 17. Postulated hydrogen-bonding pattern in a paired intermediate where the strand switch is coincident with triple helix formation.

initiating strand.[55,248-250] By determining the assembly and structural polarity of the recA filament, the initiating strand effectively dictates the polarity of the entire reaction.

ATP is hydrolyzed by the recA-ssDNA nucleoprotein filament with the monomer k_{cat} of 25 to 30 min^{-1} typical of the ssDNA-dependent reaction described above. Formation of the first pairing intermediate is accompanied by an abrupt 30% drop in the rate of ATP hydrolysis, and this new rate of ATP hydrolysis is maintained through the remainder of the strand exchange reaction and well after it is complete.[96] The lower rate (k_{cat} = 20 to 22 min^{-1}), perhaps coincidentally, is that typical of recA-dsDNA complexes. The continuation of ATP hydrolysis at a uniform rate throughout strand exchange (after pairing is complete) and other results described below indicate that recA protein remains DNA-bound during the reaction.

The reaction is progressive with the branch moving slowly through the available homology. The rate at which branch movement occurs is widely reported to be 3 to 6 bp s^{-1}, a rate averaged over all molecules in a reaction.[251,252] Since the reactions in different DNA molecules are probably not as well synchronized as often assumed, the actual rate in a given molecule may be as much as 10 to 20 bp s^{-1}. The latter rates have been reported in a few experiments under apparently optimal conditions.[253]

To the extent it is coupled to strand exchange, chemical energy is used inefficiently in this process.[125,243,251] ATP is hydrolyzed uniformly throughout the filament during strand exchange with a monomer k_{cat} of 20 to 25 min^{-1}.[77,96] Since ATP is hydrolyzed everywhere but the branch is moving at only one point, over 100 ATPs may be hydrolyzed for every base pair of heteroduplex DNA generated by a typical recA filament composed of 2000 monomers.

As already indicated, recA protein-mediated strand exchange proceeds past mismatches, DNA lesions, and short heterologies quite readily.[54-56] The reaction can also proceed past a break in DNA2, but not in DNA1.[96,254,255] The latter result reflects the asymmetry in the interaction with DNAs 1 and 2 and provides a further argument that only one filament (formed on DNA1) is involved in the process.

2. A Model Based on Minimal Use of Chemical Energy

Based on results already discussed, the simplest model that can be proposed is that of Kowalczykowski and co-workers.[29] This is based on their important observation that in three-strand exchange reaction nearly half as much heteroduplex DNA can be generated with ATPγS under some conditions as is observed in ATP-mediated reactions.[29] In this view, strand exchange requires little or no ATP hydrolysis. The nucleoprotein filament facilitates the formation of a triple helix that represents a transition state for strand exchange. This is resolved to products by recA dissociation mediated by ATP hydrolysis. The excess ATP hydrolyzed in this case would be wasted. In the case of reactions with ATPγS, the triple helix collapses at random to either substrates or products when recA is removed by SDS treatment.[29] Another interpretation of these results, in which the triple helix resembles products, has been outlined above. In either case, the function of ATP hydrolysis would be to facilitate dissociation of recA protein to resolve the triplex into products and/or to recycle the protein. Most of the energy derived from ATP hydrolysis would be unproductive. A model of this type is sufficient to explain strand exchange between homologous DNA substrates *in vitro*. In particular, it is clear that a strand switch can occur entirely or in large measure without ATP hydrolysis. What then remains as a function for ATP hydrolysis is more speculative, as outlined below. Whether or not dissociation after triple helix formation represents the only function of ATP hydrolysis, the ATPγS reaction observed by Menetski et al.[29] serves to focus any discussion of energetics in this system. In fact, the function of ATP hydrolysis in the recA system may ultimately be defined by an understanding of what the ATPγS-mediated reaction can or cannot do.

3. Energetics of DNA Strand Exchange: Why Does RecA Protein Hydrolyze ATP?

Although chemical energy may appear superfluous to strand exchange, it must be kept in mind that ATPγS is not present in the cell and a considerable amount of ATP hydrolysis occurs that must logically serve some purpose. When viewed strictly as an

enzymatic activity, the ATPase activity of recA protein is unimpressive. The optimal k_{cat} is only ~30 min^{-1} (0.5 s^{-1}) and k_{cat}/K_M is at most 10^4 M^{-1} s^{-1}, four orders of magnitude below the diffusion controlled limit for an enzymatic reaction.[256] As outlined below, this reaction is unlikely to have a significant effect on cellular metabolism. On the other hand, recA hydrolyzes more ATP, by several orders of magnitude, than can be accounted for by dissociation of recA monomers from filaments under most conditions. How can this be rationalized?

Here we take the view that to address this problem it is necessary to again consider the cellular function of recA protein and in particular the process of recombinational repair. The ATPγS reaction occurs with relatively short (5 to 7000 bp) DNA substrates that are homologous and unhindered with respect to the rotary diffusion that must take place. Recombinational repair requires an efficient reaction in an environment filled with topological and structural barriers.

Since extensive strand exchange can clearly occur with ATPγS, any role of ATP hydrolysis in the process must be regarded as an augmentation of the reaction that addresses problems external to the strand switch itself. ATP hydrolysis could contribute to the effectiveness of strand exchange in a cellular environment in at least three ways: (1) by promoting dissociation at one end to recycle the protein, (2) by rendering the reaction unidirectional and thereby ensuring that a DNA lesion is efficiently repaired even if thousands of base pairs away from the initiation point, and (3) by providing energy to move the branch past structural barriers such as DNA lesions as required for the system to function in repair. All of these have been proposed in the literature and they are not mutually exclusive.

As already noted, dissociation does not occur in the presence of ATPγS. Recent work also indicates that the ATPγS reaction is not unidirectional.[257,258] These then clearly qualify as likely functions of ATP hydrolysis. The role of ATP hydrolysis in bypassing structural barriers has not yet been adequately addressed experimentally.

As outlined in the discussion of filament disassembly, dissociation of recA monomers from a filament is probably coupled to ATP hydrolysis. It is also largely limited to the one filament end where dissociation is sometimes proposed to occur following passage of the branch point.[110] As long as ATP is regenerated, however, measurable dissociation occurs under narrowly defined conditions.[84,110] It is absent or minimal under many conditions under which strand exchange is efficient.[94,118,230,259] In addition, strand exchange proceeds in the presence of low levels of ATPγS that completely block dissociation while allowing most ATP hydrolysis to continue.[84,110,131] Thousands of ATPs may be hydrolyzed for every detected monomer dissociation, and this absence of a correlation between the two processes makes it unlikely that dissociation is the only function of the hydrolytic reaction. It is likely to be important, nevertheless, at least for recycling the recA nucleoprotein filaments.

Dissociation rates are greatly enhanced when the ADP/ATP ratio approaches 1.0[119,125] (see Section III.C), suggesting that ATP hydrolysis could also promote disassembly indirectly by increasing ADP levels at the expense of ATP. The 1200 recA monomers in a typical cell,[259a] however, can use no more than 0.1% of the chemical energy expended by the 10,000 to 20,000 ribosomes in a typical *E. coli* cell. The impact of recA-mediated reactions on cellular ATP pools is unlikely to be significant, even under S.O.S. conditions.

An examination of the ATPγS and ATP reactions offers some insights into the possible advantages of unidirectionality. Under optimal conditions, the ATPγS reaction produces heteroduplexes averaging 2.4 to 3.4 kb in length.[29] The efficiency of heteroduplex formation falls off at points more than 3 kb from the initiation point and full-size products are not observed with DNA substrates derived from bacteriophage M13.[29,258] The ATP-mediated process extends strand exchange efficiently through all the available homology, and the efficiency remains essentially constant throughout the length of the homologous sequences. A unidirectional reaction is likely to be a substantial advantage to the cell whenever a DNA lesion to be repaired is several thousand base pairs from the point where recombination is initiated.

Despite the reaction evident with ATPγS, several results suggest that strand exchange is coupled to ATP hydrolysis under normal reaction conditions. First, the facility of the reaction to proceed through insertions, mismatches, and DNA lesions in the presence of ATP[54-56] suggest an actively driven process. Second, the ATPγS and ATP reactions differ qualitatively in that long heteroduplexes averaging over 2 kb are formed within a few minutes with ATPγS,[29] whereas strand exchange is slower and progressive with ATP. This suggests that rapid rotary diffusion is actually blocked in the ATP reaction, with the rotation itself directed and controlled. A direct link between ATP hydrolysis and rotation is indicated by the additional observation that the long paranemic joints formed between circular single strands and linear duplexes with heterologous ends in the presence of ATP (Figure 16) are reduced to point contacts when ATPγS is used, even under conditions optimal for the ATPγS reaction.[258] This indicates that the interwinding of the DNAs required to form the long joints is an actively driven process. The heterologous ends evidently represent a topological barrier that blocks rotary diffusion and with it the ATPγS reaction, but is easily overcome when ATP is hydrolyzed. These results suggest that ATP hydrolysis promotes rotation of the DNAs during strand exchange. The strand switch itself is facilitated by binding energy within the filament. A directed rotation will result in unidirectional branch movement as noted in Figure 10. The reaction proceeds even when rotation of one DNA about the other is blocked, so that rotation of the DNAs about their longitudinal axes as illustrated in Figure 10 is sufficient.[260]

4. Where Is RecA Protein Located During Strand Exchange?

Another aspect of the mechanism of strand exchange that has evoked much experimentation and discussion is the status of the filament during the reaction. Of particular interest is the

question of whether recA assembly or disassembly processes play a direct role in facilitating branch movement. It is also important to know which DNA strands are bound by recA protein at different points in the reaction. The answers to these questions obtained experimentally reflect known properties of recA protein and also vary somewhat depending on reaction conditions and the techniques used for measurement. It is therefore necessary not only to identify assembly, disassembly, and binding processes that are present, but also to determine which are actually required for strand exchange.

The most direct approach for examining filaments during strand exchange is electron microscopy. The three-strand exchange reaction of Figure 1A has been followed in the electron microscope in several studies that have produced similar results.[64,89,90] The recA nucleoprotein filament is formed on the ssDNA circle, and the linear duplex binds and disappears into the filament over an extended region. RecA protein is absent on the heteroduplex (or a three-stranded intermediate) that is formed, indicating dissociation. When SSB is present, recA protein is entirely absent in the DNA after products have been formed.[64,90] The SSB is seen bound to the displaced linear ssDNA, while the heteroduplex circle is unbound. When SSB is absent, recA is generally bound to the displaced single strand and the heteroduplex is again unbound.

These studies are in general agreement with a variety of solution measurements except in one respect, i.e., the dissociation of recA protein from heteroduplex DNA. Following the initial pairing step ATP is hydrolyzed at a constant rate during and after strand exchange reactions carried out with SSB as long as ATP is regenerated.[96] In the same experiments, the heteroduplex products are extensively underwound well after the reactions are complete.[94] Both results are unambiguous demonstrations that the complete recA dissociation from heteroduplex (and all else when SSB is present) seen in the electron microscope does not occur. There is no demonstrable correlation between strand exchange and rates of either assembly or disassembly of recA filaments.[110] The heteroduplex is also protected from DNAse and restriction enzyme cleavage to an extent consistent with extensive recA protein binding.[95] (see Section III.B for a description of expected levels of nuclease protection when recA protein is bound to duplex DNA in the presence of ATP). Finally, in reactions carried out in the presence of SSB, the heteroduplex DNA product is inactive in subsequent reactions unless steps are taken to dissociate recA protein.[259] Although models developed from the E.M. observations interpret the absence of recA on the heteroduplex as dissociation, these results are not necessarily in conflict with the solution measurements, since the E.M. studies were done using fixation protocols that have been shown[82,121] *not* to preserve recA complexes on dsDNA in the presence of ATP.

Overall, the evidence supports the notion that little or no dissociation of recA protein is *required* for DNA strand exchange, although it may occur under some conditions. The recA filament therefore can, and usually does, remain essentially intact throughout the reaction. As indicated in the following section, there is no molecular event in strand exchange that requires dissociation of recA protein. Dissociation, in this view, is not intimately coupled to any essential process in strand exchange but occurs simply to recycle the protein. A treadmilling-like process that might occur in the cell could also serve to move the filament along the DNA concurrently with the branch.

It is important to note several caveats here. The effects of low levels of ADP on ATP hydrolysis, recA protein binding to DNA, and strand exchange (see Section III.C) suggest that ADP may modulate a process like treadmilling. The enhanced efficiency of recA-mediated DNA strand exchange at ADP/ATP ratios of ~ 0.3[125,243] requires further investigation. Another important caveat is that even when ATP is regenerated a small degree of dissociation might occur at the branch point followed closely behind by reassembly on the heteroduplex in a local treadmilling type of process that the measurements described above might miss.

Models for the reaction that do not invoke association or dissociation in the fundamental mechanism of strand exchange have been referred to as stable filament models by Griffith.[25] Two stable filament models are presented in the next section.

5. Models for ATP-Facilitated DNA Strand Exchange

The models presented here build on detailed models presented in the literature, adjusted as required by new evidence. They serve to illustrate the primary mechanistic possibilities for coupling ATP hydrolysis to strand exchange. The assumption implicit in this presentation is that dissociation is not the primary function of ATP hydrolysis, although some ATP hydrolysis may bring about dissociation in order to recycle the protein and/or resolve a triplex recombination intermediate. A coupling of ATP hydrolysis to strand exchange is proposed as a mechanical augmentation of the reaction that renders it unidirectional and facilitates movement past structural and topological barriers as required in recombinational repair. The hydrolysis of ATP, even when strand exchange is not underway, is viewed as an energetic contingency that reflects the importance of these processes to cell survival.

The models share many features that are cognizant of the helicity of DNA and the requirement for rotation, and the apparent presence of a right-handed triple helical pairing intermediate. Based on results and arguments outlined above, it is assumed that dissociation of recA protein is not required in the reaction and that the strand switch is promoted within the triple helix by binding energy available in the filament groove. In both cases the rotation of the DNAs is facilitated so that the reaction can proceed in environments where rotary diffusion is inhibited or blocked. Either model is consistent with the idea that ATP hydrolysis is coordinated into unidirectional waves in the filament (see Section III.C), although this is not a mechanistic requirement in either case.

The first model is an adaptation of the model of Howard-

Flanders and co-workers,[88] and envisions that the key molecular events in facilitated branch movement occur at a single point in the filament. In this variation, a segment of triple helix is formed by homologous alignment, followed by some rotary diffusion to generate the structure in Figure 18 (top). At the head of the triple helix, ATP-mediated conformation changes draw the duplex (DNA2) into the groove, where recA binding interactions separate the strands and effect a strand switch. The conformation changes proceed unidirectionally through the filament, perhaps in the form of a coordinated wave of ATP hydrolysis. ATP hydrolyzed elsewhere in the filament is wasted. The energy is effectively applied longitudinally through the helical filament, and rotation is one necessary result. The rotation is illustrated as an axial rotation of both DNAs in place, as indicated by the results of Honigberg and Radding.[260] The rotation, in turn, moves the triple helical region to the right as shown in Figure 18. Resolution of the triple helix may be facilitated by the binding of SSB or recA protein to the displaced strand (not shown).

Note that an alternative formulation of this model can be constructed that is consistent with the ideas of Menetski et al.[29] as outlined in Section IV.C.2. It is necessary only to assume that ATP hydrolysis is needed only to facilitate a dissociation event that is required for recycling the protein and resolving the triplex intermediate. In many ways, this would be closer to the original version of Howard-Flanders and co-workers.[88]

The second model (Figure 19) is based on that of Cox et al.[239] and more recent variants[225,261] and has recently been outlined in some detail.[225] It starts at the same point as the previous model, but rotation is more directly coupled to ATP hydrolysis by means of interactions between DNA2 and the external surface of the filament. The putative DNA binding sites described as site 3 are used (and, in fact, required) in this model as the site of the coupling mechanism linking ATP hydrolysis and strand exchange. These sites are envisioned to extend longitudinally along the entire length of the filament and a given site 3 is made up of a pocket contributed by every sixth monomer in the helical filament (see Figure 12). A DNA2 bound in one of these six available extended binding sites outside the filament is moved sequentially and unidirectionally from one site 3 to the next in a rotational motion driven by ATP hydrolysis. Each transfer of DNA2 from one site 3 to the next would move it 1/6 of the way around the circumference of the filaments, with six transfers required for each complete rotation. The ratchet-like coupling mechanism could result in either rotation of one DNA about the other or longitudinal rotation of each in place, as dictated by the topological situation.

A key feature of this second model is that rotation is mediated by molecular events occurring throughout the filament rather than just at the branch point. Movement of the branch is simply a byproduct of the rotation and the strand switch itself is mediated by binding interactions as DNA2 is spooled into the groove. This would explain why the rapid rotary diffusion that produces long heteroduplexes in a few minutes in the ATPγS

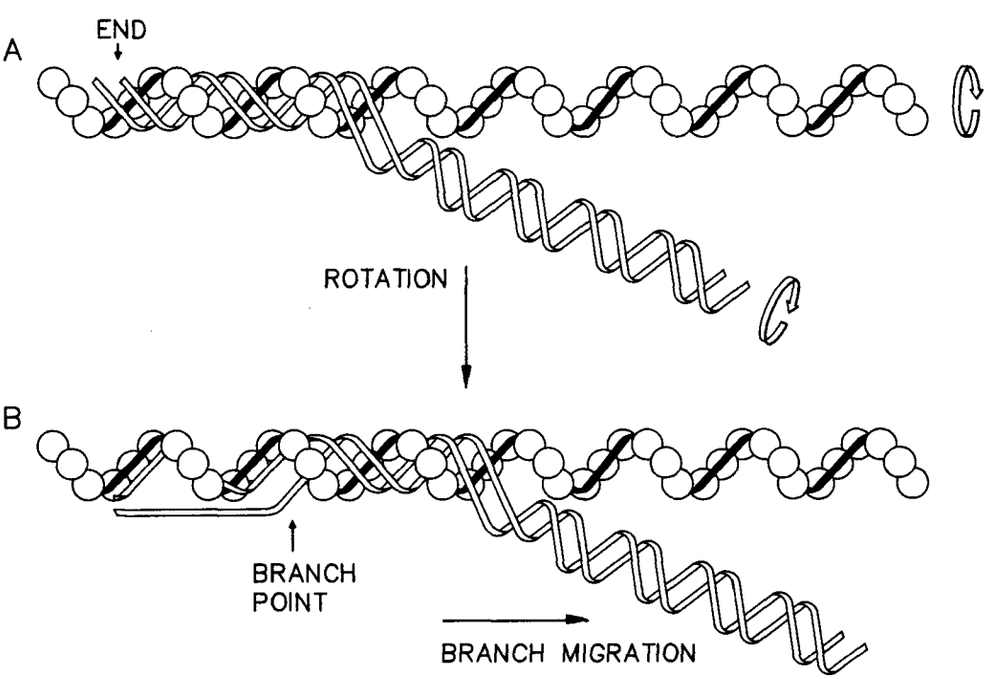


FIGURE 18. A model for strand exchange based in part on the ideas of Howard-Flanders et al.[88]

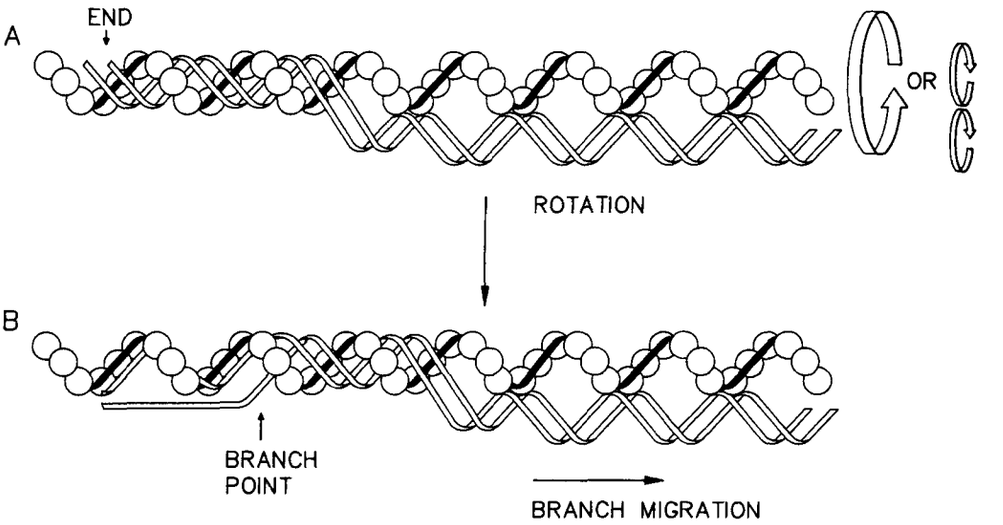


FIGURE 19. The ATP-driven rotation model for recA protein-mediated strand exchange. (See text and References 261 and 225 for details.)

reaction are not seen when ATP is present. The ATP-mediated strand exchange in this model would be constrained as well as directed, with the controlled rotation producing a relatively slow and progressive exchange of strands.

In this model, virtually all ATP hydrolysis is used productively. In effect, the ATP hydrolysis is used to generate a mechanical torsional stress sufficient to force the branch past structural barriers. If the DNA2 is bound along the entire filament, every monomer in the filament would have to hydrolyze one ATP for each 360° rotation. In a 2000 monomer filament, this would result in the expenditure of 2000 ATPs to move the branch by 18 base pairs, generating reaction efficiencies very close to what is generally observed in *in vitro* reactions. Furthermore, a k_{cat} for ATP hydrolysis of 25 min^{-1} would translate into 25 rotations min^{-1} or 25 × 18 = 450 bp min^{-1} (7.5 s^{-1}), again consistent with the rates of strand exchange observed *in vitro*. As described elsewhere,[261] this model also provides a mechanism for the strand exchange through short heterologous insertions in DNA2 observed by Bianchi et al.[56]

V. PROSPECTS

Many important questions remain about the mechanism of recA-mediated DNA strand exchange, including the structure of recombination intermediates, the use of chemical energy in the system, the interaction of recA with other proteins in recombination, and even the cellular function of the system. A better understanding of the structure of the triple-helical pairing intermediate is needed. To distinguish between the various models proposed for strand exchange, a better understanding of several key experimental observations will be needed. Perhaps foremost among these are the filament assembly and disassembly reactions, the strand exchange mediated by ATPγS, and the strand exchange that occurs through heterologous insertions in one or the other DNA. Much work also remains with respect to the mechanism of recA functions not covered here, such as the induction of S.O.S. and mutagenesis.

The prospect of a solved three-dimensional structure for recA protein should facilitate all aspects of this work and make possible a more detailed examination of structure-function relationships in this system. Once a general model for strand exchange is elucidated, another level of detail that will remain involves gaining an understanding of the conformational changes that occur during ATP hydrolysis, their effects on neighboring monomers, and their functional significance.

For the foreseeable future, recA protein is likely to remain a rich experimental system for studies of protein-DNA interaction, DNA recombination and repair, biochemical energetics, filament function and dynamics, and many other issues of biological importance.

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