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BIOCHEMISTRY OF GENETIC RECOMBINATION: Energetics and Mechanism of DNA Strand Exchange

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PERSPECTIVES AND OVERVIEW

The exchange of DNA strands between duplex DNA molecules is an event central to mechanisms of genetic recombination. How this process occurs was unknown until the discovery that the RecA protein of *Escherichia coli*,

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a protein essential for homologous recombination in *E. coli*, promotes the pairing and exchange of strands between homologous DNA molecules. The biochemical mechanism of the RecA protein-promoted DNA strand exchange reaction has proven to be an elaborate multistep reaction that is yet to be fully understood.

The RecA protein (37,842 daltons) is remarkably multifunctional. It is a DNA-dependent (ssDNA or dsDNA) ATPase; it is a ssDNA- and ATP-dependent protease that displays high specificity for several *E. coli* and phage proteins; it catalyzes the renaturation of ssDNA; and it promotes the homologous pairing and exchange of DNA strands. As a consequence of its central role in genetic recombination, SOS induction, and mutagenesis, and because of the diversity and mechanistic complexity of the reactions catalyzed, RecA protein has been the subject of much scrutiny and of many review articles. These review articles have emphasized the role of RecA protein in either genetic recombination (14, 91, 106, 107) or SOS induction (64, 114), the biochemistry of the DNA strand exchange reaction (19, 50, 92, 93, 117), evolutionary significance (80), and the structure of RecA protein and its complexes with DNA (25, 36, 108). Rather than duplicate the content of other reviews, and to provide a different perspective, this review focuses on both biophysical and mechanistic aspects of DNA strand exchange, with a particular emphasis on energetic requirements.

The RecA Protein–Promoted DNA Strand Exchange Reactions

The homologous pairing and exchange of DNA strands can be studied in vitro by using the DNA substrate pairs depicted in Figure 1. The substrate pair in Figure 1*A* demonstrates the formation of a classic *D-loop*, or displacement loop, in a reaction called joint molecule formation. For this pair, the extent of DNA strand exchange is limited because of topological constraints imposed by the supercoiled dsDNA, unless a topoisomerase is present. The substrates in Figure 1*B* have no topological restriction, permitting complete exchange and separation of DNA strands. The final pair (1*C*) are similar to those in 1*B* except that, after initiation in the single-stranded gapped region, strand exchange occurs between two duplex DNA molecules (i.e. symmetric four-stranded DNA exchange); such substrates may be more representative of physiological substrates (117). Though the biochemical behavior of each pair of substrates may differ somewhat in detail, they share a number of common requirements: (*a*) one of the DNA substrates is single-stranded or possesses a region of ssDNA, (*b*) the ssDNA is complementary to a region of the dsDNA, and (*c*) one of the DNA substrates is linear.

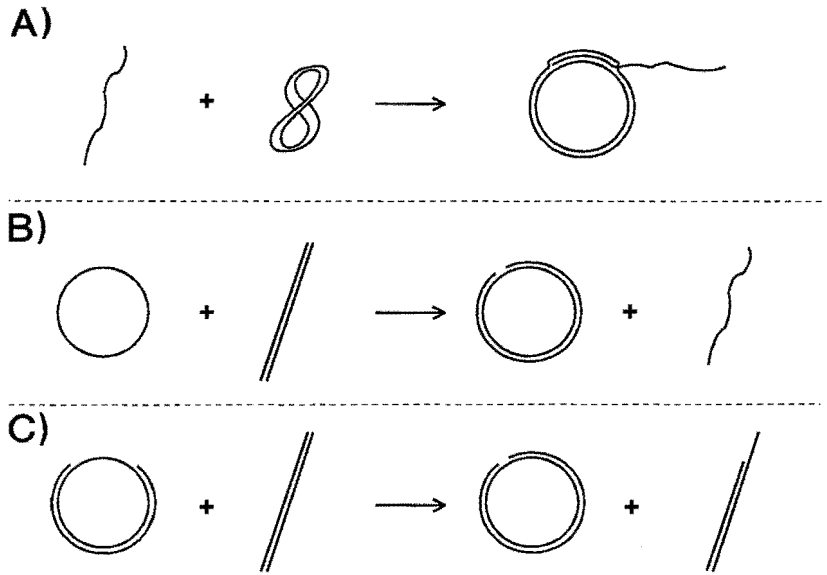

Figure 1 Typical DNA substrates used for in vitro homologous pairing and strand exchange reactions.

The DNA strand exchange reaction involving the substrates depicted in Figure 1*B* has been studied extensively because both reaction intermediates (joint molecules) and products (nicked or gapped dsDNA and linear ssDNA) can be readily detected using an agarose gel assay. The pairing and complete exchange of DNA strands occur in a series of kinetically definable steps (Figure 2). The first, referred to as *presynapsis*, requires the formation of a stoichiometric complex of RecA protein and ssDNA (1 RecA protein monomer per ~3–4 nucleotide residues); formation of this presynaptic complex is facilitated by the presence of a single-stranded DNA binding protein [e.g. *E. coli* single-stranded DNA binding (SSB) protein or bacteriophage T4 gene 32 protein]. The presynaptic step is followed by synapsis of the RecA protein–ssDNA complex with dsDNA; the first contacts are necessarily nonhomologous in nature, resulting in the formation of nonhomologously paired complexes known as *coaggregates*. This step is followed by homologous alignment and the formation of joint molecules. Joint molecules can be either *paranemic*, i.e. the incoming ssDNA is not topologically intertwined with its complement in the dsDNA, or *plectonemic*, i.e. the incoming ssDNA winds freely around its complement in the dsDNA. A paranemic joint forms when pairing occurs at an internal dsDNA site, whereas a plectonemic joint forms when pairing

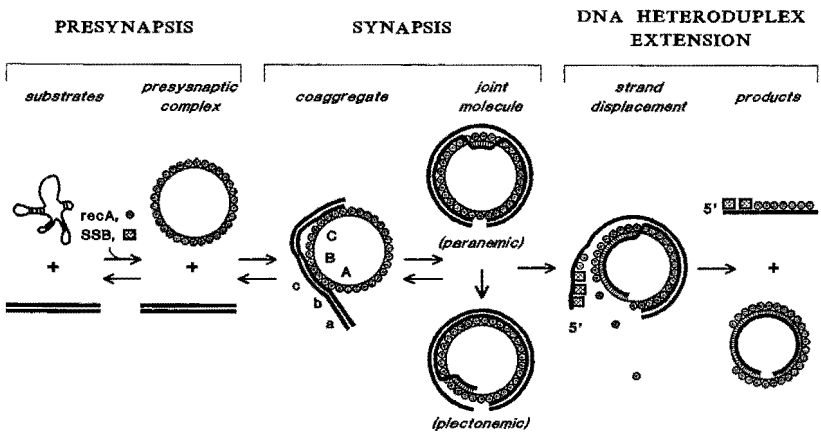

Figure 2 Illustration of the DNA strand exchange reaction promoted by RecA protein. Shown is the reaction between circular ssDNA and homologous dsDNA in the presence of SSB protein. Upon complete exchange of DNA strands (and deproteinization), the products formed are linear ssDNA and nicked (or gapped) circular dsDNA.

occurs at a dsDNA end. The final step is RecA protein–mediated branch migration, which is the unidirectional extension (in a 5′ to 3′ direction relative to the displaced strand) of the heteroduplex region in the joint molecule. Though they differ in detail, these kinetic steps also apply to the other reactions displayed in Figure 1.

Why is RecA Protein an ATPase?

The RecA protein–promoted reactions summarized in Figure 1 require the presence of ATP (except as noted below) and, normally, ATP hydrolysis coincides with the pairing and exchange of DNA strands. Consequently, researchers assumed that ATP hydrolysis is essential for DNA strand exchange. Upon formation of the presynaptic complex, RecA protein–dependent ATP hydrolysis occurs at a rate of $\sim$25–30 min^{-1} (3, 55, 78). However, this hydrolysis occurs in the absence of dsDNA (i.e. in the absence of pairing and strand exchange), demonstrating that ATP hydrolysis is not obligatorily coupled to DNA strand exchange. Early measurements of the total amount of ATP hydrolyzed relative to the number of base pairs exchanged demonstrated that, energetically, DNA strand exchange appeared to be quite inefficient. Upwards of 1000 ATP molecules can be hydrolyzed per base pair exchanged (18). However, owing to improved reaction conditions, this value decreases to $\sim$100 [if SSB protein is added (22)], $\sim$16 [if ADP is added (22)], and to $\sim$1 [if only dsDNA-

dependent activity is considered (99)]. Each of these calculations was logical, but each depended on an assumption that was naive.

The simplistic nature of these estimates was demonstrated by studies utilizing the essentially nonhydrolyzable ATP analogue, ATP-γ-S [adenosine 5′-*O*-(3-thiotriphosphate)]. Joint molecule formation can occur in the presence of ATP-γ-S (17, 39, 72, 98). In fact, up to 3.4 kilobase pairs (kb) of heteroduplex DNA are formed with less than 0.003 molecules of ATP-γ-S hydrolyzed per base pair of DNA heteroduplex formed (72). This result implies that ATP hydrolysis is not essential for the exchange of DNA strands and obviously raises questions regarding the role of ATP hydrolysis in RecA protein–promoted DNA strand exchange reactions.

Prior to 1986, the suggestion that protein-promoted joint molecule formation and exchange of DNA strands could occur in the absence of ATP hydrolysis would have been perceived as iconoclastic. This view began to change with the isolation of homologous pairing proteins from eukaryotic organisms that not only promote joint molecule formation, but do so without the need for ATP (42, 46). Thus, exceptions to the RecA protein paradigm were discovered (see 29a). The observation that DNA strand exchange can occur with no net input of free energy does not violate any thermodynamic principles and should not be surprising because DNA strand exchange is isoenergetic, i.e. the free energy of the substrates and products shown in Figures 1*B* and 1*C* is essentially identical because the number of base pairs is conserved through the reaction. Since there is no thermodynamic need for the input of free energy in DNA strand exchange, ATP hydrolysis must relieve kinetic constraints in the reaction pathway rather than overcome thermodynamic limitations.

ATP Hydrolysis Permits Modulation Between Structures with Different DNA Affinities

In this review, I attempt to put into perspective several related issues: (*a*) how can RecA protein promote the exchange of DNA strands in the absence of ATP hydrolysis; (*b*) at which steps in the RecA protein–mediated reaction is ATP hydrolysis important; and (*c*) how can the class of ATP-independent homologous pairing proteins function in the complete absence of energy input?

The simple answer to the question of why RecA protein is an ATPase is that ATP hydrolysis permits alternation between functionally different conformations. Only ATP binding is essential for DNA strand exchange. The binding of ATP to RecA protein induces an allosteric transition to a conformation referred to as the *high-affinity DNA binding* state (73); this is the conformation of RecA protein that promotes DNA strand exchange. ATP hydrolysis is needed for dissociation of the RecA protein–DNA

complex. ATP hydrolysis accomplishes two tasks: (*a*) it destroys the effector molecule, ATP, and (*b*) it produces a new effector molecule, ADP. The bound ADP induces a RecA protein conformation that has the lowest affinity for DNA (*low-affinity DNA binding state*), thereby promoting dissociation. Thus, the crucial event in the DNA strand exchange step is ligand (ATP) binding, with the free energy derived from this binding.

Induction of the high-affinity state is essential for three molecular events that require the input of free energy: (*a*) displacement of SSB protein from ssDNA; (*b*) "opening" (unwinding) of dsDNA; and (*c*) pairing and stabilization of a three-stranded DNA intermediate. Induction of the low affinity state (via ATP hydrolysis) is important for: (*a*) redistribution of bound RecA protein molecules within the presynaptic complex; (*b*) dissociation from the DNA products; and (*c*) directionality.

Given this view for the roles of ATP binding and ATP hydrolysis in the RecA protein–promoted reaction, the behavior of the ATP-independent homologous pairing proteins can be readily rationalized. Those proteins exist permanently in a conformation equivalent to the high-affinity state of RecA protein, and dissociation from DNA is catalyzed by a different (perhaps ATP-dependent) protein. Below, each of the individual steps comprising the DNA strand–exchange reaction are discussed, with particular emphasis on how each step is affected by ATP binding or hydrolysis in an effort to determine the degree to which the premises listed above are consistent with experimental data.

DNA BINDING: MODULATION OF THE STRUCTURE AND AFFINITY OF THE RECA PROTEIN–DNA COMPLEX BY NUCLEOTIDE COFACTORS

Fundamental to its ability to pair homologous DNA, the RecA protein must bind both ssDNA and dsDNA. However, an unanticipated observation is that both the structure and the stability of these protein-DNA complexes are substantially affected by nucleotide cofactor binding. The properties of these complexes are reviewed in this section.

ssDNA Binding: Formation of the Presynaptic Complex

ATP AND ADP AFFECT THE STRUCTURE OF THE RECA PROTEIN–DNA COMPLEX The RecA protein–ssDNA complex can be visualized using electron microscopy (see 25, 36, 108 for reviews). The structure is distinctly filamentous, and its morphology depends on the nucleotide cofactor present (37). In the absence of any nucleotide cofactor or in the presence of ADP (37, 108), a compact helical structure is observed with a pitch ranging from

55–70 Å, a diameter of ~120 Å, and an internucleotide spacing of 1.8–2.1 Å, which corresponds to about 30 nucleotides per helical turn. In contrast, in the presence of either ATP or ATP-γ-S, a distinct extended structure appears. The helical repeat increases to 90–95 Å; the diameter of the filament is ~100 Å; the ssDNA is extended 1.5- to 1.6-fold relative to the length of dsDNA (~5 Å per nucleotide); and one helical turn contains ~6 molecules of RecA protein per 18 nucleotide residues.

Though slight differences in morphology result from the fixation procedures used for electron microscopy, solution measurements support the existence of two different structures. Small-angle neutron scattering studies detect a compact structure when either DNA or ATP is absent; the structure has a pitch of 70 Å, a cross-sectional radius of gyration of 40 Å, and ~5 RecA protein monomers per turn (26, 27). In conjunction with the electron microscopy data, the scattering data suggest that the compact structure may contain only one RecA protein monomer per six nucleotide residues, a value that is only half that for the extended structure (26). In the presence of ATP-γ-S, the dimensions change to a pitch of 95 Å, a radius of gyration of 33 Å, and about 6 RecA protein monomers per turn of 18 nucleotides. The extended structure requires the binding of both ATP and DNA, demonstrating that the ATP-dependent transition to the extended state does not occur in the DNA-free structure.

ATP AND ADP AFFECT THE EQUILIBRIUM BINDING PROPERTIES OF THE RECA PROTEIN–DNA COMPLEX A DNA binding assay useful for quantitative studies with RecA protein takes advantage of the fluorescent properties of M13 ssDNA that is modified with chloroacetaldehyde (referred to as etheno M13 DNA) (9, 73, 104, 105). Upon binding of RecA protein, the fluorescence of the etheno M13 DNA increases. Because modification of the cytidine and adenosine residues disrupts base pairing, binding parameters are not affected by DNA secondary structure.

Saturation of the etheno M13 DNA fluorescence enhancement occurs at an observed stoichiometry of six to seven nucleotide residues per RecA protein monomer (9, 73, 104, 105). This value is unaffected by the presence of ATP, ATP-γ-S, or ADP. The ratio of the fluorescence of the saturated RecA protein–etheno M13 DNA complex relative to the protein-free DNA (referred to as the relative fluorescence increase or RFI) depends on the nucleotide cofactor present (73, 104). Two characteristic complexes are formed: in the absence of nucleotide cofactor or in the presence of ADP, the RFI is low (1.5–2.2, depending on DNA preparation and conditions); in the presence of either ATP or ATP-γ-S, the RFI is higher (2.2–2.6). Interconversion between states occurs readily upon addition of ATP or by allowing ATP hydrolysis to produce ADP (73). Because etheno M13 DNA

fluorescence increases upon nucleotide base unstacking (61) (as would occur upon extension of the polynucleotide backbone), these observations suggest that the observed fluorescence changes correspond to the different structures seen using electron microscopy and neutron scattering. All methods detect either a compact or an extended structure that depends on the nucleotide cofactor present.

In addition to the conformational effects, nucleotide cofactors affect the stability of RecA protein–ssDNA complexes substantially (73). This effect can be quantified by measuring the salt concentration required to dissociate one-half of the RecA protein–DNA complex [referred to as the salt titration midpoint or STMP (57)]. For example, the STMP of the unliganded complex is ~260 mM NaCl. The addition of 0.5 mM ATP increases the STMP to at least 450 mM NaCl; 0.5 mM ATP-γ-S results in a complex that cannot be dissociated by up to 2.5 M NaCl; and 0.5 mM ADP decreases the STMP to 160 mM NaCl. These changes are ligand binding–specific because they display ligand concentration dependence that is hyperbolic for ADP (73) or sigmoidal for ATP (78). Because of hydrolysis, measurements in the presence of ATP necessarily reflect the steady-state behavior of the various species (ATP-, ADP-, and nucleotide-free) present and are not equilibrium measurements. ATP hydrolysis is reduced by 96% upon substituting Ca^{2+} for Mg^{2+} and yet the relative stabilities described above are retained (78). Thus, relative to the unliganded complex, ATP or ATP-γ-S increase whereas ADP decreases the stability of the RecA protein–ssDNA complex.

Direct measurement of equilibrium binding parameters confirm the salt titration experiments. The equilibrium binding constant determined at 200 mM NaCl for the binding of nucleotide-free RecA protein to M13 etheno DNA is more than 20-fold greater than the binding constant for the ADP-bound form; because of differences in salt sensitivity of the binding constant for these two different complexes, the difference in affinity is expected to be substantially greater at lower NaCl concentrations (73, 78). Both in the presence and in the absence of ADP, the binding to ssDNA is cooperative. The cooperativity parameter, ω, for monomer-monomer interaction is ~50 at 25°C and ~125 at 37°C. However, analysis of binding parameters for RecA protein is complicated by self-association. Under most conditions, RecA protein exists as a heterogeneous indefinite aggregate (4, 121). Attempts to account for this self-association assume that the binding species is a multimer (111). This assumption naturally leads to a higher estimate for ω because the assumed binding species has a larger site size [e.g. ω increases to 1500 if the cooperative binding species is a 30-mer (111); however, this value reflects cooperativity between 30-mers and not between monomers (110)]. Unfortunately, which species

(i.e. monomer, dimer, etc) binds to the DNA is unknown; aggregates themselves might not bind DNA but rather may dissociate to monomers before binding. The parameters derived for monomer binding, though perhaps simplistic, at least do not introduce additional untested assumptions.

A TWO-STATE MODEL FOR DNA BINDING The binding data taken together with the structural data suggest (at least) two distinct DNA binding states for RecA protein: (*a*) a high-affinity state induced either by ATP or ATP-γ-S and characterized by the extended physical structure, the high RFI value, and the high stability of the RecA protein–ssDNA complex and (*b*) a low-affinity state induced by ADP and characterized by the compact physical structure, the lower RFI, and low stability (26, 28, 37, 73). The nucleotide-free protein may represent a third state but its characteristics are typical of the low-affinity state. During ATP hydrolysis, the RecA protein–ssDNA complex potentially passes through each of the different conformations (Figure 3). Upon binding ATP and ssDNA, the high-affinity state is induced. After ATP hydrolysis, the ADP-bound form of the protein is produced; this is most likely the form of the protein that

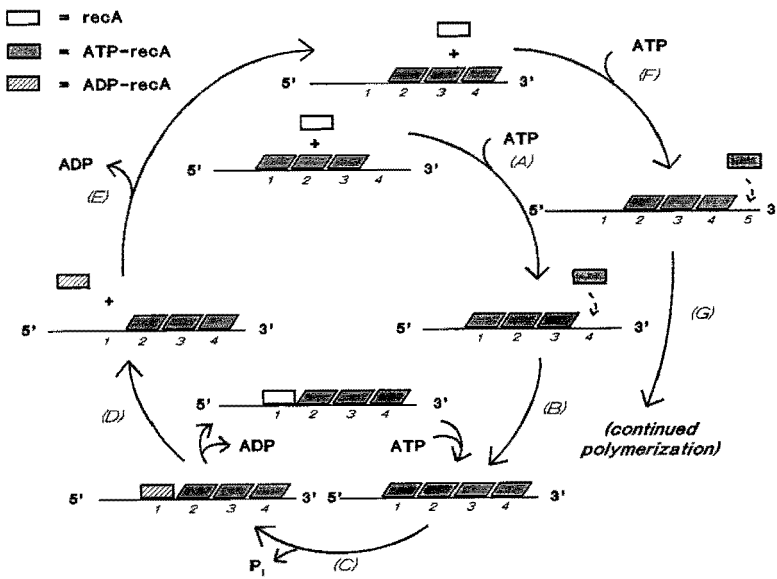

Figure 3 ATP hydrolytic cycle for RecA protein association with ssDNA. Association and dissociation are depicted as polar (5′ to 3′), resulting in net translocation of the protein filament. The inner cycle at step (*C*) represents the processive ATP hydrolysis pathway that occurs at high ATP concentrations; under these conditions, net polymerization ensues.

dissociates from the ssDNA (Figure 3, outside pathway). Alternatively, ADP can dissociate from the DNA-bound RecA protein, and RecA protein can rebind another ATP molecule without dissociating from the DNA (74, 75). This latter possibility is referred to as the processive pathway and is discussed below. Regardless of the specific pathway followed, ATP hydrolysis serves two essential functions: (*a*) destruction of the effector molecule responsible for induction of the high-affinity state and (*b*) creation of the effector ligand responsible for induction of the low affinity state. Thus, in this simple scenario, ATP hydrolysis is used for no other purpose than protein dissociation. This function, however, is not a trivial one because the requirements of DNA substrate binding (i.e. high affinity) and of rapid dissociation (i.e. low affinity) are antagonistic. ATP hydrolysis solves what was referred to as the "tight binding dilemma" for a protein that is expected to bind tightly to its DNA substrate yet be able to dissociate and subsequently act on a different DNA molecule (50). In this simple model, ATP hydrolysis is not involved in any relative movement (e.g. filament movement, translocation of protein along DNA, etc).

KINETIC PROPERTIES OF RECA PROTEIN–DNA COMPLEXES The equilibrium experiments predict that the kinetic lifetime of the RecA protein–ssDNA complexes follows the order: ATP-γ-S complex > ATP complex > unliganded complex > ADP complex. Kinetic studies measuring the rate at which RecA protein transfers from one DNA molecule to another confirm this expectation (7, 69, 74, 75, 85). The measured kinetic parameters are sensitive to experimental conditions, but, to provide a sense of the lifetimes of the various RecA protein–ssDNA complexes, some representative halflives are given (74, 75). In the presence of ATP-γ-S, nearly 70% of the RecA protein fails to transfer in 24 hours. In the absence of nucleotide cofactor, two kinetic components are observed with lifetimes of 7 seconds and 1.75 minutes, respectively. In the presence of 250-μM ADP, the lifetimes decrease to less than 2.5 seconds and 30 seconds, respectively. Finally, in the presence of ATP, the results are more complex. At low ATP concentrations (below 50 μM), RecA protein transfers rapidly, behaving kinetically like an average of the unliganded and ADP-bound species. At higher ATP concentrations (e.g. 250 μM), the fast lifetime is 16 seconds and the slow lifetime is 3.9 minutes. The kinetic behavior in the presence of ATP can be readily understood in the context of Figure 3. At low ATP concentrations, the high-affinity state is not induced (75), and the low-affinity form of RecA protein dissociates rapidly from the DNA without ATP hydrolysis, or the ADP-bound species dissociates after one ATP hydrolytic event (outer pathway). At high concentrations of ATP, ATP rebinds before dissociation of RecA protein from the ssDNA (inner pathway).

The kinetic behavior in the presence of ATP suggests that ATP hydrolysis is processive; i.e. a ssDNA-bound RecA protein can hydrolyze more than one molecule of ATP before dissociating from the DNA (75, 85). At high ATP concentrations, up to 50 molecules of ATP may be hydrolyzed before a transfer event occurs, demonstrating that ATP hydrolysis is not obligatorily coupled to dissociation (75). Other studies (S. C. Kowalczykowski & R. A. Krupp, unpublished observations) suggest that the transfer reaction overestimates the processivity by approximately fivefold. A similar study using circular ssDNA, in the presence of SSB protein, yielded an estimate of ~ 200 for the number of ATP molecules hydrolyzed during exchange (85). Without detailed kinetic studies, a mechanistic explanation for this processivity is unavailable, but cooperative interactions between RecA protein monomers are certainly a factor. Because the structure of RecA protein–ssDNA filaments in the presence of ATP is remarkably homogeneous, structural constraints imposed by neighboring proteins may prevent a monomer from adopting the ADP-bound conformation, thereby restricting dissociation from the DNA and permitting ATP rebinding. Evidence for such cooperative interactions comes from several experiments, including those on the cooperative nature of ADP inhibitory effects (22, 44, 78), the activation of RecA protein GTPase activity by ATP (79) and ATPase activity by ATP-γ-S (60a), and the ability of wild-type RecA protein to confer increased stability to mutant RecA proteins in mixed protein filaments (56). Thus, though referring to the properties of a RecA protein "monomer" is convenient, the functional form is a filament and cooperative interactions certainly play a prominent role in the behavior of each subunit.

A consequence of processive ATP hydrolysis by RecA protein is that hydrolysis of ATP can occur repeatedly without performing any net DNA strand exchange. Almost certainly, RecA protein can hydrolyze ATP both before and after the point of DNA strand exchange (Figure 2), which means that any estimate of the number of ATP molecules hydrolyzed per base pair of DNA exchanged using total amount of hydrolyzed ATP is likely to overestimate the actual utilization during DNA strand exchange.

BINDING TO ssDNA IS A POLAR POLYMERIZATION PROCESS A significant discovery regarding the association of RecA protein with ssDNA is that the binding is polar, occurring in a 5′ to 3′ direction (96). Because of the random nature of nucleation, 3′ termini are 10-fold more likely to be covered with RecA protein than 5′ termini. The polarity of RecA protein assembly on ssDNA implies that polarity in disassembly also occurs in a 5′ to 3′ direction but initiates at the opposite end of the filament (Figure 3). This conclusion follows, in part, because the cooperativity of binding

predicts that dissociation of a RecA protein monomer from the interior of a filament is 125-fold less likely than from an end of a filament. Recent experiments are consistent with a polar dissociation mechanism (62, 63). Thus, not only does RecA protein form a distinct filamentous structure when bound to ssDNA, it also associates and dissociates with a definite polarity. Both of these properties, together with NTP hydrolysis, are characteristic of other self-associating, energy transducing proteins such as actin and tubulin. This observation raises the question of whether RecA protein–promoted DNA strand exchange involves a treadmilling mechanism (20). The original treadmilling mechanism proposed that only one hydrolytic event occurs per polymerized protomer (32), which is inconsistent with processive ATP hydrolysis by RecA protein. Also inconsistent with the treadmilling model is the observation that the apparent rate of ATP hydrolysis is unaffected by the number of RecA protein "ends" [obtained by varying either the length of the RecA protein–DNA complexes (3) or the fractional saturation of DNA (55)]. However, modification of the treadmilling model to permit multiple rounds of ATP hydrolysis uncoupled from dissociation is a tenable alternative.

EFFECT OF OTHER NUCLEOSIDE TRIPHOSPHATES AND DIPHOSPHATES ON THE TRANSITION BETWEEN LOW-AFFINITY AND HIGH-AFFINITY STATES RecA protein can hydrolyze most of the other NTPs in a DNA-dependent manner (69, 71, 78, 79). Yet it promotes DNA strand exchange only in the presence of ATP or dATP, demonstrating that hydrolysis of a NTP is insufficient for DNA strand exchange. Also, the lambda repressor cleavage reaction displays a similar heirarchy of effectiveness for various NTPs (87, 116). At one level, an explanation for this heirarchy can be found in the effect that each NTP has on the affinity of RecA protein for ssDNA. Of all the NTPs, only ATP and dATP induce formation of the high-affinity state with the characteristic high STMP and high RFI (78). In constrast, GTP, dGTP, and TTP induce formation of the low-affinity state, and UTP and CTP only partially induce the high-affinity state: the STMP increases but the RFI increases only partially (78). These results imply that the high-affinity state is essential for DNA strand exchange but not for NTP hydrolysis. Finally, dCTP induces the high-affinity state but at a 10-fold higher concentration than required with ATP. This nucleotide cofactor may support DNA strand exchange at nucleotide concentrations 10-fold higher than typically used, but this possibility has not been tested. Two nonhydrolyzable ATP analogs that do not support joint molecule formation (70) also fail to induce the high-affinity state (7, 73). AMP-P-C-P binding results in the formation of a low-affinity state as judged by STMP and RFI measurements (73); thus it behaves like ADP. AMP-P-N-P bind-

ing yields a complex that has a longer lifetime (7) and higher STMP, but the RFI is low as with ADP (73). Thus, the failure of a NTP to induce the high-affinity state correlates with the absence of RecA protein–promoted DNA strand exchange and repressor cleavage (Figure 4).

All of the nucleoside diphosphates induce the low-affinity state of RecA protein; however, quantitative differences are substantial (78). For example, dGDP and TDP lower the affinity of RecA protein–etheno M13 DNA the most and dADP the least. In addition, the dADP–RecA protein–etheno M13 DNA complex has a RFI (2.2) more typical of a high-affinity complex than of a low-affinity complex, suggesting that the structure is somewhat extended and is more stable than that induced by the other NDPs. If the dADP–RecA protein–DNA complex is both longer lived and more like the extended (functional) structure, then dATP might be a better cofactor for certain RecA protein–dependent activities (78). RecA protein indeed is more proficient in SSB protein displacement and repressor cleavage in the presence of dATP (67, 76).

RELATIONSHIP BETWEEN ATP BINDING, ATP HYDROLYSIS, INDUCTION OF THE HIGH-AFFINITY STATE, AND DNA STRAND EXCHANGE The binding affinity of the adenine-containing nucleotides parallels their effectiveness in inducing the high-affinity state of RecA protein: ATP-γ-S > dATP > ATP > AMP-P-N-P ≫ AMP-P-C-P (49). The remaining NTPs show a

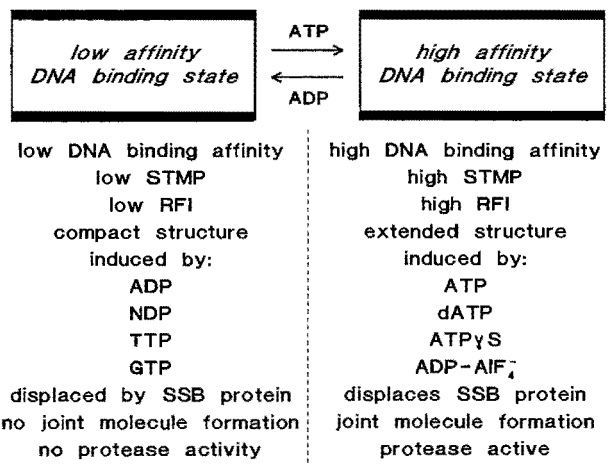

Figure 4 Biochemical properties of the low- and high-affinity states of the RecA protein–ssDNA complex. Representative nucleotide cofactors responsible for induction of their respective states are indicated.

similar parallel (16, 49, 73, 78). This hierarchy implies that a quantitative relationship exists between NTP binding affinity, induction of the high-affinity state, and RecA protein strand exchange activity. These effects can be understood in the context of a model in which the free energy of nucleotide binding is linked to both DNA binding affinity and to structural transitions (78) (Figure 5). The hierarchy follows from the fact that the total free energy derived from nucleotide binding is a function of both the binding constant and the free nucleotide concentration; thus, higher

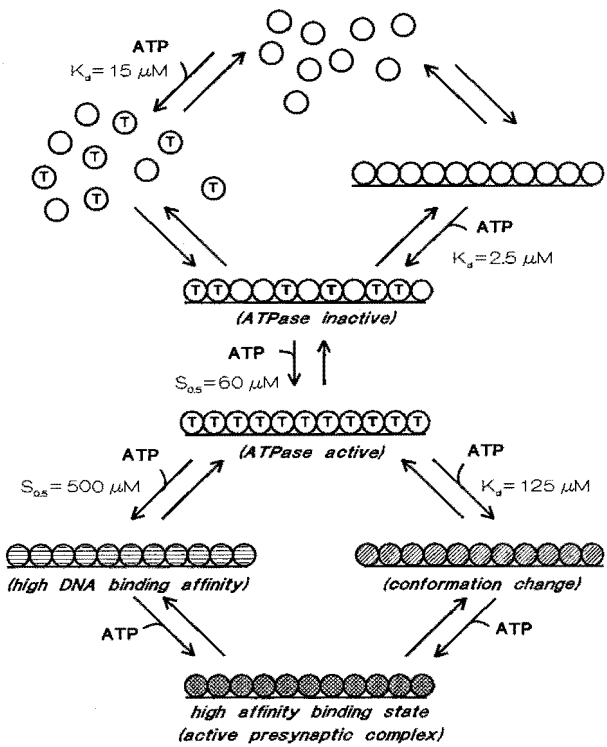

Figure 5 ATP concentration–dependent changes of RecA protein–dependent behavior. *Open circles* represent nucleotide-free RecA protein; circles labeled *T* represent the ATP–RecA protein complex; and *shaded circles* represent different conformations of the ATP–RecA protein complex. Each step represents apparent equilibrium or steady-state properties. Apparent dissociation constants (K_d) are given for events that display a hyperbolic dependence on ATP concentration. For properties that display a sigmoid dependence on ATP concentration, the ATP concentration at the midpoint of the transition ($S_{0.5}$) is given. Though the numerical values are representative, they depend on reaction conditions, DNA, and nucleotide cofactor.

concentrations of NTP are required for a NTP that binds with less affinity. Figure 5 illustrates these relationships and transitions using ATP as the ligand. The binding of ATP can occur either to free protein ($K_d \sim 15$ μM) or to RecA protein–ssDNA complex ($K_d \sim 2.5$ μM) in a thermodynamically linked cycle (49); ADP has a similar cycle except that the affinity for ADP decreases by at least threefold in the presence of ssDNA (16). ATP hydrolysis, however, shows a sigmoid dependence on ATP concentration ($S_{0.5} \sim 60$ μM) and is hardly detectable until the concentration is at least 25-μM ATP (75). Upon increasing the ATP concentration further, the stability (78) and structure (75, 109) of the RecA protein–DNA complex changes. The transition to a conformation that has a higher affinity for ssDNA is sigmoid, requiring at least 100-μM ATP, with a progressively more stable complex forming at higher ATP concentrations; below 100 μM, ATP decreases the stability of the complex even though little or no ATP hydrolysis occurs (78). The RFI increases hyperbolically with increasing ATP concentration (apparent $K_d \sim 125$ μM) to the value observed with the fully extended structure. These changes must involve independent structural changes because their dependence on ATP concentration differs and because certain NTPs and mutant proteins (see below) can display one change but not the other. Apparently all of these transitions are required (though are not necessarily sufficient; see below) for active presynaptic complex formation.

The ligand-induced transitions depicted in Figure 5 provide a semiquantitative explanation for most nucleotide dependent effects observed. For example, because UTP and dCTP bind to RecA protein with reduced affinities compared to ATP, higher concentrations are required for both NTPase activation (350 μM and 660 μM, respectively) and the structural changes (>9 mM for both NTPs). However, some uncertainties remain. For example, why low concentrations of ATP decrease the stability of the RecA protein–ssDNA complex is not clear. Also, why must more than 90% of the RecA protein–DNA complex be saturated with ATP for significant ATP hydrolysis to occur? One hypothesis is that ATP hydrolysis requires a cluster of as many as 15 contiguous ATP–RecA protein molecules bound to ssDNA; interruption of this cluster by an unliganded RecA protein molecule disrupts ATPase activation (49). The need for 15 or more contiguous RecA protein monomers may reflect the cooperative formation of a nucleus involving two to three turns of the RecA protein–DNA filament (112). An alternative explanation suggests that although RecA protein possesses only one NTP binding site per monomer, each RecA protein protomer has two types of NTP binding sites. This hypothesis implies that the functional unit is a dimer or larger in which one NTP site acts formally as an allosteric effector site whereas the other is the

catalytic site (79). Both models present attractive explanations for the cooperative activation of ATPase activity. The former model emphasizes the filamentous nature of the RecA protein–DNA complex but may conflict with data that demonstrate that oligonucleotides as short as 25 nucleotides can activate ATPase activity [unless one RecA protein filament can bind multiple oligonucleotides (97)], although a 50-mer is 50-fold more effective (3). The latter model requires that the RecA protein active unit be at least functionally a heterodimer. Growing evidence supports this requirement (see below).

THE PRESYNAPTIC FILAMENT IS FUNCTIONALLY ASYMMETRIC A curious feature of the ATP–RecA protein–ssDNA complex is that the observed binding stoichiometry depends on the assay used. When the fluorescence change accompanying etheno M13 DNA binding is monitored, the apparent binding stoichiometry is six to seven nucleotides per RecA protein, but, if ATPase activity is measured, the observed stoichiometry is three to four (76). This apparent discrepancy can be explained by the observation that the fluorescent signal originates from the DNA, whereas the ATP hydrolysis originates from the protein. However, this explanation implies that the RecA protein–ssDNA complex exists in two different stoichiometric complexes. Consistent with this view, two distinct binding stoichiometries for RecA protein–DNA complexes were first noted using nuclease protection assays; in the presence of ATP-γ-S or AMP-P-N-P, protection of eight nucleotides per monomer occurs, but decreases to four in the absence of nucleotides or in the presence of ATP (7). ATPase assays yield apparent binding stoichiometries of either three or six, depending on conditions (76). Flow linear dichroism studies with ATP-γ-S complexes of RecA protein provide spectroscopic evidence for two different stoichiometric complexes that saturate at either three or six nucleotides per RecA protein (110). Finally, centrifugation of RecA protein-poly(dT) complexes demonstrates the sedimentation of complexes containing either three nucleotides per RecA protein in the presence or absence of ADP, or six nucleotides per RecA protein in the presence of ATP (123). To further complicate the picture, complexes of RecA protein and ssDNA formed at a 3:1 stoichiometry (nucleotides: RecA protein) display subunit nonequivalence with regard to inhibition of ATPase activity by ATP-γ-S (i.e. there are two populations of RecA protein with different NTP binding affinities) (58).

Figure 6 presents several models that can account for most of the observations (58). Models A and C propose that one RecA protein monomer can bind six nucleotides of ssDNA but that a second protein can be incorporated into the complex; model B proposes that RecA protein has

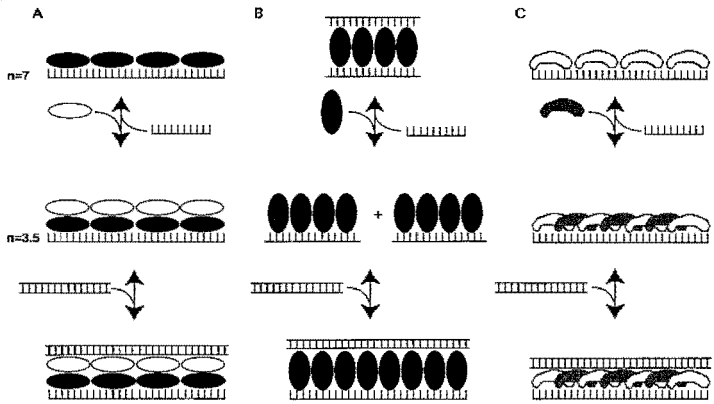

Figure 6 Three models that accommodate the existence of different stoichiometric forms of the RecA protein–ssDNA complex. For models *A* and *C*, asymmetry is intrinsic to the model. For all models, the 3:1 (nucleotides:RecA protein monomer) complex can bind homologous dsDNA and is the active form in DNA strand exchange.

an intrinsic site size of three nucleotides, but can bind two strands of DNA (7, 110). In model A, protein binding to ssDNA saturates at a stoichiometry of 6:1. A second layer of protein can associate with the first protein layer and is activated for ATPase activity without contacting the DNA; hence, apparent stoichiometries (for DNA binding versus ATPase activity) differing by a factor of two are observed. In model B, RecA protein has two binding sites that can each accommodate three nucleotides of DNA. ATPase activity is saturated when only one site is filled and, hence, saturates at three nucleotides per RecA protein, although ssDNA binding saturates at six nucleotide per RecA protein. In model C, the binding of one monomer per six nucleotides is sufficient to cause the structural change detected in the fluorescence studies, but the binding of a second monomer between the first monomers (without further change in DNA fluorescence) can be accommodated, resulting in additional ATP hydrolysis activation. For each model, the 3:1 complex represents the functional presynaptic complex that additionally can bind dsDNA. An important facet of models A and C is the nonequivalence of the RecA protein monomers intrinsic to the 3:1 complexes; one-half of the protein monomers make contacts with the ssDNA different from the remaining half. Though entirely unequivocal data in favor of any one model are unavailable [and more complex variants invoking negative cooperativity are possible (58)], the subunit nonequivalence results require models for the RecA protein presynaptic complex that are not only consistent with biochemical and structural data, but

that also incorporate subunit nonequivalence into the 3 : 1 complexes (e.g. model C or model B modified to include negative cooperativity).

dsDNA Binding: Distortion of the Duplex DNA Structure

Although the structures of RecA protein–dsDNA complexes have been extensively investigated using electron microscopy (see 25, 36, 37, 108), quantitative equilibrium and kinetic binding data are sparse relative to the data for complexes with ssDNA. Nevertheless, many parallels exist and the principal characteristics of the two-state allosteric model likely apply to RecA protein–dsDNA complexes (37). In the presence of ATP or ATP-γ-S, the structure of the RecA protein–dsDNA complex is similar to that with ssDNA. The helical RecA protein–dsDNA filament is 50% longer than B-form DNA and the structure has the characteristic 95-Å pitch. The DNA has 18.6 bp per turn and 6.2 RecA protein monomers, resulting in a stoichiometry of 3 bp per RecA protein (equivalent to the 3 nucleotides per monomer for ssDNA). The RecA protein–dsDNA is highly unwound, possessing an average rotation per bp that is only $\sim$60% that of B-form DNA (108). Nucleotide cofactors elicit the same effects on RecA protein–dsDNA stability as they do for complexes with ssDNA: ATP-γ-S binding results in a complex most resistant to dissociation by salt; ADP binding results in a complex least resistant; and an intermediate stability is observed in the absence of cofactor (1, 71).

The most notable difference between RecA protein complexes with dsDNA versus ssDNA is their kinetic behavior in ATPase assays. With ssDNA, the steady-state rate of ATP hydrolysis is achieved within a few minutes. In contrast, with dsDNA, a distinct lag phase lasting up to several hours can precede steady-state hydrolysis (53, 76, 88, 89, 99). The duration of this lag phase is increased by factors that contribute to dsDNA stability, such as increased Mg^{2+} and NaCl concentration, decreased A-T content, and decreased temperature (53, 89). The lag cannot be eliminated by incubation of RecA protein with dsDNA in the absence of ATP, demonstrating the need for ATP binding (53). Also, the lag decreases with increasing ATP concentration (53) and is shorter for an equivalent concentration of dATP (76), suggesting that the high-affinity state is required to overcome this kinetic barrier. Finally, the lag phase is decreased substantially at pH 6.2 (71, 88, 89), by regions of ssDNA attached to the dsDNA (102, 120), or by exogenous homologous ssDNA (W. M. Rehrauer & S. C. Kowalczykowski, unpublished observations).

The nucleation and growth model shown in Figure 7 accommodates these observations (53, 88). The lag phase corresponds to a rate-limiting nucleation of RecA protein onto the dsDNA. Formation of the stable nucleus is associated with a structural deformation of the dsDNA referred

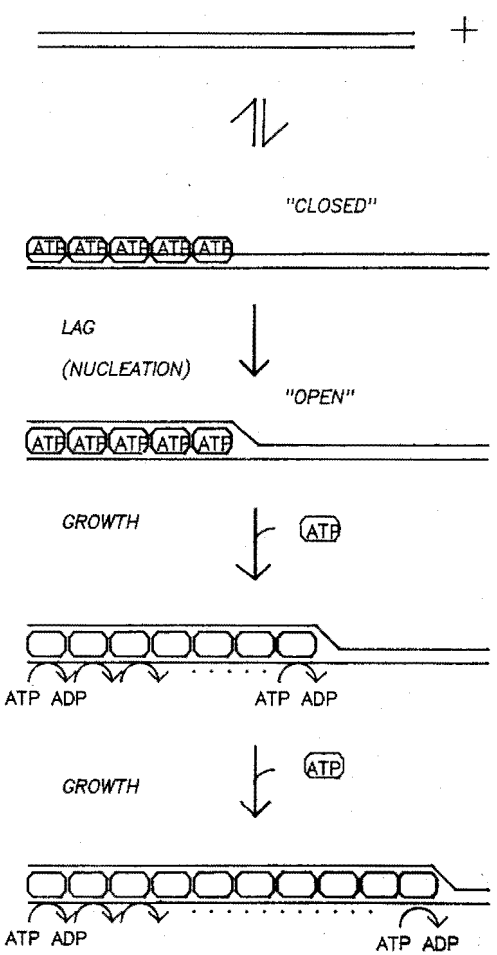

Figure 7 Model for the dsDNA-dependent ATPase activity of RecA protein. In the "open" form, the dsDNA is extensively unwound. Reprinted with permission from Reference 53.

to as opening (53) and results from unwinding of the dsDNA (71, 90). Nucleation is facilitated by factors that either destabilize the dsDNA or that enhance a property of RecA protein, most likely the rate of association with DNA (59). After this rate-limiting nucleation step, a rapid growth phase ensues, resulting in steady-state ATP hydrolysis nearly equivalent to the rate obtained with ssDNA. ATP hydrolysis is a consequence of this DNA structural deformation and not vice versa, consistent with the

interpretation that the free energy derived from binding of the ATP–RecA protein to dsDNA is responsible for the thermodynamically unfavorable unwinding. Because unwinding of the dsDNA is required at the time of homologous pairing, the high-affinity state probably promotes this energy-requiring DNA unwinding step. Consistent with this view, mutant RecA proteins (see below) defective in induction of the high-affinity state are also defective in both dsDNA-dependent ATPase activity (but not necessarily ssDNA-dependent ATPase activity) and homologous pairing activity.

THE ROLE OF SSB PROTEIN IN DNA STRAND EXCHANGE

Since the finding that *E. coli* SSB protein stimulated the DNA strand exchange activity of RecA protein, considerable discussion (19, 36, 50, 92, 93, 117) has focused upon the precise mechanism by which this stimulation occurs.

A Competition Model for SSB Protein Function

Studies using the three-stranded DNA strand exchange reaction suggested that SSB protein stabilized the RecA protein–ssDNA presynaptic complex via protein-protein interactions (23). However, SSB protein is not needed for the four-stranded exchange reaction (119). Other studies pointed to a role for SSB protein that involved the disruption of ssDNA secondary structure, which impeded the binding of RecA protein (55, 84, 113). The binding of RecA and SSB proteins was found to be competitive, and no stabilization of the RecA protein–ssDNA complex was detected by direct equilibrium or kinetic experiments (54). Under typical DNA strand exchange conditions, RecA protein displaced SSB protein from ssDNA (54, 55, 112). The most graphic evidence came from electron microscopy, which clearly demonstrated a time-dependent displacement of SSB protein from ssDNA by RecA protein (112). Finally, several studies demonstrated that other heterologous single-stranded DNA binding proteins [e.g. bacteriophage T4 gene 32 protein (55, 103), various plasmid encoded SSB proteins (30), bacteriophage N4 SSB protein (S. C. Kowalczykowski & R. A. Krupp, unpublished observations), and a *Saccharomyces cerevisiae* SSB protein (38)] could substitute for *E. coli* SSB protein, making the likelihood of specific protein-protein interactions remote.

From these studies, the following mechanism for SSB protein function has emerged (Figure 8). Regions of ssDNA secondary structure prevent the complete saturation of the ssDNA by RecA protein (55, 84, 113); SSB protein binds to these regions and, by virtue of its preferential affinity for

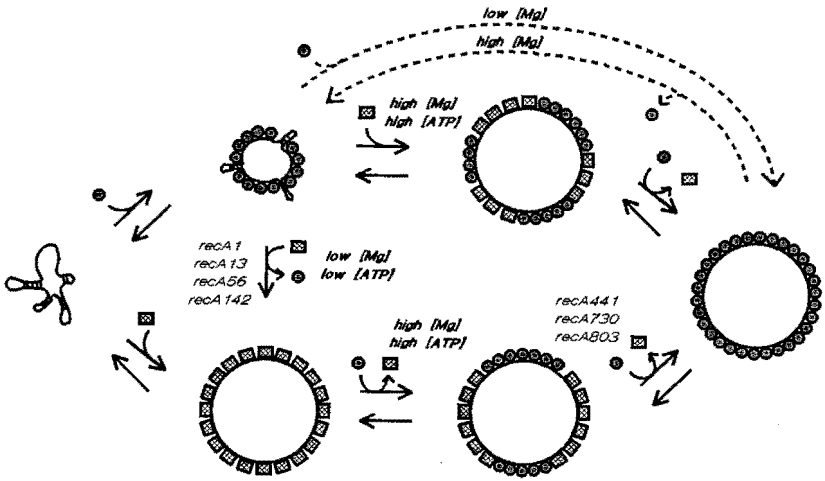

Figure 8 Competition model for the binding of RecA and SSB proteins to ssDNA. Shown are the pathways if RecA protein (*circles*) is bound to ssDNA first (*upper*) or if SSB protein (*squares*) is bound to ssDNA first (*lower*). The effect of magnesium ion concentration (in the absence of SSB protein) is depicted by the *dashed lines*. The behavior of mutant RecA proteins is indicated. In all cases, the effect of ADP is antagonistic to that of ATP.

ssDNA (see 51), destabilizes the secondary structure. RecA protein then displaces the SSB protein, allowing formation of a saturated RecA protein–ssDNA presynaptic complex. Upon RecA protein dissociation, DNA secondary structure reforms, requiring the repeated action of SSB protein (55, 82) and explaining the requirement for near-saturation amounts of SSB protein; if SSB protein action were required only once to initiate RecA protein binding at each site of secondary structure, then catalytic amounts of SSB protein would suffice. Figure 8 also shows that the need for SSB protein can be obviated by formation of RecA protein–ssDNA complexes under conditions that destabilize DNA secondary structure [e.g. low Mg^{2+} concentrations (55, 81a, 82, 84, 113)]. Such complexes can promote joint molecule formation after a shift to appropriate conditions (84, 113). However, because of RecA protein dissociation and reformation of secondary structure, these complexes decay to the type of complex formed at high Mg^{2+} concentration in the absence of SSB protein (55, 81a, 82, 84). From equilibrium considerations, RecA protein should melt DNA secondary structure if it can displace SSB protein that has melted and bound to these regions. Thus, there must be a kinetic impediment to the melting of DNA secondary structure by RecA protein that does not affect SSB protein

displacement. With this model in mind, one can explain the behavior of these proteins in the context of the different DNA affinity states introduced above.

THE HIGH-AFFINITY STATE OF RECA PROTEIN IS REQUIRED FOR DISPLACEMENT OF SSB PROTEIN Direct DNA binding studies establish a simple relationship regarding the competitive binding of RecA and SSB proteins to ssDNA. In the absence of nucleotide cofactor or in the presence of ADP, SSB protein can displace RecA protein from ssDNA, whereas, in the presence of ATP-γ-S, RecA protein can displace SSB protein from ssDNA (54). In the presence of ATP, the situation is more complex (54, 55, 82). Because of ATP hydrolysis, the outcome is determined not only by the underlying thermodynamic constraints but also by the steady-state kinetic properties; the rates of protein association, ATP hydrolysis, ATP/ADP exchange, and protein dissociation become critical parameters. Nevertheless, in the presence of ATP, RecA protein will displace most of the SSB protein on a steady-state basis, provided that ADP does not accumulate, the ATP concentration is greater than ~ 200 μM, the concentration of RecA protein is sufficiently high, and the magnesium ion concentration is greater than 1 mM (55, 59, 60, 76). These are the same experimental conditions that favor DNA strand exchange. The minimum ATP concentration required is more than that required for ATP hydrolysis but is just enough for induction of the high-affinity state (Figure 5); hence, ATP hydrolysis alone is insufficient for SSB protein displacement. Higher concentrations of RecA protein are required under suboptimal conditions (e.g. 4-mM Mg^{2+}), consistent with the competition scenario (55, 59, 60). The requirement for high magnesium ion concentrations is not completely understood because, although the high-affinity state is induced at 1-mM Mg^{2+}, SSB protein displaces RecA protein from ssDNA (55, 59, 60); consequently, induction of the high-affinity state, although necessary, is not sufficient for SSB displacement. One explanation is that, despite induction of the high-affinity state, the binding affinity of RecA protein for ssDNA is less than that of SSB protein. An alternative explanation is that, despite a higher affinity of RecA protein for ssDNA, the rate of RecA protein binding to ssDNA is too slow to permit significant net displacement. Consistent with the latter view, the rate of RecA protein association with ssDNA demonstrates slow, nucleation-limited behavior with a rate that decreases with decreasing Mg^{2+} concentration (10).

Both the quantitative and qualitative issues relating to the induction of the high-affinity state by NTPs other than ATP also apply to displacement of SSB protein by RecA protein. For example, RecA protein can displace SSB protein more rapidly and more completely in the presence of dATP

than in the presence of ATP (76). At the other end of the scale, GTP fails to induce the high-affinity state and, as expected, GTPase activity is completely inhibited by SSB protein (79). These observations demonstrate the need for the high-affinity state (and not just NTPase activity) in order for RecA protein to resist displacement from ssDNA by SSB protein.

THE DISPLACEMENT OF SSB PROTEIN IS POLAR: IMPLICATIONS FOR DNA STRAND EXCHANGE REACTION The sequence of events that occur when RecA and SSB proteins are added simultaneously to ssDNA was studied using electron microscopy (112). The micrographs are visually striking and show that presynaptic filament formation occurs in three steps: first, SSB protein binds rapidly to the ssDNA; second, RecA protein nucleates onto this SSB protein–ssDNA complex; and, third, a rapid cooperative polymerization of additional RecA protein onto the ssDNA occurs concurrently with SSB protein displacement. The resultant saturated RecA protein–ssDNA complexes contain fewer than 10 SSB protein tetramers per M13 DNA molecule. In agreement with DNA binding studies and ATPase assays, the rate of presynaptic complex formation is optimal at stoichiometric amounts of RecA protein (1 RecA protein per 3 nucleotide residues), at ATP concentrations greater than 1 mM, and at substoichiometric concentrations of SSB protein (1 tetramer per 72 to 144 nucleotide residues). Complex formation decreases with higher SSB protein concentrations; the net rate of RecA protein polymerization decreases from a maximum of 970 monomers per minute at a ssDNA to SSB protein ratio of 144 (nucleotides : tetramer) to 240 per minute at a ratio of 18.

An important consequence of the 5′ to 3′ polarity of RecA protein polymerization is that the 5′ terminus of linear ssDNA is free of RecA protein because random nucleation is rate-limiting (112). This tendency is accentuated in the presence of SSB protein, resulting in a 5′ terminus coated with SSB protein (96). Because strand exchange proceeds 5′ to 3′, joint molecule formation and DNA strand exchange involving linear ssDNA will be inhibited because, effectively, only a less stable paranemic joint molecule can form. This polarity is most likely responsible for the bias observed in the pairing of linear ssDNA with supercoiled dsDNA: pairing is at least 10-fold more likely if the 3′ terminus, rather than the 5′ terminus, is homologous (47, 48). Similarly, pairing between linear dsDNA and linear ssDNA that is homologous only at its 5′ end does not occur, unless an extra length of 33–45 nucleotides is added to the 5′ end of the ssDNA (B. B. Konforti & R. W. Davis, unpublished observations). The use of circular ssDNA as a substrate eliminates the end-binding deficiency but introduces a new consideration: the products of a reaction between circular ssDNA and linear dsDNA are linear ssDNA and nicked dsDNA

(Figure 1). The linear ssDNA product cannot participate in the reverse reaction if its 5′ end is coated with SSB protein or is simply free of RecA protein. This kinetic difficulty in coating the 5′ end of ssDNA is the probable explanation for the observation that under certain conditions, the reaction between circular ssDNA and linear dsDNA is irreversible (99). Under conditions in which displacement of SSB protein is more efficient, RecA protein can coat enough of the displaced ssDNA to promote re-invasion and the formation of homology-dependent DNA networks (13, 60). Thus, the dynamics of RecA protein polymerization play an important role in substrate utilization.

BOTH THE IN VIVO PHENOTYPE AND THE IN VITRO DNA STRAND EXCHANGE ACTIVITY OF MUTANT RECA PROTEINS CORRELATE WITH THEIR ABILITY TO INDUCE THE HIGH-AFFINITY STATE The importance of SSB displacement activity is highlighted by the biochemical properties of mutant RecA proteins (see 50a). In all cases, mutant RecA proteins (RecA1, RecA13, RecA56) that display a null phenotype (complete loss of recombination function and SOS induction) fail to displace SSB protein and fail to adopt the high-affinity state (S. D. Lauder & S. C. Kowalczykowski, unpublished observations). Though these proteins bind ssDNA (6, 101), they have little or no enzymatic activity, providing little information regarding essential activities.

A second class of defective RecA proteins shows impaired in vivo properties but possesses partial activity in vitro. The RecA142 protein has nearly normal ATPase activity at low salt concentrations, binds to ssDNA, and forms a complex with ssDNA that is stable to higher concentrations of NaCl than the nucleotide-free complex; however, RecA142 protein fails to achieve the characteristic high RFI of the high-affinity state (52, 56). Owing to its inability to fully induce the high-affinity state, RecA142 protein displays three enzymatic defects: its NTPase activity is completely inhibited by SSB protein; it lacks M13 dsDNA–dependent ATPase activity; and it cannot promote homologous pairing. A second mutant protein in this category, RecA G160N protein also retains ATPase activity in vitro but, as with RecA142 protein, is inhibited by SSB protein (5). The ssDNA binding properties of this mutant are unknown, but it probably also fails to induce the high-affinity state. A third partially defective mutant protein is the RecA430 protein. In contrast to the RecA142 and RecA G160N proteins, RecA430 can displace SSB protein and promote DNA strand exchange, but only at elevated concentrations of NTP or RecA430 protein (77). The RecA430 protein is also distinctly more active in the presence of dATP (77). Consistent with its reduced functionality, RecA430 protein displays STMP values that are lower than those for wild-type

protein but has identical RFI values. The alterations in the high-affinity binding state associated with these mutations could result from perturbation of either the ATP binding site, the ssDNA binding site of the high-affinity conformation, or the sites involved in the structural transition itself.

A third class of mutant RecA proteins displays enhanced biological activity. Consistent with their phenotype, the RecA441 protein (59, 60), RecA730 protein (P. E. Lavery & S. C. Kowalczykowski, unpublished observations), and RecA803 protein (68; M. V. V. S. Madiraju, P. E. Lavery, S. C. Kowalczykowski, & A. J. Clark, unpublished observations) display both enhanced SSB protein displacement activity and DNA strand exchange activity. This enhancement is particularly pronounced at suboptimal reaction conditions. Each protein induces a high-affinity state that is indistinguishable from that of the wild-type protein. Therefore, these equilibrium binding properties do not explain the enhanced activity of these proteins. The only enhanced physical property associated with these proteins is an increased rate of association with ssDNA. Thus, as speculated above, the rate of nucleation-limited association with ssDNA is probably an important determinant of RecA protein function. The molecular nature of the rate-limiting step is unknown, but two possibilities are that either nucleation is inhibited by aggregates that are nonproductive for nucleation [e.g. the bundles seen using electron microscopy (108)] or nucleation requires formation of a specific self-aggregate. In the former, the mutant proteins would be defective in this nonproductive aggregation; in the latter, the mutant proteins would possess enhanced nucleation-specific self-association.

Do The Different Binding Modes of SSB Protein Play a Specific Role in the DNA Strand Exchange Reaction?

The binding of SSB protein to ssDNA is complex, involving several different binding modes whose equilibrium distribution is sensitive to both temperature and ionic composition (65, 66). At 37°C, over the range of 4- to 10-mM Mg^{2+}, SSB protein binds in a high-salt mode referred to as SSB_{56} (the subscript indicates the binding stoichiometry in nucleotides per tetramer) (8). Below 4-mM Mg^{2+} an equilibrium mixture of both the SSB_{56} and the SSB_{40} (low-salt) modes exists, and approximately equal amounts of each are present at 1-mM Mg^{2+}. Coincidentally, above 4-mM Mg^{2+}, SSB protein stimulates both the M13 ssDNA–dependent ATPase and the DNA strand exchange activities of RecA protein (55, 99); below 1- to 2-mM Mg^{2+}, SSB protein inhibits all wild-type RecA protein activity (55, 113). These observations lead to the possible conclusion that the SSB_{40} mode of SSB protein inhibits RecA protein function whereas the SSB_{56} mode is stimulatory and, consequently, important for RecA protein func-

tion. However, this correlation is invalid for several reasons, especially when the behavior of mutant RecA proteins is considered. First, in contrast to rATPase activity, the M13 ssDNA–dependent dATPase activity of RecA protein is stimulated by SSB protein at all Mg^{2+} ion concentrations (from 1–15 mM) (76); therefore, the low-salt mode of SSB protein is not always inhibitory to wild-type RecA protein. Second, the M13 ssDNA-dependent ATPase activity of the third class of mutant proteins discussed above [e.g. RecA441 (59, 60)] is also not inhibited at 1-mM Mg^{2+}. Third, SSB protein can inhibit the ATPase activity of wild-type RecA protein, even at 10-mM Mg^{2+}, if different polynucleotides, such as poly dT, are used (55), demonstrating that the high-salt mode of SSB protein is not always stimulatory. Fourth, the M13 ssDNA–dependent ATPase activity of RecA142 (52) and RecAG160N (5) proteins is similarly inhibited by SSB protein at 10-mM Mg^{2+}. And fifth, the other single-stranded DNA binding proteins (e.g. T4 gene 32 protein) that stimulate RecA protein activity (30, 38, 55, 103) are not known to bind ssDNA in different modes. Thus, although changes in the quantitative aspects (i.e. binding affinity, cooperativity, kinetic rate constants, etc) of SSB protein binding (or of any other single-stranded DNA binding protein) to DNA are important parameters that affect the outcome of the ssDNA binding competition, the different binding modes themselves do not appear to play a specific role in RecA protein function in vitro. The critical parameter, therefore, is the affinity of RecA protein for ssDNA relative to that of SSB protein.

DNA PAIRING AND STRAND EXCHANGE

The mechanisms of homologous alignment, joint molecule formation, and subsequent extension of the heteroduplex joint are the least-understood aspects of the DNA strand exchange reaction. To carry out these steps, RecA protein must bind two DNA molecules that are initially aligned nonhomologously and permit homologous pairing. The ability to bind two DNA molecules has been inferred from many assays, and recent studies clearly demonstrate that a RecA protein presynaptic filament can accommodate up to one equivalent of heterologous ssDNA or dsDNA (83, 110). However, the issues of whether the homology search is facilitated by RecA protein (either actively or passively), what the role of ATP hydrolysis is in these steps, and how DNA strand exchange occurs are considered below.

Homologous Pairing of DNA Molecules

The kinetic mechanism by which DNA molecules find the singular orientation that permits homologous pairing is still unclear. Part of the reason

for this uncertainty is the complexity of the RecA protein–promoted pairing reactions and part is because of real differences in experimental outcome (15, 33–35, 43). Kinetic analysis has focused on the question of whether either additional homologous or heterologous DNA sequences affect the rate of joint molecule formation. What complicates interpretation of published results is that these added DNA sequences affect not only the observed reaction rate, but they also affect the extent (yield) of the reaction. Consequently, the initial rate of pairing may change, but the apparent rate constant for pairing will not (43). Therefore, knowledge of DNA pairing rates, without knowledge of the extents of reaction, is insufficient for interpretation of the kinetic data. For this reason, only those results that provide this information are discussed.

Increasing the length of the homologous duplex DNA increases the extent and the apparent rate constant for both the three-stranded (43) and the four-stranded pairing reactions (15). These results suggest a two-step kinetic model involving a rapid pre-equilibrium step to form an intermediate and a subsequent rate limiting step to form the species that is detected in the joint-molecule assay (43). These steps might represent the rapid formation of a nonhomologously paired intermediate followed by a rate-limiting homology search or, alternatively, the rapid formation of homologously aligned DNA molecules followed by the slower conversion to a stable joint molecule. Given the absence of any effect by heterologous DNA under these conditions, the latter mechanism seems likely (43).

The effects of heterologous DNA sequences have been contradictory (compare 15, 43, and 33–35) and may depend on experimental protocols. Under certain conditions, heterologous DNA attached to homologous sequences does not affect either the three-stranded (43) or the four-stranded pairing reactions (15); for the former reaction, no change occurs if the heterologous DNA is not covalently attached to the homologous DNA. However, under other conditions, heterologous DNA increases the yield of products by threefold, without increasing the apparent rate constant for the reaction (34). The simplest interpretation of these data is that heterologous DNA does not affect the rate-limiting step of the reaction (stable joint molecule formation, perhaps) but, under certain conditions, increases the number of DNA molecules that can participate in productive joint molecule formation. RecA protein apparently facilitates this latter function through the formation of coaggregates (12). These coaggregates effectively sequester the DNA and, once formed, do not readily exchange bound DNA with free DNA, effectively limiting the DNA that can participate in pairing (34). Thus, though heterologous DNA can influence the pairing reaction, it seems to mostly affect the extent of the reaction and

not the rate-limiting step. Clearly, further clarification of these effects is required.

Joint Molecule Formation in the Absence of ATP Hydrolysis

DNA HETERODUPLEX FORMATION IN THE PRESENCE OF ATP-γ-S Understanding the role of ATP hydrolysis is crucial to understanding the mechanism of the DNA strand exchange reaction. Because ATP hydrolysis is not required for presynaptic complex formation, it is logical to ask whether hydrolysis is required for joint molecule formation. When ATP-γ-S is substituted for ATP, homologously paired joint molecules can form (17, 39, 98). However, whether pairing is accompanied by exchange of DNA strands was not clear. Recent research showed that, under optimal conditions, not only can RecA protein promote the formation of stable plectonemic joint molecules in the presence of ATP-γ-S, but extensive DNA heteroduplex formation occurs (72). Up to 3.4 kb of DNA heteroduplex are formed and fewer than 0.003 molecules of ATP-γ-S are hydrolyzed per base pair of DNA exchanged (72). These results demonstrate that ATP hydrolysis is not essential for either the DNA homology search, the formation of paired DNA molecules, or the exchange of DNA strands. Thus, models that require ATP hydrolysis for these steps can be eliminated.

To explain how pairing and exchange of DNA strands could occur in the absence of ATP hydrolysis, Menetski et al (72) suggested that ATP binding is essential for induction of the high-affinity state of RecA protein and that this conformation of RecA protein stabilizes a transition state complex consisting of three homologously paired DNA strands. Because this putative three-stranded intermediate must resemble a structure on the pathway to product formation, the Watson-Crick hydrogen bonding of the dsDNA substrate must be strained and the incoming ssDNA must be paired via non-Watson-Crick hydrogen bonding interactions. With the DNA strands poised for exchange, dissociation of the protein results in resolution of this intermediate to final product. Dissociation of the protein normally occurs after ATP hydrolysis, but in the case of the ATP-γ-S–dependent reaction, dissociation occurs upon denaturation of the protein with SDS. The transition-state structure was initially depicted as symmetric, i.e. halfway between substrate and product because approximately one half of the input DNA was converted to heteroduplex product (72). However, recent data suggest that the observed 3.4 kb of exchange reflect a limit imposed by the reaction conditions, probably owing to protein discontinuities in the presynaptic filaments. If dsDNA that is 54 nucleotides in length is used, complete exchange is observed (100);

similarly, if dsDNA 2.8 kb in length is used, complete exchange of DNA strands is also observed (S. C. Kowalczykowski & R. A. Krupp, unpublished observations). Thus, the structure of the transition state intermediate probably resembles the products more than the substrates.

JOINT MOLECULE FORMATION DOES NOT REQUIRE A NTP COFACTOR An implication of the ATP-γ-S–dependent DNA strand exchange activity and of the properties summarized in Figure 4 is that induction of the high-affinity state (with a sufficient affinity for DNA) may be the sole requirement for homologous pairing and exchange. If true, then effector molecules other than NTP might promote the transition to the high-affinity state and, consequently, support joint molecule formation. ADP·AlF_4^- is such an effector. This complex mimics the ground-state ligand, ATP, but lacks the β-γ high-energy phosphodiester bond (11), and, consequently, no free energy can be derived from hydrolysis. In the presence of this nucleotide, RecA protein can promote the cleavage of the LexA repressor protein, suggesting that the high-affinity state can be induced (81). Image analysis demonstrates formation of the same characteristic structure in the presence of ADP·AlF_4^- that is induced by ATP or ATP-γ-S (122). Consistent with these observations, etheno M13 ssDNA binding studies confirm that the high-affinity state is induced by ADP·AlF_4^- and, most significantly, joint molecule formation occurs in the absence of ATP when ADP·AlF_4^- is present, with no detectable ATP (or ADP) hydrolysis (S. C. Kowalczykowski & R. A. Krupp, unpublished observations). These collective data demonstrate that the presence of NTP is not required to activate normally NTP-dependent activities; in principle, any suitable effector molecule can substitute.

JOINT-MOLECULE FORMATION BY A MUTANT RECA PROTEIN DEFECTIVE IN NTP HYDROLYSIS If ATP hydrolysis is not essential for RecA protein's pairing activity, then one should be able to construct a mutant RecA protein that binds NTP, fails to hydrolyze NTP, yet still promotes joint molecule formation. Such a mutant RecA protein (RecA72 protein) was recently made by substitution of an arginine residue for the lysine residue present at position 72 (W. M. Rehrauer & S. C. Kowalczykowski, unpublished observations). This region of the protein contains a Walker A-type consensus site for ATP binding (115) and is believed to be involved in polyphosphate binding. The structure of the Ras p21 protein confirms that the invariant lysine residue at the equivalent position in the GTP binding site contacts the β-γ phosphates (86). As expected, RecA72 protein binds NTP but its hydrolysis activity (k_{cat}) is reduced by at least 500-fold relative to the wild-type protein (W. M. Rehrauer & S. C. Kowalczykowski, unpublished

observations). In addition, dATP induces the high-affinity state and supports joint molecule formation. Thus, joint molecule formation does not require NTP hydrolysis.

A MODEL FOR JOINT MOLECULE FORMATION The facts described are accommodated by the model depicted in Figure 9 (72), which is essentially a detailed version of a model proposed earlier (40). In step *a*, the binding of ATP and ssDNA induce the high-affinity state of RecA protein. The resultant filamentous presynaptic complex can homologously pair dsDNA in such a way that the initial hydrogen bonding is weakened or disrupted and the complementary strand is paired with the invading ssDNA (*b*). ATP hydrolysis results in the formation of an ADP·P_i complex (*c*) that retains the properties of the ATP complex. Upon dissociation of the P_i, the resultant ADP induces the low-affinity state (*d*). Dissociation of the product ssDNA occurs (*d*), followed by dissociation of RecA protein from the heteroduplex DNA product (*e*); these last two steps have been visualized with electron microscopy (95, 109). Finally, the cycle continues

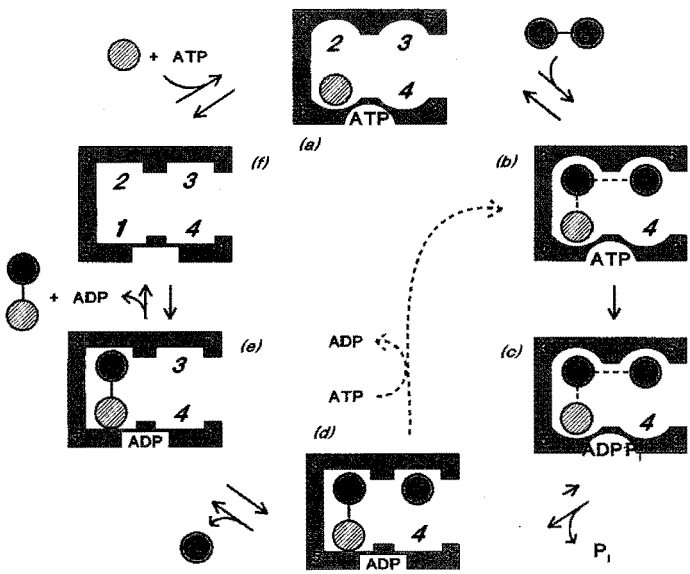

Figure 9 Model for the mechanism of DNA strand exchange promoted by RecA protein. Shown is a cross-sectional view for the pairing of one set of homologous DNA strands within the functional RecA protein promoter. If the promoter is a monomer, then only one nucleotide-binding site is required. If the protein is a dimer, then an additional nucleotide-binding site is implied.

upon rebinding of ATP and reassembly onto ssDNA (*f*). The dashed line going from *d* to *b* illustrates that ATP hydrolysis is not obligatorily linked to the cycle and that exchange of ADP for ATP can occur. In this case, additional ATP hydrolysis would occur with no additional strand exchange; such nonproductive ATP hydrolysis could also occur at steps *a* through *e* but, for clarity, are not shown. The model depicts four contact sites for DNA strands; only three are essential for three-stranded exchange but the fourth may be involved in four-stranded exchange reactions. The sites are numbered in order of expected occupancy in the high affinity-state (site 4 may have little or no DNA binding affinity), and proper polarity must be observed. Image reconstruction data place the ssDNA within the ssDNA–RecA protein filament in the same location as dsDNA in the dsDNA–RecA protein filament (i.e. site 1) (29). Also, the occupancy of sites 1 and 2 (*d* and *e*) by dsDNA should result in a complex that is nonproductive in DNA strand exchange (i.e. strand exchange between *d* and *b* is not reversible). Finally, this model explains the ATP hydrolysis–independent formation of heteroduplex DNA by proposing that ATP binding provides the free energy required to stabilize the three-stranded DNA intermediate and that ATP hydrolysis serves only to dissociate and recycle the RecA protein (72). This view readily explains the observation that many eukaryotic homologous pairing proteins do not require ATP (42, 46; see 29a) these proteins effectively exist only in the high-affinity state (*a–b*) in the absence of nucleotide.

THREE-STRANDED DNA STRUCTURE Though RecA protein can hold two DNA molecules in homologous register, the nature of the DNA-DNA contacts in the homologously paired structure are unclear. The suggestion that a kinetic intermediate in the reaction is a three-stranded DNA molecule (72) raises the question of whether stable, protein-free three-stranded DNA exists. Electron microscopy has detected regions of DNA, devoid of protein, where three strands of DNA coincide (95, 109) but whether this is a triple-stranded DNA structure is unclear. Recent nuclease-protection data suggest that formation of a protein-free, three-stranded region of DNA, several thousand bases in length, precedes strand displacement (94). In addition, the products of a pairing reaction involving short regions of homology possess unusual temperature stability, suggesting the formation of a unique DNA structure (41). The proposed structure is three-stranded, and a potential model was suggested (41). The actual structure of this interesting reaction intermediate is unknown, but it may represent a novel DNA structure that plays a key role in the recognition of homologous sequences.

RecA Protein–Promoted Extension of the DNA Heteroduplex Joint

Joint molecule formation by RecA protein occurs within a few minutes. This rapid step is followed by a slower step in which the nascent heteroduplex joint is unidirectionally extended (5′ to 3′) to produce completely exchanged product molecules and which takes 30–60 minutes to traverse 7 kb (17, 24, 118). This slow step was referred to as RecA protein–mediated branch migration and could be stopped by the addition of ATP-γ-S, suggesting the need for ATP hydrolysis (17). In the ATP hydrolysis–independent reactions described above, joint molecules form rapidly (1–2 min) but show little or no change with time (72). This suggests that postsynaptic heteroduplex extension requires ATP hydrolysis.

The first of two limiting views of this behavior is that joint molecule formation and nascent DNA strand exchange are both kinetically and mechanistically distinct from the subsequent DNA strand exchange process. In this view, plectonemic joint molecule formation occurs in an ATP hydrolysis–independent manner as depicted in Figure 9, but subsequent strand exchange requires a mechanistically distinct ATP hydrolysis–dependent reaction. This ATP hydrolysis–dependent reaction may be similar to the rotation model that was elaborated elsewhere (19–21); in brief, the rotation model proposes that DNA heteroduplex extension results from active rotation of the dsDNA about the presynaptic filament in an ATP hydrolysis–dependent reaction. This model explains the requirement for ATP hydrolysis during strand exchange, rationalizes the apparent inefficiency of ATP utilization during DNA strand exchange, and offers an explanation for how strand exchange proceeds through insertions and deletions in the DNA (2). However, if this mechanism is operative, RecA protein must switch from a simple ATP hydrolysis–independent mode of DNA strand exchange early in the reaction to a complicated and apparently unrelated ATP hydrolysis–dependent rotation mode.

The second limiting view is that both phases of the DNA strand exchange reaction are mechanistically identical but are separated in time by a slower kinetic step, resulting in kinetically distinct phases. All DNA strand exchange events occur by the same mechanism, as depicted in Figure 9, and DNA strand exchange after initial heteroduplex DNA formation requires ATP hydrolysis for a different reason. ATP hydrolysis would be needed for additional rounds of dissociation and reassociation in order to correct discontinuities in the presynaptic filament that impede subsequent DNA heteroduplex formation. The initial length of nascent heteroduplex DNA formed in the first few minutes may be limited by the continuity of the RecA protein filament. Discontinuities would arise from the necessarily

stochastic nature of nonspecific binding to ssDNA. In the limit of irreversible binding (which is approached with ATP-γ-S complexes), DNA cannot be saturated with protein unless dissociation is permitted (31). This obstruction suggests that, for the ATP-γ-S–dependent reaction, the presynaptic filament is contiguous for 3.4 kb, on average. The discontinuities (39) that are present cannot be corrected because dissociation does not occur. In the presence of ATP, the contiguous region may be much shorter because of dissociation resulting from ATP hydrolysis, but repolymerization is possible. Extension of the nascent heteroduplex joint would then require filling of these protein voids by slow dissociation and reassociation of RecA protein. Voids that are not multiples of RecA protein's site size would require complete disassembly of the filament that is downstream (i.e. to the 3′ side) of the growing filament. A similar mechanism (based on the treadmilling model) was proposed for DNA strand exchange promoted by the bacteriophage T4 UvsX protein (45). Though this dissociation-dependent view has the advantage of mechanistic economy, one troubling result is that the exchange of DNA-bound RecA protein with free protein pools is not detected (85). This observation is curious given that RecA protein can exchange between DNA molecules within a few minutes (7, 74, 75, 85); it may suggest either that direct transfer between DNA strands is a mechanistically important dissociation pathway or that free RecA protein in solution is unable to associate rapidly with DNA [perhaps because of slow disassembly from an aggregate (85)]. However, consistent with the occurrence of dissociation, excess heterologous ssDNA inhibits DNA heteroduplex growth, implying that the RecA protein–DNA complex is indeed labile (17). The arguments in favor of either the rotation model or the dissociation-association model and their derivatives are currently largely circumstantial. Understanding the mechanism of this important step of the DNA strand–exchange reaction requires further inquiry.

CONCLUDING REMARKS

I have attempted to present a relatively simple and coherent view of the mechanism and energetic requirements of the DNA strand–exchange reaction promoted by the *E. coli* RecA protein. This view is that RecA protein exists in two different conformational states whose induction is controlled by the bound nucleotide cofactor. The high-affinity state of RecA protein is functionally important in DNA strand exchange and repressor cleavage. Neither DNA strand–exchange nor repressor-cleavage activity require NTP hydrolysis, only the binding of a suitable effector molecule. In this sense, RecA protein displays classic allostery, and much

of the thinking applied to other allosteric proteins is certainly applicable to RecA protein. Similarly, the biochemistry of RecA protein function possesses elements common to other proteins: self-assembly (actin, dynein, tubulin); energy transduction (myosin, membrane ATPases); NTP hydrolysis–induced conformation changes (G-proteins, EF-Tu); and nucleic acid structural changes (DNA gyrases, DNA helicases). Approaches useful in the study of these proteins will certainly be applicable to RecA protein.

Though I have repeatedly made the point that the exchange of DNA strands does not require NTP hydrolysis, that is not to say that NTP hydrolysis is unimportant to the overall DNA strand exchange reaction. The absence of DNA heteroduplex extension in the absence of ATP hydrolysis demonstrates this point clearly. NTP hydrolysis must play a crucial role in permitting dissociation of DNA-bound RecA protein molecules and thereby allowing rearrangement to the final steady-state distribution. This function certainly plays a role in the formation of a contiguous presynaptic complex and may play a role in postsynaptic redistribution of the protein. The hydrolysis of NTP also ensures that DNA strand exchange is irreversible, and it permits directional polymerization of RecA protein, resulting in many of the polar aspects of DNA strand exchange.

Is the simple two-state view of RecA protein adequate to explain its behavior? The answer is almost certainly not. Upon closer examination, we will likely find that other intermediate conformations are invoked; in fact, current observations that some mutant RecA proteins need to be defective in only one of the hallmarks of the high-affinity state suggest that intermediate conformations exist. Is induction of the high-affinity state sufficient to promote DNA strand exchange? The answer to this question is also negative. There are conditions (such as low magnesium ion concentrations) in which induction of the high affinity occurs but joint molecule formation does not. This observation implies either that another property important for function remains unknown or that there is a quantitative deficiency (e.g. the complex cannot bind dsDNA with sufficient affinity). Is there a need for NTP hydrolysis beyond its role in dissociation? Why is NTP hydrolysis needed for extension of the DNA heteroduplex joint? What are the recognition elements involved in the homologously paired three-stranded DNA structure? The answers to these questions are still to come.

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