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HOMOLOGOUS PAIRING AND DNA STRAND-EXCHANGE PROTEINS

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CONTENTS

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PERSPECTIVES AND SUMMARY

The ability to pair two DNA chromosomes homologously and to exchange DNA between them lies at the heart of all models for general recombination. This process requires that sequence similarity between two DNA molecules is searched, homology is recognized, and individual DNA strands are mutually exchanged. The complexity of this molecular recognition process has hampered mechanistic analysis, but recent concerted effort has resulted in significant understanding of this elaborate series of events. The biochemical features of this process and of the proteins that promote it are reviewed here.

Major insight came with the discovery that the *Escherichia coli* RecA protein, known from genetic analysis to be crucial to recombination (1), promoted the homologous pairing and exchange of DNA strands (2–6). It is, perhaps, surprising that a single protein can carry out such a complicated biochemical process, but RecA protein is a remarkably complex entity (see Figure 1; details are explained below). RecA protein binds both ATP and DNA, and acts not as a monomer or a limited assemblage of monomers, but rather as a helical filament of indefinite length polymerized on DNA. This nucleoprotein complex, the presynaptic complex, requires ATP binding to attain its striking functional form and is the active species during the homology search and DNA strand exchange. Despite the need for ATP binding in filament assembly and in the homologous alignment of DNA, neither the homology search nor DNA strand exchange requires ATP hydrolysis (see below), further highlighting the unusual nature of this reaction.

The ubiquity of RecA-like proteins in eubacteria (7, 8) argues for conservation of the mechanism for homologous pairing and DNA strand exchange. The extension of this mechanism to eukaryotes is supported by a growing list of proteins that are structurally similar to the *E. coli* RecA protein (see below). Thus, the paradigms established from studies of RecA protein can be tested for their generality.

A hallmark of RecA protein–promoted DNA strand exchange is its ATP dependence. However, a class of eukaryotic pairing proteins can function in the absence of ATP. Although they were initially thought to be a limited case of the RecA paradigm [reviewed in (9)], recent evidence argues that these proteins promote pairing and apparent DNA strand exchange by a distinct reaction mechanism. Most, if not all, of these ATP-independent proteins require nucleolytic degradation of one strand of a duplex molecule

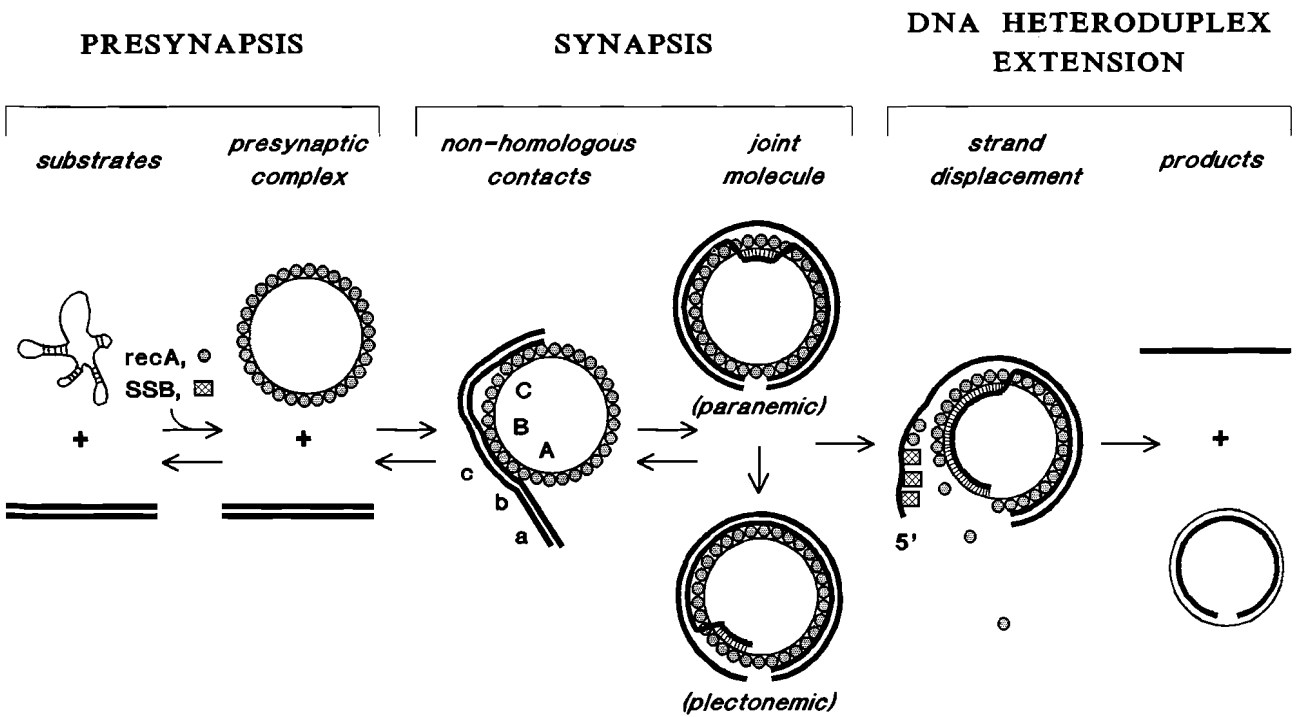

Figure 1 Model for DNA strand exchange promoted by RecA protein. See text for details. Modified from (16).

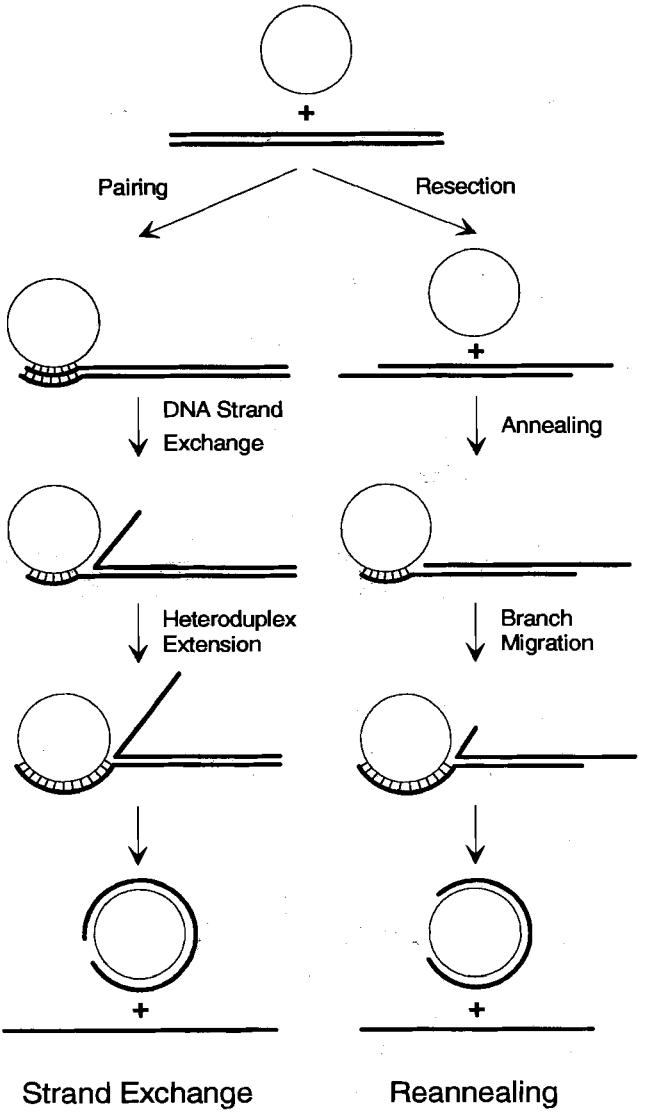

Figure 2 Models for the generation of heteroduplex DNA. *(Left)* DNA strand-exchange mechanism involving initial strand invasion of dsDNA by ssDNA followed by DNA heteroduplex extension. *(Right)* Reannealing mechanism involving renaturation of ssDNA between resected dsDNA and ssDNA molecules, followed by either thermal or protein-mediated branch migration.

as a first step (Figure 2; see below). The annealing of complementary regions of single-stranded DNA (ssDNA), rather than the invasion of double-stranded DNA (dsDNA) by ssDNA, is responsible for the observed homologous pairing. The ensuing extension of DNA heteroduplex may or may not be protein-promoted.

The existence of two different biochemical mechanisms for effecting DNA strand exchange (ATP-dependent and -independent) raises the question: Is the net input of free energy, or even the participation of proteins, necessary? While the complexity of the reaction seems to favor protein-mediated catalysis, neither condition is essential in vitro. DNA strand exchange between identical sequences is isoenergetic (i.e. an equal number of basepairs are disrupted and reformed), so DNA strand exchange is not restricted thermodynamically. This fact argues that the major mechanistic need for proteins is kinetic. Since catalysis involves lowering the activation energy of a rate-limiting step, DNA strand-exchange proteins must facilitate the formation or stabilization of a normally unstable transition-state structure, which many lines of evidence suggest is a three-stranded intermediate (10, 11; see below). In contrast, when DNA sequences are not identical (due to mismatches), then a need for energy input arises; this consideration predicts the involvement of an ATP-dependent step when sequence similarity is imperfect.

This introductory perspective has raised issues that will be elaborated below. The discussion first addresses structural, energetic, and experimental aspects of the homologous pairing of DNA molecules. This groundwork is followed by a discussion of the ATP-dependent class of pairing proteins and the mechanism by which they promote DNA strand exchange. Next, the ATP-independent class of pairing proteins and their mechanism of action are examined. A brief description of eukaryotic structural homologs of RecA protein and their potential as DNA strand-exchange proteins follows. Finally, protein-independent renaturation, pairing, and strand exchange are compared to the protein-promoted reactions. Table 1 summarizes pertinent information about the proteins that are discussed. Other perspectives on DNA strand-exchange proteins and homologous recombination are found in (7, 12–22). This article is an elaboration of a previous overview of this topic (9).

PRINCIPLES OF HOMOLOGOUS PAIRING AND DNA STRAND EXCHANGE

Homologous Pairing

The problem of homologous recognition between DNA molecules is, in principle, no different than that of site-specific recognition by DNA-binding proteins. There are typically few appropriate targets in the entire genome,

Table 1 Properties of homologous pairing and DNA strand-exchange proteins[a]

ATP-dependent proteins											
Protein	Organism	M_r[b]	ATPase activity	Stoich.[c]	Mode[d]	Pairing end bias[e]	Joint extension[f]	Renat.[g]	Aggreg./ coagg.[h]	Accessory factors[i]	Assays[j]
RecA	*E. coli*	38	Y	3	S	none[k]	5'→3'	Y	Y/Y	SSB	em, fb, jm, ns, se
RecA	*P. mirabilis*	38	Y	1.5	S	—	—	—	—		fb, jm, se
RecA (RecE)	*B. subtilis*	42	Y[l]	—	S	—	—	—	—	SSB	jm, se
RecA	*T. aquaticus*	36	Y	—	—	—	—	—	—	SSB?	jm
UvsX	phage T4	44	Y	3–5	S	5'	5'→3'	Y	Y/Y	G32P; UvsY	em, fb, jm
RecA-like	*P. sativum*	40	?[m]	—	S?	—	—	—	—		em, jm
Rec1	*U. maydis*	70?	Y	200	C?	3'	3'→5'	Y	—		fb, jm, ns
ATP-independent proteins											
Protein	Organism	M_r	ATP binding	Stoich.	Mode	Nuclease activity[n]	Extension polarity[o]	Renat.	Aggreg./ coagg.	Accessory factors	Assays
RecT	*E. coli*	33	N	13	S	N[p]	none[q]	Y	—		em, jm
β	phage λ	28	N	6[r]	S	N[p]	—	Y	—		—[r]
Sep1/Xrn1	*S. cerevisiae*	175	N	35–40	S	I (5'→3')	none[q]	Y	Y/Y	yRPA; SF1	em, jm
DPA/EF3	*S. cerevisiae*	120	—	~20	S	N[p]	none[q]	Y	—		em, jm
STPα/TFIIS	*S. cerevisiae*	38	—	—	—	Y (<0.5%)	—	Y	N/—	ySSBs	em, jm
p^{140}/ExoII	*S. pombe*	148	N	40	S	I (5'→3')	none[q]	Y	—	FAS	em, jm, ns
Rrp1	*D. melanogaster*	105	N	400	C	I (3'→5')	none[q]	Y	Y/Y		em, jm, se
HPP-1	human T-cell	130	Y[s]	25[t]	S	Y (3.3%)	—	—	—	hRPA, others	em, jm, se
v-SEP	vaccinia	—	—	—	—	Y (0.9%)	5'→3'[u]	Y	—		em, jm
ICP8	HSV-1	138	—	10	S	N (<0.01%)	—	—	—		em, jm
p53	human	53	Y	—	—	—	—	Y	—		se[v]

[a] Adapted from (9).
[b] Expressed as kDa.
[c] Optimal stoichiometry of homologous pairing protein (nt ssDNA per protein monomer) required for strand exchange in the absence of any other protein.
[d] Mode of action (stoichiometric or catalytic).
[e] Preferred end (5' or 3') of displacement of the noncomplementary strand during initial pairing.
[f] Polarity of extension of the DNA heteroduplex joint relative to the displaced, noncomplementary strand.
[g] Ability to renature complementary ssDNA.
[h] Ability to either aggregate ssDNA or coaggregate ssDNA and dsDNA.
[i] Accessory factor(s) that stimulate the homologous pairing protein.
[j] Assay used to determine activities: em = electron microscopy with visualization of the displaced strand; fb = filter-binding assay with retention of joint molecules; jm = gel assay with production of joint molecule intermediates; ns = nuclease sensitivity assay; se = gel assay with the production of form II molecules.
[k] Differing results have been obtained (see Refs. 38, 59, 92a, 93, 226–228). The discrepancies may be due to variations in substrates, reaction conditions, and experimental technique; Refs. 93 and 226 employed filter-binding assays and EM, whereas the other five references used gel assays.
[l] Although both ATP and dATP are bound by the protein, only dATP activates nucleotide hydrolysis and DNA strand exchange.
[m] The addition of exogenous ATP is required for DNA strand exchange.
[n] Presence of nuclease activity in the protein preparation: I = intrinsic to strand exchange protein (with polarity of nuclease activity indicated); Y = nuclease activity reported in protein preparation (level of detection indicated); N = no nuclease activity reported.
[o] Polarity of branch migration.
[p] Requires ssDNA tails to initiate pairing.
[q] Observed polarity is dependent on the polarity of the ssDNA overhang used to initiate pairing.
[r] Optimal stoichiometry of the homologous pairing protein required for ssDNA renaturation; no strand-exchange assay has been reported for this protein.
[s] Based on UV crosslinking by the ATP analog, 8-azido ATP.
[t] Expressed as bp dsDNA per protein monomer.
[u] This observed polarity may be dictated by the nuclease contaminant in this preparation.
[v] The gel assay for strand transfer involved linear molecules <100 nt in length.

making the mechanics of the search process seem insurmountable. Yet despite the vast excess of inappropriate sites or alignments, specific sites and homologous sequences are nevertheless located. Clearly, both recognition elements and a mechanism for identifying them exist. In contrast to site-specific DNA binding, however, protein-promoted DNA homology recognition must involve proteins sufficiently nonspecific so that they can interact with any DNA sequence, yet specific enough so that only the homologous counterpart is recognized. This is possible only if nonspecific DNA-binding proteins utilize the sequence-specific information inherent to DNA. Both the major and minor grooves of dsDNA are sources of potential recognition elements that might permit homology to be detected; once pairing is achieved, the proteins involved are imagined to provide stability to the aligned structures.

MAJOR GROOVE PAIRING The possibility of specifically paired four-stranded DNA structures involving homologous dsDNA molecules was recognized by McGavin (23, 24). It was proposed that non-Watson-Crick hydrogen bonding involving atoms in the major groove of dsDNA could provide the required specificity. The pairing scheme is specific, in that any given basepair can bond only with its homolog and not with another basepair. The bases of the resulting tetraplex structure form a near square, with the basepairs related by a dyad axis perpendicular to their common plane (Figure 3). The corners of the rectangle are ~10–11 Å apart, and the diameter of the tetraplex is essentially unchanged from that of dsDNA. Model building affirms that the structure is feasible. In the absence of charge neutralization, this DNA structure would have twice the charge density of B-form DNA, indicating that without the participation of protein or other stabilizing components, this structure would be less stable than dsDNA. The experimental observation that RecA protein binds to the minor groove of dsDNA (25, 26) suggests that major groove pairing is utilized in the RecA protein–dependent homology search.

MINOR GROOVE PAIRING Minor groove pairing schemes lack the specificity of those invoking major groove pairing (27). In addition, intercoiling of two duplexes along their minor grooves requires untwisting and unstacking of dsDNA, both of which are unfavorable processes. Interestingly, however, such perturbations are features typical of RecA protein–dsDNA complexes [see (21)]. Though interactions between the minor grooves of DNA lack specificity, it was proposed that high specificity arises from the major groove contacts that result after DNA strands are exchanged, providing a means for stabilizing the desired products (27).

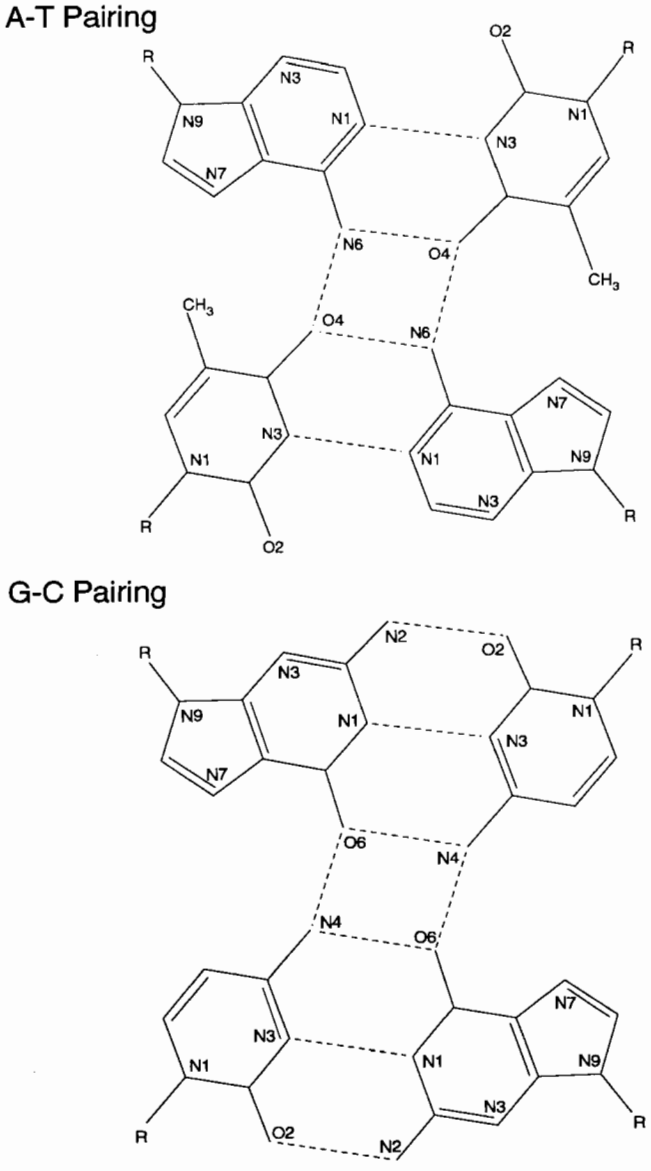

Figure 3 Tetraplex (four-stranded) DNA structure proposed by McGavin (23, 24).

TRIPLEX DNA STRUCTURES Pairing reactions promoted by RecA protein require that one of the substrate molecules be partially single stranded, which suggests that recognition involves contacts between ssDNA and its dsDNA homolog. Since only one of the molecules is basepaired, it is possible that the dsDNA is disrupted before homologous contacts are established, so that conventional Watson-Crick basepairing between the ssDNA and its complement from the dsDNA can be made. Though plausible, no evidence supports this hypothetical "dsDNA opening before pairing" scheme. Instead, experimental evidence argues for a pairing intermediate involving three juxtaposed strands of DNA [see (10, 11, 28) for a critical appraisal of this topic].

The notion of a triple-stranded intermediate in the RecA protein–promoted DNA strand-exchange reaction was advanced by Howard-Flanders and colleagues (29). Although many studies provide compelling evidence for a close association of three strands within the RecA protein–DNA filament (30–36), the precise nature of this structure remains elusive. A three-stranded structure is likely to exist, at least transiently; this transition-state complex was featured in a model to explain DNA strand exchange without the need for ATP hydrolysis (37, 38). Other studies, however, suggest that this structure (or an analogous triplex structure) persists after removal of RecA protein (33, 34, 36).

Several unique triplex DNA structures have been proposed as the recognition intermediates (Figure 4). In contrast to triplex structures formed non-enzymatically (where the two identical strands assume an antiparallel orientation), the identical strands of recombination triplexes must be in a parallel orientation (10, 11, 39). One of the first pairing schemes for recombination intermediates envisioned pairing of the ssDNA only with purine residues in the dsDNA (33) (Figure 4). Subsequently, it was found that replacement of the N7 guanine by a carbon atom in 7-deazaguanine had no effect on DNA strand exchange; this result suggested that the interactions at the N7 position were not crucial to the rate-limiting step of the pairing reaction (40). In agreement, neither was the N7 guanine protected from dimethyl sulfate under conditions that promoted pairing nor did methylation affect pairing (41, 42). Methylation of N6 adenine and N4 cytosine did lower the T_m of a possible three-stranded structure, however, leading to a proposal for an alternative structure that retained some of the characteristics of the McGavin pairing schemes and that did not involve bonding with the N7 position (42) (Figure 4). Finally, energy minimization analysis led to yet a third structure that differs somewhat from the second model (43); the authors' calculations suggest that the triplex structure results from an electrostatic recognition code, although one of the basepairing arrangements involves interaction with an N7 atom (Figure 4). At this time it is not clear which, if any, of these triplex pairing schemes represent stable intermediates of homologous pairing reactions and, hence, these structures

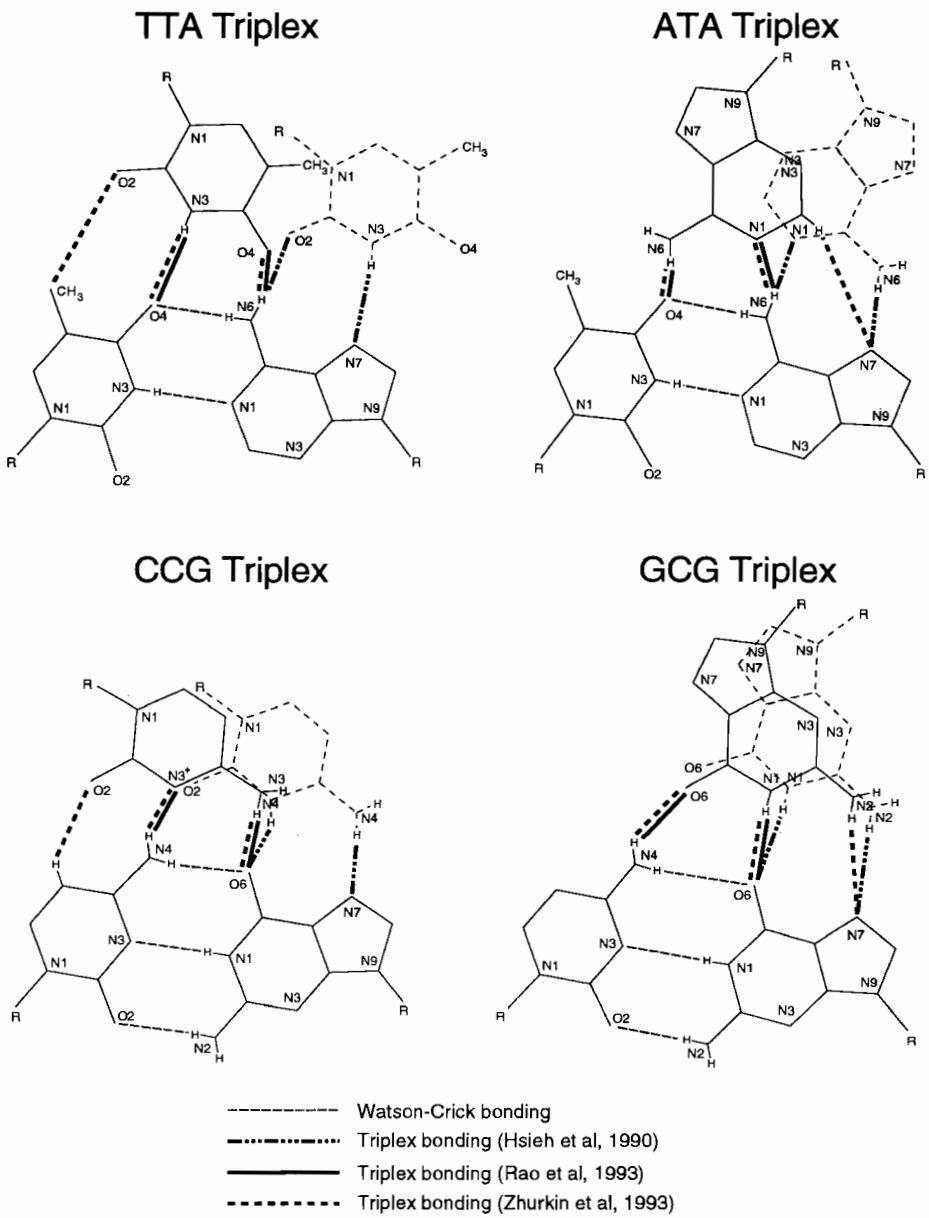

Figure 4 Proposed pairing schemes for RecA protein–mediated triplex (three-stranded) DNA structures. The two bases at the bottom of each structure are bonded by typical Watson-Crick pairing. The third base at the top of each structure is bonded by non-Watson-Crick pairing. Three models of non-Watson-Crick pairing are shown, as indicated in the legend. For clarity, the third base in the Hsieh et al model (33) is composed of a dashed line, while that for the Rao et al (42) and Zhurkin et al (43) models is a solid line. These diagrams are for comparison only and are not meant to imply specific bond angles or lengths.

should be viewed as hypothetical. In fact, a recent examination of the disposition of the three homologous strands of DNA within the RecA protein filament reveals that they are nearly identical to that expected for products of the reaction rather than substrates or intermediates (41). This result implies that the three-stranded DNA intermediate of the enzymatic reaction is short lived, and that upon homologous recognition, it is rapidly converted to a structure with exchanged DNA strands (41). The reason for the detection of apparently stably paired three-stranded species after deproteinization of joint molecules formed at the ends of the linear dsDNA remains unknown.

DNA Strand Exchange

After pairing is achieved, the resultant joint molecules can exchange homologous strands. Conceptually, homologous pairing and DNA strand exchange are separable events, but experimentally, strand exchange may be instantaneous. The act of DNA strand exchange can lead to two types of structures—plectonemic and paranemic—that possess different stabilities.

PLECTONEMIC JOINT MOLECULES In a plectonemic structure, the DNA strands of the heteroduplex are intertwined. Consequently, a free homologous end must be present on one of the DNA molecules involved in plectonemic joint molecule formation. This normally requires that one of the molecules be linear or that a topoisomerase be present to introduce a transient break. Figure 5*A* shows DNA substrate pairs capable of plectonemic joint molecule formation. Once formed, plectonemic joint molecules are stable in the absence of protein and, being stabilized by conventional basepairing, have a T_m characteristic of dsDNA. In the absence of topological constraints, the length of the DNA heteroduplex region is unlimited; for typical in vitro substrates (6–7 kilobases, or kb, in length), a fully displaced DNA strand is readily detected.

PARANEMIC JOINT MOLECULES In a paranemic structure, net intertwining of DNA strands is prevented, which for covalently closed molecules results in no net change in the linking number for the joint molecule. Paranemic joint molecules result when the invading DNA strand is unable to rotate freely around its complement (i.e. when the DNA molecules are topologically constrained). An example of this is pairing that initiates away from the ends of the linear DNA or between two circular DNA molecules (Figure 5*B*). Experimentally, homologous pairing can be limited to regions internal to the dsDNA by introducing heterologous DNA sequences on either the ssDNA or the dsDNA. Despite being basepaired, the topological strain imposed on paranemic joint molecules makes them wholly dependent on the binding of protein for stability (44). Paranemic joints are kinetically convertible to

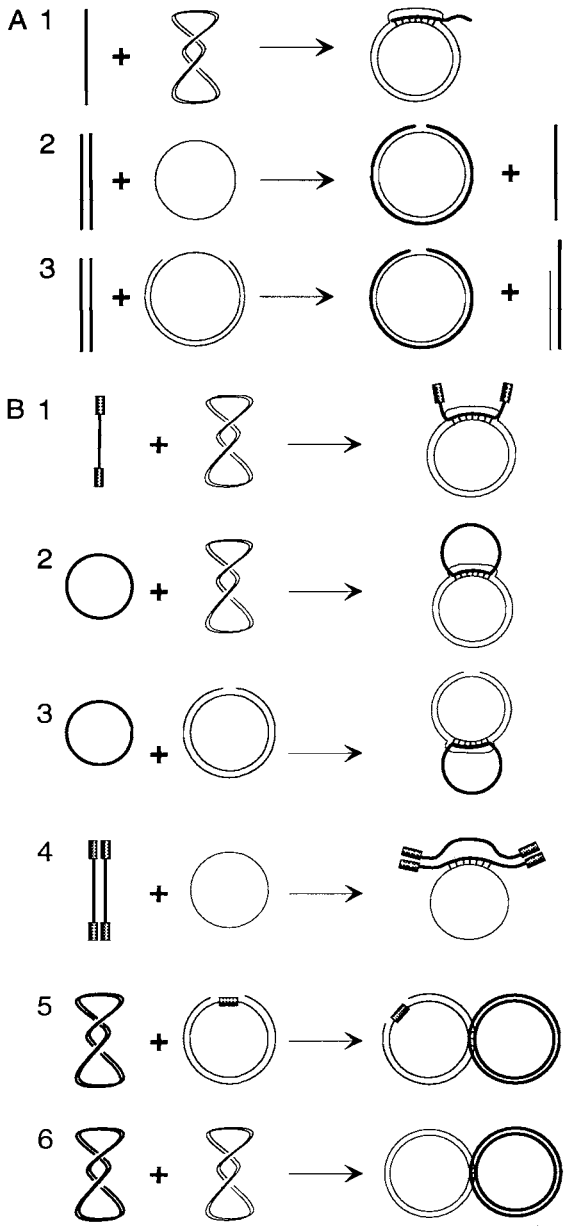

Figure 5 Substrates used to characterize homologous pairing and DNA strand-exchange proteins. Reactions that result in the production of plectonemically intertwined product molecules are shown in (*A*); those that are restricted to forming paranemic, non-intertwined molecules are shown in (*B*). Regions of nonhomology are indicated by shaded cylinders.

plectonemic joints when pairing reaches the end of a linear DNA molecule, or when they are acted upon by a topoisomerase (45).

Energetic Considerations

Because DNA strand exchange between identical sequences is isoenergetic, there is no thermodynamic requirement for energy input in the protein-catalyzed reaction. This rather obvious statement seemed to fly in the face of observations that anywhere from 1 to 1000 ATP molecules were hydrolyzed per basepair (bp) of DNA exchanged [see (16) for discussion]. However, various studies showed that both homologous pairing and DNA strand exchange (resulting in formation of up to 3.4 kb of DNA heteroduplex) could occur in the presence of the essentially nonhydrolyzable ATP analog, ATPγS (37, 38, 46–48). In fact, a nucleoside triphosphate is not needed for limited (800–900 bp) DNA strand exchange (SC Kowalczykowski, RA Krupp, in preparation), and a mutant RecA protein (RecA K72R) that reduces NTP hydrolysis by more than 600-fold is nevertheless capable of 1.5 kb of DNA strand exchange (50). Collectively, these results demonstrate that the free energy derived from ATP hydrolysis is not linked to the physical exchange of DNA strands.

Instead, ATP hydrolysis is important for the dissociation of RecA protein upon completion of strand exchange. This seemingly trivial role for ATP hydrolysis is readily explained by considering ATP as an allosteric effector (16). Binding of ATP induces a functional state of RecA protein that has high affinity for DNA, whereas ADP, the product of ATP hydrolysis, induces a nonfunctional state that has low affinity for DNA (51). The ATP hydrolytic cycle therefore serves an important function from an enzymatic perspective: It allows alternation between high- and low-affinity states, thus enabling successive rounds of protein binding and dissociation. Since the substrates and products of DNA strand exchange are nearly identical, such modulation permits RecA protein to bind the substrates with a sufficiently high affinity needed for DNA strand exchange and, at the same time, prevents product dissociation from becoming rate limiting. Thus, even though ATP hydrolysis and dissociation are not directly coupled for RecA protein (see Refs. 7, 16), ATP hydrolysis resolves the "tight-binding dilemma" faced by enzymes that must act on DNA yet dissociate with sufficient rapidity.

Physiological substrates contain regions of DNA sequence nonhomology that may be as small a single basepair mismatch or as large as several kilobasepairs. RecA protein can promote DNA strand exchange across such heterologies, but the reaction requires continual ATP hydrolysis (52, 53). A heterologous region as short as six nucleotides (nt) is sufficient to block the ATPγS-dependent reaction (52), whereas the ATP-dependent reaction

can traverse heterologies in either ssDNA or dsDNA as large as 1308 nt, albeit inefficiently when present in the dsDNA (54). Consequently, the existence of heterologies introduces a thermodynamic need for energy input. It is noteworthy that in the four-stranded reaction, DNA heteroduplex extension also does not occur in the presence of ATPγS, despite the absence of any heterology (55); the requirement for ATP hydrolysis in this case may reflect either a need for rotation of the two dsDNA molecules or an inability to bind another molecule of dsDNA when dsDNA is bound irreversibly to the RecA protein filament.

These observations define additional roles for ATP hydrolysis beyond dissociation of RecA protein from DNA: to bypass heterologies, to promote DNA heteroduplex extension between DNA duplexes, and to impart a directionality to the DNA strand-exchange process (see below). The ability to bypass heterologies and to exchange DNA between duplex molecules seemed a reasonable justification for extensive ATP hydrolysis by RecA protein when it was known to be the only protein capable of promoting branch migration. Recently, however, two proteins essential to homologous recombination, the RuvAB and RecG proteins, have been shown to promote ATP-dependent branch migration [see (18, 56)]. This raises the question of which protein promotes DNA heteroduplex extension in vivo and whether this particular property of RecA protein is essential to cellular function.

Experimental Assays

Identification of homologous pairing and DNA strand-exchange proteins requires reliable in vitro assays. Several assays have been developed, chiefly to examine the properties of RecA protein–promoted DNA strand exchange. However, although RecA protein can both homologously pair and exchange DNA strands, other proteins may conceivably only pair DNA molecules, with responsibility for DNA strand exchange being relegated to a second factor. Hence, it is worth noting explicitly what each of these assays measures (i.e. only homologous pairing or both pairing and strand exchange).

NITROCELLULOSE FILTER–BINDING ASSAY The first assay used to detect joint molecule formation was the nitrocellulose filter–binding (displacement loop or D-loop) assay (57). Using conditions that minimize retention of DNA on the filter by protein, it is possible to selectively retain DNA with single-stranded character. Experimentally, the reaction contains unlabeled ssDNA and homologous labeled dsDNA (supercoiled or linear). When pairing occurs (producing joint molecules having either ssDNA tails or ssDNA in the D-loop), the labeled DNA is retained on the filter. A recent variation of this assay uses ssDNA immobilized on a filter to detect pairing (58). Although generally reliable, these assays assume that the DNA is

completely deproteinized, which depends on the treatment used (44, 48). The assays are subject to artifacts introduced by nucleases or helicases, which can convert part or all of the labeled dsDNA to ssDNA; this ssDNA is retained, either independently of homologous ssDNA or by renaturation with the ssDNA. Another potential problem is retention of labeled DNA as part of a large aggregate, but this can also be detected by the absence of a requirement for homologous ssDNA. The filter-binding assay measures homologous pairing and not necessarily the exchange of DNA strands since, unless short oligonucleotides are used, the "tail" of the homologously paired ssDNA can account for retention on the filter.

AGAROSE GEL ASSAY Perhaps the most informative assay is the agarose gel assay (46). In this assay, the reaction products are deproteinized and analyzed by gel electrophoresis. Joint molecule intermediates appear as species with lower mobility than either of the DNA substrates. In the absence of topological constraints, the DNA strands can be completely exchanged, resulting in the formation of discrete product molecules. Thus, two potentially distinct phases of the reaction, initial pairing and DNA heteroduplex extension, can be simultaneously analyzed.

The favored substrate pair employed in this assay consists of circular ssDNA and linear dsDNA, because these substrates are easily obtained from ssDNA phages, and because the substrates, intermediates (joint molecules), and products are easily discerned. However, as with any other assay, this assay is subject to potential artifacts, the most prominent of which is nucleolytic digestion of the linear dsDNA by a strand-specific exonuclease. Should this occur, any protein or treatment capable of renaturing ssDNA will produce intermediates with a mobility comparable to that of joint molecules, but that in fact are reannealed molecules. Processive degradation by an exonuclease can result in the production of a species indistinguishable from the gapped circular product, but with complete loss of the displaced strand. This artifact is easily controlled for by individually labeling the ends of the DNA strand that is to be displaced (i.e. the strand in the dsDNA that is identical to the invading strand) and verifying that each end is intact in the joint molecules; it is highly recommended that this control become an absolute requirement in the characterization of any new pairing protein.

DNA helicases can contribute to artifactual pairing if the unwinding of the dsDNA is coupled to the action of a DNA renaturation protein. This artifact would not be revealed by the labeling experiment described above. However, both this artifact and that introduced by nucleases can be avoided by using covalently closed, supercoiled DNA. Joint molecule formation between supercoiled DNA and homologous ssDNA is readily detected,

although extension of the DNA heteroduplex joint is limited by the topological constraint of using covalently closed DNA (59).

NUCLEASE SENSITIVITY ASSAY The most direct assay for DNA strand exchange involves measuring the displacement of ssDNA from a linear dsDNA molecule (46). Using uniformly labeled dsDNA, the existence of a displaced DNA strand can be assayed by adding a ssDNA-specific nuclease (e.g. S_1 or P_1) to deproteinized samples. This assay is perhaps the easiest to quantify and generally yields the most accurate kinetic data and measurements of DNA heteroduplex length. In addition, if the specific activity of the labeled DNA is sufficiently high, the presence of contaminating DNA exonuclease activity can be monitored by intentionally omitting the nuclease. If the nuclease assay conditions do not perturb the joint molecules, this assay is a direct measure of DNA strand exchange.

ELECTRON MICROSCOPY Electron microscopy (EM) provides the most visual evidence of pairing and DNA strand exchange. Micrographs of DNA strand exchange taken before removal of protein can provide striking displays of the pairing process (30, 60), while micrographs of deproteinized samples can demonstrate the presence of a displaced DNA strand [see (14, 21)]. Provided that the spreading procedures do not select for a specific subclass of molecules, EM is a direct assay for both pairing and DNA strand exchange. However, it is neither the most convenient nor the most accessible assay and is subject to the same nuclease and helicase artifacts.

THREE-STRANDED VS FOUR-STRANDED REACTIONS The most common substrates are a ssDNA molecule and a homologous, fully dsDNA molecule (Figure 5*A, reactions 1 and 2*); this is the three-stranded reaction. Because of the enzymatic requirements imposed by the properties of RecA protein, pairing between intact duplex substrates does not occur. However, DNA strand exchange can occur between duplex DNA pairs if one molecule has a homologous ssDNA region 37–52 nt in length (61–63); this is the four-stranded reaction (Figure 5*A, reaction 3*) (64, 65). Pairing and exchange initiate in the ssDNA region, and DNA heteroduplex then extends into the double-stranded region. The characteristics of both reactions are similar, but some differences exist. Most notably, ssDNA-binding protein (SSB protein) is not needed for joint molecule formation when the regions of ssDNA are short (<162 nt) or for exchange between regions of dsDNA (65). In addition, an intermediate in the four-stranded reaction is a bona fide Holliday junction rather than a D-loop joint molecule (66). These particular characteristics may prove useful in the identification of novel pairing proteins.

DETECTION OF PARANEMIC JOINT MOLECULES Every pair of DNA substrates designed to detect plectonemic joint molecule formation is also capable of forming paranemic joint molecules (32, 44, 61–63, 67–69). Paranemic pairing can be studied directly using DNA substrates that either are covalently closed or are prevented from pairing at DNA ends by the presence of heterologous DNA sequences (typical substrate pairs are shown in Figure 5*B*). Since paranemic joint molecules are unstable when deproteinized, any of the assays described above can be used as long as bound proteins are not removed. Alternatively, paranemic pairing can be detected by treating the closed circular molecule containing the paranemic joint with a topoisomerase; a homology-dependent perturbation of the linking number confirms the presence of pairing (61–63, 69). Since the presence of bound protein can affect the accuracy of all of the aforementioned assays, dependence on DNA sequence homology must be absolute, even though transient interactions with heterologous DNA may result in a much smaller but still detectable unwinding of supercoiled DNA (70).

POTENTIAL ARTIFACTS All pairing reactions are susceptible to artifacts, because any activity that generates ssDNA can yield a positive result due to renaturation of complementary regions. Although a potentially interesting reaction in itself, this does not constitute DNA strand exchange. Biochemical activities that contribute to such artifacts include strand-specific dsDNA exonucleases, helix-destabilizing proteins, and helicases. Trace amounts of strand-specific dsDNA nuclease activity can generate sufficient ssDNA in the dsDNA substrate to permit reannealing with the ssDNA; results with ATP-independent pairing proteins demonstrate that 20 nt or less of homologous ssDNA are sufficient (see below). Thus, nucleolytic degradation corresponding to as little as 0.3% of a 6 kb dsDNA substrate would suffice to produce pairing by DNA renaturation rather than by DNA strand exchange. Helix-destabilizing proteins (e.g. *E. coli* SSB protein) can potentially lower the T_m of dsDNA below the assay temperature. Upon deproteinization of the assay mixture prior to analysis, the free DNA strands can spontaneously renature to give the appearance of ATP-independent DNA strand exchange. The presence of helicase activity is particularly misleading because unwinding requires ATP hydrolysis; thus, helicases also introduce artifactual ATP dependence to an apparent DNA strand-exchange reaction. The artifactual results caused by these activities are compounded when deproteinizing conditions that enhance renaturation are used. For example, drying of the DNA following ethanol precipitation led to an incorrect assignment of DNA strand-exchange activity to histone H1 (71, 72). Phenol extraction in the presence of salt is another example that led to the detection of an artifactual pairing activity in *S. pombe* nuclei that resulted from nuclease activity (73).

For these reasons, DNA strand-exchange assays of partially purified fractions, particularly those using linear dsDNA, can be notoriously unreliable. Most, but not all, of these artifacts can be minimized by using covalently closed dsDNA as one of the substrates. Since DNA shorter than ~400 bp is particularly susceptible to denaturation (73a), DNA substrates greater than this length should be used.

ATP-DEPENDENT DNA STRAND-EXCHANGE PROTEINS

The *E. coli* RecA protein was the first DNA strand-exchange protein discovered; consequently, its properties have served as a benchmark against which all newly discovered proteins are compared. Genes encoding proteins with high degrees of similarity to RecA protein have been identified in every prokaryote examined. Thus, it is likely that the biochemical properties of RecA protein are characteristic of a broad and ubiquitous family of DNA strand-exchange proteins.

Escherichia coli *RecA Protein*

The RecA protein (M_r 37,842) was discovered as a DNA-dependent ATPase and as a DNA- and ATP-dependent coprotease (74, 75, 75a). Subsequently, the RecA protein was found to possess ATP-stimulated DNA renaturation and ATP-dependent DNA strand-exchange activities (2–6). The unique DNA strand-exchange activity almost certainly reflects the protein's intracellular recombination function, although a role for its DNA renaturation activity in vivo cannot be eliminated [see (76)]. The DNA strand-exchange activity of RecA protein consists of three major phases: presynapsis, synapsis, and DNA heteroduplex extension [Figure 1; see (7, 12, 14, 16, 17, 19, 21, 22)].

PRESYNAPSIS The first step of DNA strand exchange is the assembly of RecA protein on ssDNA to form a right-handed helical structure known as the presynaptic complex. The assembly of RecA protein on ssDNA is polar, with association and dissociation occurring in the 5′→3′ direction (77, 78). This structure has 6.2 monomers per turn, a pitch of ~95 Å, and a diameter of ~100 Å [(14, 21, 79, 80) and references therein]. The most unusual characteristic of this complex (as well as the one formed with dsDNA) is that the DNA is extended ~50% relative to B-form DNA, increasing the axial spacing between basepairs to 5.1 Å, and unwinding the DNA to 18.6 bp per turn. Assembly into the active form requires ATP, dATP, or ATPγS and a saturated complex (one monomer per 3–4 nt). Thus, for the typical ssDNA substrate used in vitro, the functional form of RecA protein in the

homology search is a filament of approximately 2000 protein monomers. This complex is capable of hydrolyzing ATP at a modest rate (k_{cat}) of 25–30 min^{-1} (81–83). ATP hydrolysis—though it accompanies this and the subsequent steps—is not required for presynaptic complex formation, the homology search, or DNA strand exchange (37, 38, 46–48, 53; SC Kowalczykowski, RA Krupp, in preparation).

SYNAPSIS The presynaptic filament is capable of rapidly searching for DNA sequence homology. Although the details of the homology search remain unclear [see (16) for discussion of the limitations of existing data regarding the kinetics of this process], it is certain that the first step involves the formation of random nonhomologous contacts. These interactions typically result in large, easily sedimented complexes of nonhomologously paired ssDNA and dsDNA called coaggregates (84, 85); they are detected under many, but not all, conditions that support DNA strand exchange (86–89). The heterologous contacts are promiscuous, being independent of orientation of the DNA strands and capable of recognizing either complementary or identical sequences (90), and they lead to a transient unwinding of the dsDNA (70). The minimum length of homology required for recognition in vitro can be as low as 8 nt (91). Iteration of these random collisions is envisioned ultimately to align a region of homology; thereafter, the two DNA molecules pair homologously along their length. Since this process occurs with equal efficiency and rate in the absence of ATP hydrolysis, the mechanism of the homology search must be completely passive. The recognition of DNA sequence homology results in formation of a region of nascent DNA heteroduplex estimated to range from 100 to 300 bp in length (32, 35, 92). Plectonemic joint molecule formation occurs at the homologous ends of the DNA substrate pairs (Figure 5*A*). Pairing between circular ssDNA and linear dsDNA occurs at either end of the dsDNA; however, pairing between linear ssDNA and supercoiled dsDNA occurs preferentially at the 3′ end of the ssDNA (59), primarily as a consequence of the polarity of RecA protein assembly/disassembly (see Ref. 92a for discussion).

DNA HETERODUPLEX EXTENSION The region of DNA heteroduplex formed in the synaptic phase can enlarge, provided there is no topological constraint. In the RecA protein–promoted reaction, this process is not random but, instead, is protein-mediated. The direction of DNA heteroduplex formation is 5′→3′ relative to the displaced ssDNA (or the invading ssDNA) (6, 93–95), which is the same direction as RecA protein polymerization (77). This phase of DNA strand exchange requires ATP hydrolysis (37, 46), and introduces torsional strain into the dsDNA (96, 97). Under typical reaction conditions, RecA protein–promoted DNA heteroduplex extension occurs at

a rate of 2–10 bp s^{-1} (46, 93, 94), leading to the complete exchange of DNA strands between substrates 7 kb in length.

STIMULATORY FACTOR: SSB PROTEIN DNA strand exchange is stimulated by the *E. coli* SSB protein (65, 98); other ssDNA-binding proteins from both prokaryotic and eukaryotic sources function similarly (82, 99–102). Each of these stimulatory proteins binds cooperatively and preferentially to ssDNA (103). Thus, the stimulatory effects of SSB protein are mediated through its interaction with ssDNA rather than through specific protein-protein interactions. The binding of RecA and SSB proteins to ssDNA is competitive, with the outcome determined by reaction conditions (82, 104–106).

The stimulatory effects of SSB protein are manifest both pre- and postsynaptically (Figure 6). Presynaptic complex formation is impeded by the presence of DNA secondary structure, to which RecA protein cannot bind (82, 104, 105). SSB protein removes this impediment to complete presynaptic complex formation by removing the secondary structure. About one SSB protein monomer per 15 nt ssDNA is required for optimal presynaptic complex formation. In addition, SSB protein eliminates aggregation of ssDNA caused by RecA protein binding (85). Thus, joint molecule formation is stimulated by eliminating DNA secondary structure, which permits formation of a continuous filament, and by preventing nonproductive aggregation. Excess SSB protein often inhibits RecA protein–dependent activities (98).

Postsynaptically, SSB protein serves two functions. The first is to prevent formation of homologously paired networks of DNA that result from intermolecular reinvasion events (107). The single strand displaced from one DNA molecule can reinvade another dsDNA molecule in an infinite pattern, causing formation of extensive, basepaired DNA networks. Because RecA protein polymerizes 5′ → 3′ (77) and nucleates binding randomly, SSB protein can bind to the 5′ end of the displaced linear ssDNA and hinder its utilization by RecA protein [although under conditions where displacement of SSB protein from ssDNA by RecA protein is enhanced, network formation still occurs (87, 108)]. Beyond this sequestration role, SSB protein plays a direct role in joint molecule formation. This is detected under conditions where the presynaptic role is completely bypassed through the inclusion of volume-excluding agents (e.g. polyethylene glycol or polyvinyl alcohol) (109, 110). Under these conditions, the requirement for SSB protein is directly proportional to the amount of ssDNA produced by DNA strand exchange, and not to the amount of ssDNA initially present in the reaction. The binding of SSB protein to the displaced ssDNA directly

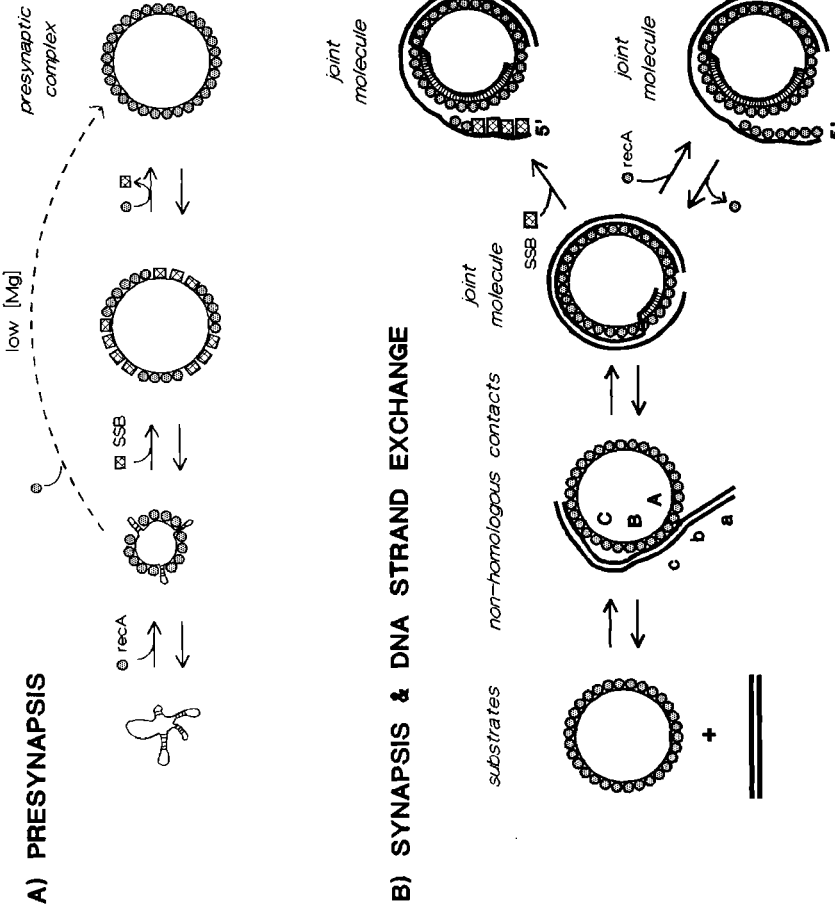

Figure 6 Models for the stimulatory effects of SSB protein on RecA protein–mediated DNA strand exchange during both presynaptic (*A*) and postsynaptic (*B*) steps. See text for details. From (110).

stimulates, by almost 10-fold, the observed rate of joint molecule formation, presumably by preventing the reverse reaction.

DNA RENATURATION ACTIVITY The ability of RecA protein to renature ssDNA was recognized before its DNA strand-exchange activity. Renaturation is optimal at lower molar ratios of RecA protein to ssDNA (1 monomer per 30 nt), where most of the ssDNA is devoid of protein (2, 111, 112). This renaturation is unusual because it is stimulated 2–3-fold by ATP, but it retains the characteristics of the ATP-independent reaction (112). In contrast to many protein-promoted renaturation reactions [e.g. by SSB and T4 gene 32 proteins (G32P) (113, 114)], the reaction is first order rather than second order in DNA concentration. DNA renaturation coincides with conditions that promote extensive aggregation of ssDNA, and both aggregation and renaturation activities of RecA protein are inhibited by SSB protein (85, 111). Thus, the mechanism of DNA renaturation most likely involves a rapid condensation of ssDNA into aggregates; the bimolecular reaction becomes unimolecular because of the high effective DNA concentration. The products of the reaction are normally large intermolecularly basepaired networks, but in the absence of ATP, simple unit-length dsDNA can be obtained (115). The biological role of RecA protein–promoted renaturation remains an open question, because there currently exists no mutant RecA protein differentially affected in its DNA strand-exchange and DNA renaturation activities (76).

Proteus mirabilis *RecA Protein*

The RecA protein from *P. mirabilis* (M_r 38,176) is 73% identical to that of *E. coli* (116). Not surprisingly, it complements an *E. coli recA* mutation (117) and has all the activities of the *E. coli* protein: ssDNA-dependent ATPase (118), DNA strand exchange (118), and LexA repressor cleavage (119) activities. The protein can promote DNA strand exchange using many of the substrate pairs used by the *E. coli* protein, including ssDNA and supercoiled DNA, circular ssDNA and linear dsDNA, and gapped and linear dsDNA substrates. In contrast, *E. coli* SSB protein reduces the ATPase activity of *P. mirabilis* RecA protein by about 80%, which suggests that *P. mirabilis* RecA protein can only partially resist displacement by SSB protein from ssDNA. Consistent with this inhibitory effect of SSB protein, *P. mirabilis* RecA protein does not complete the exchange of DNA strands between circular ssDNA and linear dsDNA when *E. coli* SSB protein is present, limiting the reaction to formation of intermediate joint molecules (118). The behavior of *P. mirabilis* SSB protein in these reactions is untested.

Bacillus subtilis *RecA (RecE) Protein*

The *B. subtilis* RecA (RecE) protein (M_r 38,300), which displays 60% identity with the *E. coli* protein (120), is encoded by the gene previously known as *recE* (121). RecA protein has both DNA strand-exchange and *E. coli* LexA protein cleavage activities (122). Despite these parallels, the *B. subtilis* protein is unable to hydrolyze ATP; it can, however, hydrolyze dATP at a rate about 65% that of *E. coli* RecA protein. DNA strand exchange requires dATP, and ATP is an inhibitor of both the dATPase and the DNA strand-exchange activities. In the presence of dATP and *E. coli* SSB protein, 60% of the linear dsDNA and circular ssDNA is converted to complete DNA strand-exchange product. The requirement for dATP is unusual, but this characteristic is mimicked by a mutant *E. coli* RecA protein, RecA K72R protein, which also requires dATP for DNA strand-exchange activity (50), of which ATP is also a competitive inhibitor (WM Rehrauer, SC Kowalczykowski, unpublished observation).

Thermus aquaticus *RecA Protein*

A RecA protein homolog was isolated from the thermophilic eubacteria, *Thermus aquaticus* (50a; JG Wetmur, DM Wong, B Ortiz, J Tong, F Reichert, DH Gelfand, personal communication). The protein has 59% identity and 78% similarity to the *E. coli* RecA protein. Binding to ssDNA requires ATPγS for detection, is optimal at about 55°C, and is detectable to about 70°C. The protein possesses an optimum for DNA-dependent ATPase activity above 70°C. Joint molecule formation occurs at the optimal temperature of 65°C. The yield of joint molecules formed is about 4–5-fold less efficient than that promoted by *E. coli* RecA protein, and DNA heteroduplex formation is limited (i.e. the complete exchange of DNA strands between M13 DNA substrates does not occur) (50a). The failure to promote extensive DNA heteroduplex formation may have been due to the use of *E. coli* SSB protein in the reactions. RecA proteins were also isolated from three other thermophiles: *Thermus thermophilus, Thermotoga maritima,* and *Aquifex pyrophilus* (JG Wetmur et al, personal communication); the cognate SSB proteins are yet to be isolated.

Bacteriophage T4 UvsX Protein

The bacteriophage T4 analog of *E. coli* RecA protein is encoded by the *uvsX* gene. UvsX protein (M_r 43,760) bears many biochemical similarities to RecA protein, despite being the most divergent of the prokaryotic RecA-like proteins; it has only 23% identical and 15% similar residues (123). Most of the identities cluster in the ATP-binding site, with the remaining conserved residues being primarily hydrophobic amino acids

important to tertiary or quaternary structure (124). Next to *E. coli* RecA protein, UvsX protein is the best characterized DNA strand-exchange protein. The mechanism of UvsX protein–promoted DNA strand exchange, while different in some important details from that of RecA protein, is the same globally as that of RecA protein: Presynaptic complex formation results in a helical UvsX protein–ssDNA filament; synapsis results in both paranemic and plectonemic joint molecules; and DNA heteroduplex extension results in the complete exchange of 6–7 kb of DNA. However, UvsX protein appears to be more dynamic in its kinetic behavior than RecA protein and, perhaps most significantly, interacts directly with a novel auxiliary factor, the UvsY protein (see below).

UvsX protein has ssDNA-dependent NTPase, DNA renaturation, and DNA strand-exchange activities (125–128). The ATPase activity of UvsX protein is distinctive among RecA-like proteins, producing both ADP and AMP, and its rate (k_{cat}) is ~15-fold greater than that of RecA protein (240 ADP and 145 AMP min^{-1}) (127). UvsX protein binds both ssDNA and dsDNA cooperatively with a stoichiometry of one monomer per 3–5 nt, and forms a presynaptic filament with a structure similar to that made by RecA protein (129). ATP or ATPγS binding stabilizes the UvsX protein–ssDNA filament (125), suggesting that the appropriate nucleoside triphosphate induces a transition to a higher-affinity state. Joint molecule formation, which is poor in the absence of stimulatory factors (see below), yields both paranemic and plectonemic molecules (130). The DNA strand-exchange reaction displays an optimum in UvsX protein concentration: lower concentrations are suboptimal for presynaptic complex formation, and higher concentrations (which exceed saturation of the ssDNA) reduce joint molecule formation due to binding of UvsX protein to dsDNA (130). Branch migration by UvsX protein (15 bp s^{-1}) is somewhat faster than that promoted by RecA protein (131, 132). Because of the higher rate of ATP turnover and the concomitant increased rate of protein dissociation, the UvsX protein–ssDNA filament is more dynamic than the RecA protein–ssDNA filament (132, 133).

STIMULATORY FACTOR: GENE 32 PROTEIN DNA strand exchange promoted by UvsX protein is enhanced by the T4 phage–encoded ssDNA-binding (helix-destabilizing) protein, G32P. This protein is a 33.5-kDa analog of SSB protein that binds preferentially and cooperatively ($\omega \approx 10^3$) to ssDNA (103). G32P increases both the rate and the yield of joint molecule formation (134). The optimal concentration of G32P (one monomer per 8–10 nt) needed for DNA strand exchange, when UvsX protein is present at suboptimal concentrations, represents the amount needed to saturate the ssDNA. Maximal joint molecule formation in the presence of G32P can occur at

subsaturating concentrations of UvsX protein (one monomer per 8 nt) (125, 134, 135), but excess G32P inhibits both the ATPase and the joint molecule formation activities of UvsX protein. SSB protein can substitute for G32P, although the rates of joint molecule formation and of complete exchange between DNA substrates are more than 5- and 15-fold slower, respectively (127, 134). In contrast to the *E. coli* system, where SSB protein typically acts in both the pre- and postsynaptic phases, G32P is not needed in presynapsis when UvsX protein is in large excess over the DNA concentration (135). Under these conditions, G32P seems to function only in the postsynaptic phase by stabilizing the displaced strand (135), in much the same way that SSB protein functions (110).

The mechanism of the homology search is not well understood. Like RecA protein, UvsX protein can coaggregate nonhomologous ssDNA and dsDNA, but these coaggregates are not detected under optimal conditions in the presence of G32P (134). The polarity of pairing and DNA strand displacement is also 5′→3′ relative to the displaced strand (125, 132), and is stimulated 5–10-fold by G32P (132). DNA strand exchange promoted by UvsX protein between circular ssDNA and linear dsDNA commonly results in the formation of homology-dependent DNA networks that fail to enter an agarose gel; though not typically reported for RecA protein, homology-dependent networks are the major product under conditions that enhance the ability of RecA protein to displace SSB protein (87, 108, 109). This parallel in network formation argues that UvsX protein can more effectively remove G32P from the displaced strand of DNA and utilize this ssDNA in subsequent invasion events. As with RecA protein, DNA heteroduplex extension is stopped by the addition of ATPγS to an ongoing reaction, but, unlike RecA protein, a brief acceleration in the rate is seen (127, 132). This transient increase was interpreted to mean that DNA strand exchange could not only occur in the presence of ATPγS, but that the stabilizing effect of ATPγS actually provided a burst of enhanced exchange; due to the inability of UvsX protein to redistribute itself in the presence of ATPγS, further DNA strand exchange was prohibited. A similar conclusion was reached for RecA protein, based on very different experiments [see (16)].

STIMULATORY FACTOR: UVSY PROTEIN The T4 phage recombination system is unique in that both genetic and biochemical data demonstrate the need for an accessory protein that, as yet, has no counterpart in any other system. This protein, UvsY protein (M_r 16,000), binds cooperatively to both ssDNA and dsDNA (136). It interacts directly with UvsX protein in a 1:1 molar ratio (137). UvsY protein increases the rate of UvsX protein–dependent ATP hydrolysis by 2–3-fold under suboptimal conditions and DNA strand exchange by ~3-fold (133, 136, 137). Because UvsY protein increases the

apparent affinity of UvsX protein for ssDNA, UvsX protein has increased resistance to displacement from ssDNA by G32P (136, 137). All of these important stimulatory effects are specific to UvsX protein and are not observed with RecA protein.

A complex series of interactions are proposed for the reaction containing all three proteins (138). UvsY protein interacts with the carboxyl terminus of G32P, and this interaction is necessary to load UvsY protein on the ssDNA. Once bound to the DNA, UvsY protein promotes the binding of UvsX protein to the DNA, presumably through direct protein-protein interactions. It appears that not all of the G32P is displaced by the binding of UvsX and UvsY proteins; it has been suggested that G32P remains associated with the DNA-bound UvsX-UvsY complex via protein-protein interactions (139). The T4 phage system represents the best example of functionally important specific protein-protein interactions in a DNA strand-exchange reaction; if there is any counterpart to the UvsX-UvsY protein interaction, it may be that of the *S. cerevisiae* Rad51 and Rad52 proteins (140) (see below).

Pisum sativum *RecA Protein*

A 39-kDa protein that is immunologically related to *E. coli* RecA protein was identified in pea (*Pisum sativum* L.) chloroplasts (141) and, consistent with this observation, genomic (but not chloroplast) DNA of pea hybridizes to a *Synechococcus recA* probe (142). Extracts of these chloroplasts were subsequently shown to possess DNA strand-exchange activity (142a), which is both ATP- and Mg^{2+}-dependent. Both linear dsDNA by circular ssDNA and linear ssDNA by supercoiled DNA substrate pairs form joint molecules. Because the linear DNA in each case was end-labeled, these assays were controlled for the potential occurrence of exonuclease-dependent renaturation. EM analysis confirms the existence of a displaced strand (142a).

Ustilago maydis *Rec1 Activity*

The first, and so far only, eukaryotic ATP-dependent homologous pairing activity to be purified is from *U. maydis*. It was initially called Rec1 protein because its activity was not detectable in preparations from a *rec1* mutant strain (143, 144); it is now clear that this protein is not the product of the *REC1* gene (145–147), which encodes a 3′→5′ exonuclease lacking pairing activity (148). The protein that encodes this pairing activity is not known, but recently it was found that the *U. maydis* Rec2 protein (M_r 84,000) bears similarity to RecA protein (BP Rubin, DO Ferguson, WK Holloman, personal communication). Homology resides in the regions required for nucleotide binding and, in this respect, it is similar to other eukaryotic structural homologs of RecA protein (see below). Until the genetic identity

of the Rec1 activity is determined, the term "Rec1 is maintained to indicate the first pairing activity identified in *U. maydis* (149).

The Rec1 activity (estimated molecular weight of 70,000) catalyzes ssDNA-dependent ATP hydrolysis, renaturation, and DNA strand exchange (143, 149, 150). Somewhat unexpectedly, the protein neither binds ssDNA cooperatively nor forms filaments, although it does bind Z-DNA with greater affinity [2–6-fold (151) to 20–75-fold (152)] than it binds B-DNA. The ATPase activity is cooperative in protein concentration (Hill coefficient of 1.8), and the specific activity ($\sim$225 min^{-1}) is comparable to that of UvsX protein but is $\sim$10-fold greater than that of RecA protein (143, 149). Like the case of RecA protein, the DNA renaturation activity is first order in DNA concentration, is stimulated by ATP (10–15-fold), and is optimal at substoichiometric concentrations (1 monomer per 300 nt); unlike the case of RecA protein, the amount of renatured DNA product is proportional to the amount of protein present, suggesting that the protein does not turnover in this reaction (149).

Rec1 activity homologously pairs the same kinds of substrates used by RecA protein (143, 150) and, in addition, pairs fully duplex DNA substrates (143, 150, 153, 154). Unlike the reaction with the prokaryotic proteins, a fraction (40%) of the joint molecules detected using ssDNA fragments and supercoiled DNA are formed independently of ATP, but DNA heteroduplex extension is fully ATP dependent (143). In the presence of the nonhydrolyzable ATP analog, AMP-PNP, the joint molecules formed are apparently paranemic (150) and contain unwound dsDNA. Joint molecule formation occurs preferentially, displacing the 3′ end of the linear duplex DNA (150); this polarity is opposite that of both RecA and UvsX proteins. Unlike either RecA or UvsX protein, Rec1 activity can pair two supercoiled DNA molecules provided that a topoisomerase and either homologous ssDNA fragments or actively transcribing RNA polymerase are present (153). Thus, generation of a displaced strand in one of the DNA molecules is sufficient for pairing between intact duplex DNA molecules. Since as little as one protein monomer per 200 nt is sufficient for pairing (143), it appears that this activity is not required in stoichiometric amounts. In sum, although there are similarities to the *E. coli* model, the Rec1 pairing activity displays some notable differences.

ATP-INDEPENDENT DNA STRAND-EXCHANGE PROTEINS

The discovery of DNA strand-exchange activity in *E. coli* stimulated a search for similar activity in other organisms. Given the importance of recombination, it was not surprising that such an activity was found in

many species. But the discovery of ATP-independent DNA strand exchange, promoted by activities primarily from eukaryotic sources, seemed to controvert the well-established properties of the RecA protein–promoted reaction. The subsequent realization that ATP binding, and not hydrolysis, was sufficient for DNA strand exchange by RecA protein blunted this criticism, because it could be argued that the ATP-independent proteins represented a class of proteins that were equivalent to the active form of RecA protein that results from ATP binding [see (9)].

This encompassing explanation proved not to be accurate. Careful characterization of these ATP-independent reactions uncovered the presence of nuclease activity that, in at least a few cases, was intrinsic to the purified pairing protein (see below). Most of the assays conducted with this class of putative DNA strand-exchange proteins involved the typical circular ssDNA and linear dsDNA substrates. In several well-documented cases, it is now clear that the observed DNA pairing and/or strand-exchange activity does not occur between ssDNA and an intact dsDNA molecule, but only with digested dsDNA containing a ssDNA tail (Figure 2). Thus, pairing is initiated by reannealing of two ssDNA regions rather than by DNA strand invasion and displacement.

The model in Figure 2 illustrates a mechanism for homologous pairing and strand exchange that describes the reactions promoted by the ATP-independent class of activities. The first step requires resection by a strand-specific dsDNA exonuclease; it appears that a nuclease of either polarity will suffice and that the polarity of this degradation step determines the observed apparent polarity in the subsequent DNA strand-exchange step. The nuclease activity is intrinsic to some, but not all, pairing proteins (see below). The second step involves the protein-mediated renaturation of the complementary ssDNA. These two steps are sufficient to be interpreted as DNA strand-exchange activity in nearly any in vitro assay even though no exchange of strands has occurred. The next step is displacement of ssDNA by a process that can be referred to as DNA strand exchange but is mechanistically more akin to the DNA heteroduplex extension phase of the RecA protein–promoted reaction. This step has an inherent asymmetry attributable to the DNA degradation step. Thermodynamic considerations dictate that the reaction can proceed in only one direction so as to maximize both the number of basepairs formed and the entropy of the products. Consequently, random thermal branch migration would appear unidirectional, with a displaced strand being liberated only if exchange occurs in one direction (to the right in Figure 2). The process may be accelerated by the pairing protein, as appears to be the case for the *E. coli* RecT protein (see below) (155). This model is biochemically distinct from the model for DNA strand exchange promoted by RecA protein.

The biological role of the ATP-independent DNA strand-exchange proteins is difficult to assess because genetic analysis is either non-existent or complex. Notable exceptions include the *E. coli* RecT and λ phage β proteins, which are important to certain types of recombination events, and the *Drosophila* Rrp1 protein, which is important in DNA repair (see below).

It is too early to say whether all ATP-independent proteins act by a similar mechanism, but they obviously do not imitate the behavior of RecA protein. It is also unclear how many of these proteins actually function in recombination, precluding their description as recombination proteins. The realization that most, if not all, of these proteins act on nuclease-digested DNA calls for a re-examination of their characteristics. Until then, it would be prudent to refer to such proteins as either reannealing or homologous pairing proteins, and not DNA strand-exchange proteins, unless the displacement of ssDNA by strand exchange is demonstrated to be protein dependent.

Escherichia coli *RecT Protein*

The RecE and RecT proteins are encoded by a genetic locus that was originally designated *recE*. This locus is part of a cryptic lambdoid prophage, *rac,* and is composed of two genes, *recE* and *recT,* which encode a nuclease (exonuclease VIII) and a DNA-binding protein, respectively (156, 157). These genes bear functional, but not sequence, similarity to the bacteriophage λ recombination genes, *redα* and *redβ* (see below). The *recT* gene can complement a *recA* defect in plasmid recombination, arguing that the RecT protein must possess an activity that either alone or in concert with other proteins is functionally equivalent to one possessed by RecA protein (156, 158).

The 33-kDa RecT protein is a tetramer in solution (157). It binds to ssDNA, but not to dsDNA; half-maximal binding, as monitored by a nitrocellulose filter-binding assay, occurs at a stoichiometry of one tetramer per 80 nt. Like the β protein of bacteriophage λ, RecT protein promotes ATP-independent, partially (75%) Mg^{2+}-dependent renaturation of ssDNA (157).

In addition to renaturation, RecT protein can promote homologous pairing between circular ssDNA and linear dsDNA, provided that one strand of the linear dsDNA is digested to produce a homologous ssDNA tail (155). This pairing activity depends absolutely on prior nucleolytic function, but accepts both 3′ and 5′ ssDNA tails. Any nuclease (e.g. exoIII, exoVIII, or T7 gene 6 protein) can serve in this capacity. Thus, the reaction must initiate by renaturation of the complementary regions of ssDNA. The pairing reaction is ATP independent, is Mg^{2+} dependent, and requires at least one RecT protein monomer per 13 nt. The region of DNA heteroduplex is not restricted to the region of resected DNA, but extends into the region of intact dsDNA;

displacement of ssDNA can be detected by EM. This displacement appears to depend on a unique RecT protein activity, because another protein (histone H1) capable of promoting the initial DNA renaturation step cannot displace ssDNA (155).

Bacteriophage λ β Protein

Bacteriophage λ encodes two proteins, λ exonuclease and β, that are essential for phage-specific recombination. The β protein (M_r 28,000) has biochemical properties that partly resemble those of RecA protein and partly those of SSB protein (99, 159). β protein has neither nuclease nor D-loop formation activities, but like SSB protein, it stimulates the activity of RecA protein (99). Stimulation is particularly evident at suboptimal concentrations of RecA protein, and requires a stoichiometric amount of β protein (one monomer per 4 nt).

Under conditions of one protein monomer per 6 nt, β protein also possesses a first-order DNA renaturation activity (99, 159). It is not known whether β protein can promote a pairing reaction using resected dsDNA. The genetic and biochemical similarities between the *recET* system and the phage λ *red* system suggest that the combined actions of a strand-specific dsDNA exonuclease and an annealing protein constitute a biochemical alternative to the type of pairing reaction promoted by RecA protein. This parallel leaves open the possibility that other functionally similar proteins exist (see following sections).

Saccharomyces cerevisiae *Sep1/STPβ*

Sep1 (Strand-exchange protein 1) and STPβ (Strand Transfer Protein β) are independent isolates of a yeast protein that effects transfer between circular ssDNA and linear dsDNA (160, 161). Initially, Sep1 was purified as a 130-kDa proteolytic fragment, which accounted for the initial size discrepancy between it and STPβ (180 kDa). The gene (*SEP1/DST2*) encoding Sep1/STPβ has been identified, and it encodes a protein of 175 kDa (162, 163).

Unlike for RecA protein, the homologous pairing and DNA strand-exchange reaction promoted by Sep1/STPβ (hereafter referred to simply as Sep1) is slightly inhibited by, rather than dependent on, ATP (160). Sep1 has both DNA renaturation (164) and exonuclease activities (165). The latter activity is responsible for resection of the linear dsDNA to reveal ssDNA that is utilized by the DNA reannealing activity to produce paired complexes. As for *E. coli* RecT protein, a ssDNA tail of at least 20 nt of either polarity is required for pairing (165a); in the absence of nuclease activity (e.g. in the presence of Ca^{2+}), no plectonemic pairing occurs (165). Consequently, the initiation phase of pairing for Sep1 proceeds by an annealing, rather

than a strand invasion, reaction. Maximal pairing was reported to require about one Sep1 fragment (p^{130}) monomer per 12–14 nt or one intact Sep1 monomer per 35–40 nt (165), but recent work finds that optimal joint molecule formation occurs at about 1 Sep1 monomer per 100 nt (165b). This value is in agreement with direct ssDNA-binding studies that yield a binding site size of 70–100 nt and coincides with the amount needed to aggregate the DNA (165b). The stoichiometric requirement for Sep1 protein can be alleviated more than 10–30-fold by loading the protein onto the ssDNA ends of resected dsDNA (165b). Sep1 displays no end-bias in joint molecule formation, and strand displacement proceeds 5′→3′ (relative to the displaced strand), consistent with the polarity of strand degradation. Based on EM observations, Sep1 action can result in the net displacement of 4.1 kb (165); it is not known whether Sep1 directly promotes this exchange step. Recently, Sep1 was found to promote paranemic joint formation, in a reaction that required at least 41 bp of homology (J Chen, R Kanaar, NR Cozzarelli, personal communication). Paranemic joints between ssDNA and dsDNA were detected by both filter-binding and EM assays and, interestingly, pairing between supercoiled DNA and linear dsDNA was also observed.

The fragment of Sep1 protein (p^{130}) binds noncooperatively to both ssDNA and dsDNA (164). Its affinity for ssDNA is higher than that for dsDNA. p^{130}-ssDNA complexes are stable to 200 mM NaCl, but both ssDNA renaturation and DNA strand exchange are inhibited well below this salt concentration, arguing that a step succeeding ssDNA binding must be responsible for the salt sensitivity. As for RecA protein, optimal renaturation of ssDNA occurs at a substoichiometric concentration of p^{130} (~one monomer per 100 nt) (164).

The exonuclease activity of Sep1 has been extensively characterized. This protein was initially identified as an exoribonuclease, Xrn1, which processively degrades both poly(A)$^+$-tailed RNA and rRNA in a 5′→3′ manner (166). In addition, the protein has RNaseH activity (167). Although the early experiments did not detect nuclease activity on DNA substrates, subsequent work showed that Sep1 degrades both ds- and ssDNA (at rates of 20 and 70 mol nt per min per mol protein, respectively), although ssRNA is preferred (165, 165a, 166). The nuclease activity requires Mg^{2+}, is inhibited by Ca^{2+}, displays a pH optimum of 8.5, and has an average processivity of 45 nt. The polarity of this intrinsic exonuclease activity dictates the apparent polarity of DNA strand exchange (5′→3′) promoted by Sep1.

Mutations in the gene encoding Sep1 exhibit pleiotropic effects. In addition to its identification as an exoribonuclease (*XRN1*) (168), this gene was also identified as being involved in nuclear fusion (*KEM1*) (169) and in the maintenance of plasmids containing a defective ARS (autonomously

replicating sequence) (*RAR5*) (170). An essential gene (*RAT1/TAP1/HKE1*) with homology to *SEP1* was also isolated (171, 172, 172a). It encodes a 116-kDa 5′→3′ exoribonuclease that is implicated in mRNA trafficking and transcriptional activation. The meiotic *S. pombe* homolog of Sep1, exoII, was initially purified as a ssDNA nuclease (173). Mutations in *SEP1/DST2* have a slight defect (2–3-fold) for intragenic mitotic recombination (162, 163), but no intergenic defect (169); they do not sporulate, arrest in pachytene, and show certain defects in some, but not all, recombination assays (15, 15a, 173a). Formation and processing of dsDNA breaks occurs in *sep1* mutants, but the level of recombination is reduced. It appears that the absence of a striking recombination phenotype is at least partially due to redundant functions. The *sep1Δdmc1Δ* or *sep1Δrad51Δ* double mutants display more severe defects than any single mutation; meiotic intrachomosomal recombination was reduced more than 20-fold and meiotic interchromosomal recombination was partially reduced (D Tishkoff, B Rockmill, GS Roeder, RD Kolodner, personal communication). This complicated behavior potentially argues for a direct role for Sep1 in meiotic recombination and, in addition or alternatively, these phenotypes are indirect consequences of the pleiotropic physiological defects of *sep1* mutants.

STIMULATORY FACTOR: yRPA A number of yeast ssDNA-binding (ySSB) proteins stimulate the pairing activity of Sep1. One of these is the large subunit of a heterotrimeric protein, known as yeast replication protein A (yRPA) (174). Though normally isolated as a complex consisting of 69-, 36-, and 13-kDa polypeptides, a 34-kDa proteolytic fragment of the large subunit, encompassing the central portion of the polypeptide and containing the Zn^{2+} finger DNA-binding domain, can by itself stimulate the activity of Sep1 (101).

Addition of the 34-kDa fragment results in an 18-fold increase in the initial rate, primarily by reducing a kinetic lag in the formation of joint molecules (101). This ySSB protein does not change the sigmoid dependence on Sep1 concentration, but it does reduce (by ~2–3-fold) the amount of Sep1 required for optimal levels of DNA pairing. There is little enhancement of DNA pairing by ySSB protein at saturating concentrations of Sep1. Likewise, a variety of other ssDNA-binding proteins stimulate joint molecule formation by Sep1 (161). A 50-fold stimulation is observed at optimal concentrations, and up to 1.5 kb of heteroduplex DNA is formed. Only 2–3 molecules of Sep1 per linear dsDNA molecule are required when optimal concentrations of these ySSB proteins are present (161).

The trimeric yRPA holoprotein also stimulates Sep1, with maximal stimulation occurring at saturating concentrations of yRPA (102). yRPA binds ssDNA with a stoichiometry of one molecule per 90 nt, forming a

beaded structure similar to that formed by *E. coli* SSB protein. It binds with both high affinity ($>10^9$ M^{-1}) and cooperativity ($\omega = 10^4$–10^5). Excess yRPA inhibits pairing, but not nuclease, activity. It has been proposed that this ySSB protein stimulates the activity of Sep1 by inhibiting the aggregation of ssDNA and promoting the coaggregation of ssDNA and dsDNA molecules (102).

STIMULATORY FACTOR: SF1 SF1 (Stimulatory Factor 1) is a 55-kDa protein (originally described as a 33-kDa protein) that substantially reduces the amount of Sep1 required for DNA strand exchange (15, 175). When an optimal amount of SF1 (one monomer per 20 nt) is present, the amount of Sep1 is reduced to only one molecule per 5800 nt. The rate of DNA strand exchange is increased by at least 3–4-fold and, rather than simple joint molecules, large DNA networks are formed. SF1 can aggregate both ssDNA and dsDNA, but this property is not the basis of its stimulatory effect, since SF1 is effective under conditions that reduce its aggregation activity.

Perhaps the most significant property of SF1 is its ssDNA renaturation activity (175, 176). Since it is now apparent that pairing in the Sep1-dependent reaction initiates by reannealing of complementary ssDNA, the ability of SF1 to alleviate the amount of Sep1 required is easily understood: SF1, itself, must catalyze the renaturation step, and the few molecules of Sep1 needed provide the nucleolytic activity necessary to resect the ends of the linear dsDNA. Whether SF1 or Sep1 promotes the strand displacement step is unclear. Furthermore, the identity of SF1 is unknown; given that fatty acid synthase can stimulate the activity of the *S. pombe* homolog of Sep1 (177), the prospect of nonspecific stimulation remains open.

Saccharomyces cerevisiae *DPA Protein*

The second ATP-independent DNA strand-exchange activity (M_r 120,000) isolated from mitotic yeast cells was called DNA pairing activity (DPA) (178). Although its biochemical properties are similar to those of Sep1, it is a distinct protein (15, 179). The sequence of the gene for DPA reveals that it is identical to translation elongation factor 3 (EF3) (K McEntee, personal communication); the likelihood that EF3 is directly involved in recombination is low, and since EF3 is an essential protein, establishing a role for EF3 in recombination will be difficult.

DPA possesses ssDNA-binding, DNA aggregation, and ATP-independent DNA renaturation activities, but no nuclease activity (178). DNA renaturation is extremely rapid (<1 min), and is optimal at stoichiometric amounts of protein (one monomer per 50 nt). The yield shows a sigmoid dependence on protein. Anticipating the need for nucleolytic processing by the ATP-independent homologous pairing protein preparations, Halbrook & McEntee appreciated that DPA required dsDNA substrates with either 5′ or 3′ tails

approximately 50 nt in length in order to initiate homologous pairing via its renaturation activity; DNA substrates with only a 4-nt overhang failed to pair (178). DPA forms up to 3–5 kb of heteroduplex DNA and appears to require the continued presence of protein, but this phase of the reaction displays no preferred polarity. Thus, DPA-promoted homologous pairing initiates by renaturation and is followed by a DNA heteroduplex extension phase.

Saccharomyces cerevisiae *STPα*

STPα (Strand Transfer Protein α) (38 kDa) was isolated from meiotic yeast cells (180). It apparently catalyzes DNA strand transfer between linear dsDNA and circular ssDNA as well as DNA renaturation. Its biochemical properties bear a similarity to those of Sep1, and the presence of trace levels of nuclease activity appear to explain the apparent DNA strand transfer activity. STPα acts catalytically (2–3 molecules per dsDNA molecule) in the presence of a saturating amount of 26-kDa ySSB protein (one monomer per 6–8 nt). Pairing activity is stimulated by nonspecific agents (e.g. histone H1 and spermidine), with optimal stimulation occurring at concentrations that aggregate 50% of the DNA. The gene encoding STPα, *DST1,* has been cloned (181), and was found to be a previously identified gene (*PPR2*) encoding the transcription elongation factor, TFIIS (182, 183). A role for TFIIS in genetic recombination appears unlikely.

Schizosaccharomyces pombe *p^{140}/ExoII Protein*

The p^{140} protein (M_r 140,000) was purified from vegetative *S. pombe* cells (184). It promotes homologous pairing and DNA strand exchange (as observed by EM) between circular ssDNA and linear dsDNA, and it has an intrinsic nuclease activity. A monomer in solution, the p^{140} protein degrades ssDNA, dsDNA, and RNA in a 5′→3′ direction; activity requires Mg^{2+}, is inhibited by Ca^{2+}, and degrades these nucleic acids at rates of 180, 5, and 0.07 nt per min per molecule, respectively. These nucleolytic properties are similar to those of the *S. cerevisiae* Sep1 protein. Based on its biochemical properties, protein sequence, and antigenic behavior, this protein is identical to a polypeptide, exoII, that was purified from meiotic cells as a ssDNA exonuclease (173). As for *E. coli* RecT protein and *S. cerevisiae* Sep1, the DNA pairing activity of p^{140}/exoII requires resection of the linear dsDNA by a nuclease to reveal complementary ssDNA and is enhanced by 6% polyethylene glycol. Consistent with the expectation that pairing initiates in the ssDNA regions, p^{140}/exoII is also capable of renaturing ssDNA.

Independently, a multicomponent pairing system was partially purified from mitotic *S. pombe* cells (185). The predominant species in the active

fraction have molecular weights (100, 65, and 30 kDa) different from that of p^{140}/exoII. The 65-kDa polypeptide fraction alone promotes limited pairing and DNA renaturation, but the reaction is stimulated by the addition of fractions containing the other two proteins. It is not clear how the apparent pairing activity of this complex initiates; while it was reported that the fractions contained no nuclease activity (185), the gel assay used was not sensitive enough to detect the small amounts of nuclease activity that are sufficient to activate other ATP-independent pairing proteins.

STIMULATORY FACTOR: FATTY ACID SYNTHASE A factor ($p^{190/210}$) that stimulates the activity of p^{140}/exoII was also isolated (177). Remarkably, protein sequencing identifies $p^{190/210}$ as fatty acid synthase (FAS). This protein binds both ss- and dsDNA and is capable of renaturing DNA. Renaturation activity presumably stems from the ability of FAS to aggregate DNA when present at a ratio of about one molecule per 250 nt or bp of DNA. FAS has no nuclease activity and does not stimulate the nuclease activity of p^{140}/exoII. Furthermore, it can promote homologous pairing of resected dsDNA molecules with complementary ssDNA, as well as the subsequent displacement of ssDNA, at least as effectively as p^{140}/exoII. The amount of protein needed for this activity coincides with the amount required for aggregation. The mechanistic basis for this reaction is unclear, but must be a consequence of the ability of FAS to bind and aggregate DNA nonspecifically. As it is unlikely that FAS has any role in homologous recombination, this observation should be taken as an indication that, although renaturation and DNA strand exchange as measured by this assay may represent a genuine activity of recombination proteins, the existence of such an activity does not automatically identify a recombination protein.

Drosophila melanogaster *Rrp1*

Two ATP-independent DNA strand transfer activities, which may be promoted by the same 105-kDa protein, were isolated from *D. melanogaster* embryo nuclear extracts (186–188). Rrp1 (Recombination repair protein 1) has ssDNA and dsDNA aggregation, ssDNA renaturation, and DNA strand-exchange activities. Most significantly, the protein possesses 3′-strand specific dsDNA exonuclease and apurinic endonuclease activities (188a). In agreement, sequencing of the gene (*rrp1*) revealed strong homology to both *E. coli* exonuclease III (a 3′ exonuclease) and apurinic/lapyrimidinic endonucleases (189–191). Since the *RRP1* gene can complement *E. coli* cells defective in exonuclease III (*xth*$^-$) and endonuclease IV (*nfo*$^-$), it is likely that one in vivo role of Rrp1 is as an endonuclease (191a).

The reaction promoted by Rrp1 appears catalytic, and demonstrates a pairing bias for the 5′ complementary DNA strand, observations that are

explained by its nucleolytic activity (186, 187, 191). The maximum extent of DNA heteroduplex formation is ~600 bp (186), and joint molecule formation requires as little as 13 bp of homology (33). This result suggests that as few as 13 nt need to be resected for pairing activity, a value that is in good agreement with the *S. cerevisiae* Sep1 and DPA data. The pairing activity also correlates with the ability of the purified protein to aggregate ssDNA, conditions that would encourage renaturation of ssDNA with the resected dsDNA. Deletion of the carboxyl terminus results in a 452-amino-acid polypeptide that retains renaturation, but not nuclease, activity; this truncated protein cannot promote homologous pairing between ssDNA and linear dsDNA substrates unless ssDNA tails at least 35 nt long and of either polarity are produced by an exogenous nuclease (191). Complete strand displacement could occur if the displaced strand is <400 nt long; the observation that histone H1 did not promote DNA strand displacement suggests that Rrp1 may facilitate this branch migration phase.

Human HPP-1

A homologous DNA pairing protein (HPP-1; M_r 130,000) was purified from human T cells (192, 193). Its pairing activity is ATP independent, apparently requires a 5′ complementary strand in the dsDNA, and proceeds 3′→5′ relative to the displaced strand; all three observations can be explained by the presence of a trace 3′→5′ exonuclease activity. Joint molecule formation between circular ssDNA and linear dsDNA occurs within a few minutes, but only ~6% of the DNA is converted into a product with more than 7 kb of heteroduplex DNA. Extension of DNA heteroduplex proceeds at a rate of 2 nt s^{-1}. Optimal pairing requires as little as one monomer per 25 bp dsDNA, but both the rate and extent are dependent on the concentrations of protein and DNA (192, 193). Even though HPP-1 appears to bind ssDNA cooperatively, it does not form extensive nucleoprotein filaments.

HPP-1 is found associated in a 500-kDa recombination complex at earlier steps in the purification (192). This complex is also proficient in DNA strand exchange, but the reaction promoted by the complex fraction is both ATP-dependent and catalytic. Interestingly, HPP-1 binds the photoaffinity analog, 8-azido ATP. Thus, it is possible that, in vivo, HPP-1 functions as part of a complex that utilizes ATP (possibly that bound by HPP-1) to promote DNA strand exchange. The presumptive ATP-dependent factor may assist in HPP-1 turnover, since the rate of strand exchange in crude extracts is 10-fold faster.

One component of the 500-kDa complex has been identified as the human SSB protein, hRPA (194). Although normally isolated as a heterotrimer of 70-, 32-, and 14-kDa subunits, only the large and small subunits are present

in the 500-kDa complex. Addition of stoichiometric amounts of this protein decreases the amount of HPP-1 required for strand exchange 10-fold and increases the rate of the reaction >50-fold. The 70-kDa subunit alone stimulates the reaction, although the stimulation is somewhat greater when purified hRPA is used. Since this effect is specific (neither SSB protein, G32P, nor *S. cerevisiae* yRPA substitutes for hRPA), it is likely that direct interactions between the hSSB protein and HPP-1 facilitate the strand-exchange reaction.

Another protein that both renatures complementary ssDNA and forms joint molecules between circular ssDNA and linear dsDNA was partially purified from human B cells (195, 195a). Nonhydrolyzable ATP analogs inhibit the reaction by up to 50%. The paired DNA molecules contain limited regions of heteroduplex DNA and initiate at the 5′ end of the complementary strand in the linear dsDNA. Initial preparations degraded 30–45% of the 3′-end label, raising the possibility that the nuclease-reannealing mechanism for pairing applies to this activity as well (195); however, a recent preparation promotes homologous pairing without detectable nuclease or ATPase activity (33).

Vaccinia v-SEP

An extract from vaccinia virus–infected HeLa cells was partially purified and shown to promote ATP-independent DNA strand exchange (196). The active fraction (v-SEP) contains three predominant polypeptides with apparent molecular weights of 110, 52, and 32 kDa, but it is not known which protein(s) promote(s) the reaction. v-SEP possesses DNA renaturation and dsDNA exonuclease activities, consistent with the basic requirements common to the ATP-independent DNA pairing reactions. Presumably as a result of the polarity of the exonuclease activity, pairing proceeds unidirectionally (5′→3′). A displaced strand as long as 3 kb was detected by EM. Whether this DNA strand displacement step is protein promoted is unknown.

Herpes Simplex ICP8

The 138-kDa ICP8 was purified from herpes simplex virus-1 (HSV-1)-infected cells (197). It binds ssDNA, but not dsDNA, with a stoichiometry of ~10 nt per monomer (197, 198), and forms protein filaments in the absence of DNA (197). When dsDNA is added to ICP8-ssDNA complexes, homology-dependent pairing occurs. Strand exchange is less efficient than that promoted by RecA protein; transferred fragments of 1 kb are detected, but exchange of molecules the size of full-length M13 is not detected. Direct measurement of nuclease activity detected low levels of activity (~0.3 nt per 5′ end), but other evidence suggests that pairing proceeds by an annealing mechanism. First, blunt-ended DNA is fourfold less efficient than molecules

with 1–2-nt overhangs at forming joint molecules. Second, a maximum of ~35% of the substrate forms joint molecules. Finally, ICP8 does not form D-loops with supercoiled DNA.

Human p53 Protein

The p53 tumor-suppressor protein binds preferentially to the ends of ssDNA, and it promotes both DNA renaturation (198a, 198b) and DNA strand transfer (198b). In the presence of Mg^{2+}, p53 protein renatures both DNA and RNA. DNA renaturation is inhibited by all of the NTPs examined, suggesting that they either are allosteric effectors or occupy a DNA-binding site. DNA strand transfer between a variety of oligonucleotides up to 70 nt in length was examined in the absence of Mg^{2+} (198b). Transfer of DNA strands occurred in a protein concentration–dependent reaction that was not inhibited by DNA mismatches as long as 4 contiguous nt. As with DNA renaturation, GTP inhibited the DNA strand transfer reaction, and mutant p53 proteins that bind ssDNA poorly fail to promote DNA strand transfer. The DNA strand-exchange characteristics of this protein, particularly the need to omit Mg^{2+}, are unlike those of any ATP-independent DNA strand-exchange protein summarized herein, but the short length of oligonucleotides used combined with the preferential binding of p53 protein to ssDNA suggest the possibility that p53 protein is a helix-destabilizing protein and is simply melting the dsDNA; reannealing could occur upon deproteinization without the need for a concerted strand transfer event (see discussion in section on *Experimental Assays*). The only other report of DNA strand transfer in the absence of Mg^{2+} was by histone H1 protein, which was ultimately traced to an artifactual denaturation event (71, 72).

STRUCTURAL HOMOLOGS OF RecA PROTEIN

As indicated in the previous section, pairing activities isolated from eukaryotic cells promote homologous pairing and joint molecule formation in the absence of either ATP binding or hydrolysis. However, joint molecule formation by nearly all ATP-independent proteins is absolutely dependent on either the presence of an exonuclease activity or the resection of the ends of the duplex DNA, suggesting that the mechanism of pairing is based upon reannealing of ssDNA regions rather than upon strand invasion. This observation raises the question of whether homologous pairing and DNA strand-exchange proteins exist in eukaryotic organisms that function by a mechanism of DNA strand exchange as defined by *E. coli* RecA and T4 phage UvsX proteins.

Recently, eukaryotic proteins having sequence and structural homology to RecA protein (see below) have been identified. Their sequence similarity

Table 2 Structural homologs of RecA protein

Protein	Organism	M_r[a]	A.a.[b]	Sequence comparison		
				Region[c]	Cons.[d]	Ident.[e]
Rad51	*S. cerevisiae*	43	400	33–240	59	33
Rad55	*S. cerevisiae*	46	406	39–229	35	17
Rad57	*S. cerevisiae*	52	460	39–229	36	19
Dmc1	*S. cerevisiae*	37	334	24–262	67	26
				33–240	50	27
Rad51-like	*S. pombe*	—	365	33–240	53	30
Mei3	*N. crassa*	27[f]	266[f]	33–256	54	25
Rec2	*U. maydis*	84	781	40–85	51	40
				140–157	61	33
Lim15	*L. longiflorum*	—	349	33–240	—	29
RecA-like	*A. thaliana*	42[g]	387[g]	1–352	53	20
Rad51-like	chicken	38	339	33–240	—	26
Rad51-like	mouse	38	339	1–303	55	30
				33–240	51	28
Rad51-like	human	38	339	31–260	56	30
				33–240	51	28

[a] Expressed in kDa.
[b] Number of amino acid residues.
[c] Region of RecA protein used in sequence comparison analysis.
[d] Percent conserved (similar and identical) amino acid residues between indicated protein and RecA protein within the stated region of RecA protein.
[e] Percent identical amino acid residues between indicated protein and RecA protein within the stated region of RecA protein.
[f] These values are based on the open reading frame identified by Cheng et al (210). It has been noted that sequences upstream of the reported coding region also contain homology to Rad51 protein (R Rothstein, personal communication); therefore, it remains unclear whether the true size of Mei3 protein is larger than that reported.
[g] Estimated size of the mature product after cleavage of the chloroplast transit peptide.

to RecA protein is summarized in Table 2. While limited biochemical data is available for these proteins and none have yet been shown to possess DNA strand-exchange activity, their characteristics allude to the universality of the prokaryotic paradigm.

Saccharomyces cerevisiae *Rad51 Protein*

Mutations in *RAD51* display recombination and repair defects consistent with the involvement of this protein in DNA strand exchange [reviewed in (199)]. In a strain containing a *rad51* null mutation, the double-strand breaks with processed ssDNA tails indicative of the earliest steps of recombination are formed, but these intermediates are not processed to produce recombinants (140).

The central portion of the Rad51 protein (M_r 42,961) is 30% identical and 24% similar to that of RecA protein. Included in this region is the

nucleotide-binding fold of RecA protein, a motif that is conserved in Rad51 protein (124). Mutation of the conserved lysine residue (Lys191) in the polyphosphate-binding loop of Rad51 protein results in a null phenotype (140), as it does for *E. coli* RecA protein (50, 200) (WM Rehrauer, SC Kowalczykowski, unpublished observation).

Rad51 protein was first purified from *E. coli,* although it has subsequently been purified from yeast with no apparent difference in biochemical activity (140, 201, 201a). Like RecA protein, this protein binds both ss- and dsDNA, and the protein concentration dependence displays sigmoid behavior, which indicates that the active form of this protein may be a RecA protein–like filament. DNA binding is stimulated by the presence of ATP and saturates at a stoichiometry of 2 nt per monomer, and the structure of the Rad51 protein–ssDNA complex undergoes a conformational transition upon ATP binding (140), properties that are characteristic of RecA protein–ssDNA complexes (51). Despite these indications that Rad51 protein might form filaments on ssDNA, EM imaging showed only filaments formed on dsDNA. Image reconstruction from electron micrographs of these Rad51 protein–dsDNA complexes finds that the structures of the prokaryotic and eukaryotic filaments are highly conserved (201). The RecA protein–ATP–dsDNA filament is right handed, has a pitch of 92–97 Å, a 5.1 Å axial rise, and 18.6 bp per turn, parameters that are nearly identical for the Rad51 protein–ATP–dsDNA filament (a pitch of 99 Å, and the same 1.5-fold-increased axial rise and number of bp per turn). No structure was observed for the ~120 amino acids at the amino terminus of Rad51 protein that are not present in RecA protein, suggesting that this region of unknown function is disordered. Genetic analysis suggests that the functional form of Rad51 protein in vivo is a filament, as many mutations in *RAD51* display codominant behavior (201b), a behavior manifest by *recA* mutations (see Ref. 76). Yet, despite these similarities to RecA protein, studies have failed to identify other activities, such as DNA renaturation and (co)aggregation, that are normally associated with RecA protein function. Initially, Rad51 protein displayed no detectable ATPase activity (140); however, it was recently reported that Rad51 protein hydrolyzes ATP in a ssDNA-, but not a dsDNA-, dependent manner at a rate that is about one-fourth that of RecA protein (201a). No conditions have yet been found that allow Rad51 protein to promote DNA strand exchange (140, 201a).

A possible reason for the inability to detect Rad51 protein–mediated DNA strand-exchange activity is that the protein functions as one necessary but insufficient component of a complex. The ability of Rad51 protein to act in the presence of ssDNA-binding proteins such as yRPA has not been reported, but other studies suggest that Rad51 protein may comprise only

part of a recombination-promoting complex, perhaps similar to the T4 system. Using affinity chromatography, Shinohara et al demonstrated that Rad51 and Rad52 proteins interact (140). Genetic evidence confirms a direct functional interaction between these proteins (202) (C Bendixen, R Rothstein, personal communication; JH New, SC Kowalczykowski, unpublished observations). The *RAD52* gene lies in the same epistasis group as *RAD51* and was reported initially to have no biochemical activity (140). But recently it was found that Rad52 protein binds both ssDNA and dsDNA, and that it can promote ATP-independent renaturation of ssDNA (201a). Perhaps somewhat surprisingly, it can also promote DNA strand exchange between circular ssDNA and linear dsDNA molecules, although the efficiency is only 5% of the reaction promoted by RecA protein; the Rad52 protein–promoted reaction is not enhanced by ATP. It is possible that the interaction of one or more of the other proteins in this epistasis group (Rad50, Rad54, Rad55, Rad57) with Rad51 and/or Rad52 proteins may be required for efficient DNA strand exchange to occur. In fact, both Rad55 and Rad57 proteins (52 and 53 kDa, respectively) have homology to RecA protein (203, 203a). As with Rad51 and Dmc1 proteins (see below), the most highly conserved region of these proteins is in the nucleotide-binding fold, a region referred to as Domain II (15a, 201a). Outside of this region, Rad55 and Rad57 proteins share no additional similarity to other RecA-like proteins. No biochemistry has been reported for either protein.

Saccharomyces cerevisiae *Dmc1 Protein*

The *DMC1* gene was identified as a cDNA clone derived from a transcript that was meiosis specific and that, when disrupted, was essential for meiosis (204). It was also identified by cross-hybridization to a *Lilium longiflorum* cDNA clone, *LIM15*, which is specifically transcribed at meiotic prophase (204a). Genetic experiments indicate that *DMC1* is involved in the early stages of recombination. As in *rad51* cells, exonucleolytically processed double-strand breaks persist in *dmc1* strains. These and other effects of *dmc1* mutations are only observed in meiotic cells.

The Dmc1 protein sequence has a significant homology to RecA protein, particularly in the hydrophobic core and the nucleotide-binding domain (124, 204). Sequence similarity diverges at both the amino and carboxyl termini of the proteins. Greater similarity is found between Dmc1 and Rad51 proteins; their sequences are 45% identical, again primarily in the central region of the proteins. Even greater similarity exists between Dmc1 and Lim15 proteins (48%); similarities are found not only in the central core region [Domain II (15a, 201a)], but also in the amino-terminal Domain I, suggesting that these two proteins represent a subclass of the RecA-like proteins (15a, 201a).

Analysis of the sequence similarity between Dmc1, RecA, and UvsX proteins makes a case for the conservation of function as well (124). A 288-amino-acid region of Dmc1 protein shares 26% identity and 41% similarity with the central portion of RecA protein (204). Modeling of the Dmc1 protein sequence on the crystallographic structure of RecA protein reveals several classes of identical or conserved residues (124). This analysis suggests that the fundamental properties of Dmc1 and RecA proteins are similar, but until demonstrated biochemically, such a proposal must be viewed as a hypothesis.

Arabidopsis thaliana *RecA-like Proteins*

Two RecA protein homologs have been identified in *Arabidopsis thaliana*. One was identified on the basis of both its immunological crossreactivity to the *E. coli* RecA protein and hybridization to the cyanobacterial *recA* gene (142), while the other (Drt100) was isolated on the basis of its ability to complement partially the recombination and repair defects of *recA*$^-$ uvrC$^-$ phr$^-$ *E. coli* cells (205). Both proteins appear to be targeted to the chloroplast, but they are distinct in sequence. The former shows 61% sequence identity to the *Synechococcus* RecA protein and 52–57% identity with 20 other prokaryotic RecA proteins; the sequence conservation is primarily in the central core, with poor conservation at the amino and carboxyl termini (142). Drt100 shows no identity to RecA proteins but does possess a consensus ATP-binding site (205).

Other Rad51-like Proteins

Homologs of Rad51 protein have been identified in *S. pombe* (201a, 206, 206a), chicken (207), mouse (206, 208), and human (206, 209) cells using probes derived from *RAD51* sequences. The fission yeast protein is 30% identical and 53% similar to RecA protein, and 69% identical to Rad51 protein. The chicken protein is 68% and 49% identical to Rad51 and Dmc1 proteins, respectively, and 95% identical to its mammalian counterpart. It is found in lymphoid tissue and germ cells, suggesting that it is involved in not only DNA repair but also recombination. The mouse protein is 83% and ~55% homologous to Rad51 and RecA proteins, respectively. It is expressed in spleen and intestine as well as lymphoid tissue and germ cells, suggesting that it is involved in both immunoglobulin and general recombination. When expressed in *S. cerevisiae,* this protein partially suppresses *rad51* defects (208). The human protein is 83% homologous (67% identical) to Rad51 protein and 56% homologous (30% identical) to RecA protein. The region encompassing the nucleotide-binding fold (Domain II) of each of these homologs displays the greatest amino acid conservation. However, in addition, these proteins show conservation of their amino-terminal Domain

I sequences that is distinct from the sequences of both the Dmc1 and Lim15 protein subclass and the Rad55 and Rad57 proteins (15a, 201a). Use of the mouse *RAD51* cDNA probe reveals a single genetic homolog in sources as diverse as human, chicken, rabbit, pig, snake, turtle, frog, swellfish, sea urchin, mussel, lamprey, fruit fly, and tobacco (206, 208).

A RecA protein structural homolog has also been identified in *Neurospora crassa* (210). Mutations in the gene encoding this protein, *mei3,* have recombination and repair defects. This protein is smaller than the other RecA protein homologs (27 kDa), but retains the central hydrophobic core and nucleotide-binding domains. Mei3 protein has 27% identity to RecA protein over a 214-amino-acid region, but has much greater identity to Rad51 protein (73% over 260 amino acids).

PROTEIN-FREE DNA PAIRING AND DNA STRAND EXCHANGE

The complexity of the protein-promoted DNA strand-exchange reactions belie their physicochemical simplicity. All of the processes promoted in vivo by proteins occur in vitro in the complete absence of proteins. As for all protein-promoted reactions, the intracellular process benefits from both increased rates and control of the reactions. However, analysis of the protein-free reactions offers the benefits of simplicity and of physical insight into the underlying mechanism.

DNA Renaturation

Nearly every protein discussed above possesses the ability to renature complementary DNA strands. Protein-free renaturation of DNA is usually a second-order kinetic process that is limited by at least two important characteristics of DNA: electrostatic repulsion between charged phosphates and secondary structure [see (211)]. Proteins could mitigate either or both limitations. Charge repulsion can be minimized by reagents as simple as inorganic salts, but positively charged alkyl detergents (e.g. dodecyl- and cetyltrimethyl-ammonium bromide) are far more effective reagents. Though DNA renaturation in the presence of these detergents is still a second-order process, the rate of annealing is more than 2000-fold faster than the reaction in 1 M NaCl due to weak favorable interactions between the detergent-coated DNA molecules (212). Condensation of DNA into aggregates by agents such as poly(ethylene oxide), sodium dextran sulfate, phenol-salt emulsions, NaCl in the presence of ethanol, spermine, spermidine, and hexaminecobalt (III) ion greatly increases renaturation (213). DNA-binding proteins not only reduce charge repulsion, but eliminate secondary structure as well; the rapid second-order reactions catalyzed by SSB protein and G32P are notable

examples (113, 114). Other proteins that bind DNA [transcription factor IIIA (TFIIIA) (214), histones (215), ribonucleoprotein A1 (216), fatty acid synthase (177), and the "model" protein polylysine (215)] also renature ssDNA. Thus, agents that increase the local concentration of DNA by either miminizing repulsive interactions or by introducing weak favorable interactions enhance DNA renaturation; hence it should be no surprise that many proteins promote DNA annealing.

Since DNA renaturation is promoted by many reagents, both protein and nonprotein, it is reasonable to ask whether a special characteristic distinguishes the renaturation activity of a protein as being exclusive to (and perhaps important to) homologous recombination. Two proteins known to be essential to recombination, *E. coli* RecA protein and λ phage β protein, promote DNA renaturation by a first-order process, but this feature is not unique: TFIIIA-dependent DNA renaturation is also first order. From examination of the many proteins described in this review, the only property unique to recombination proteins is the ATP stimulation of renaturation displayed by RecA-like proteins.

D-loop Formation

The uptake of ssDNA fragments by homologous supercoiled DNA can occur independent of protein (57). This process requires an optimal temperature (75–78°C) about 5°C below the T_m. At 37°C, the rate of D-loop formation is ~100-fold slower but is nevertheless detectable. The apparent equilibrium constant for D-loop formation is favorable and has a value of about 10^6 M^{-1} (57). Thermodynamic analysis shows that the reaction is driven by the entropy increase associated with loss of superhelical turns. The rate-limiting step exhibits positive entropy and enthalpy of activation, suggesting that this step involves the unstacking of a few basepairs in the dsDNA (57). Knowledge that the rate-limiting step requires dsDNA opening suggests that this step is a candidate for acceleration by a catalyst; this prediction is consistent with the ability of RecA protein to unwind dsDNA in anticipation of the strand-exchange step.

DNA Strand Exchange

Like renaturation, DNA strand exchange requires that the impediment to bringing two DNA molecules into proximity be overcome; but unlike DNA renaturation, DNA strand exchange requires destabilization of the dsDNA. Ostensibly, DNA strand exchange would seem too concerted a process to be catalyzed at room temperature without the intervention of proteins; this, too, is not the case. Condensation of DNA into aggregates by 15% poly(ethylene oxide) and 0.3 M NaCl results in DNA strand exchange between circular viral ssDNA and duplex DNA fragments as large 2748 bp

(213). Condensation is proposed to bring DNA molecules into proximity and to cause destabilization of the dsDNA. DNA strand exchange of a 240-bp fragment is less demanding, occurring at or above either 1% polyethylene glycol or 1 M NaCl (217). It was suggested that RecA protein–promoted DNA strand exchange proceeds by a similar mechanism [i.e. RecA protein both increases the local concentration of the DNA partners and destabilizes the dsDNA, permitting exchange to occur (213)]. In agreement with this physical chemical analogy, RecA protein is highly proficient at pairing DNA molecules, regardless of sequence, within the confines of the presynaptic filament, and is also able to unwind and unstack dsDNA, distortion that clearly destabilizes duplex structure (see section on *E. coli* RecA protein).

Exchange of DNA strands as measured by branch migration of DNA joined by reannealing of ssDNA can also be promoted by a passive ingredient such as bovine serum albumin, suggesting that volume-excluding reagents may promote this kind of exchange as well (217).

Part of the problem encountered when bringing two DNA molecules into close proximity is electrostatic repulsion. Certainly one function of a DNA strand-exchange protein must be to minimize this repulsive interaction. This condition is also required for protein-independent DNA collapse. A DNA analog, polyamide nucleic acid (PNA), that has an uncharged polyamide backbone is useful in studying the effects of charge repulsion. ssPNA not only recognizes its complementary sequence in dsDNA, but it also spontaneously displaces the identical strand in the dsDNA to form a D-loop structure (218). This structure is stabilized by the high stability of PNA-DNA hybrids, and the enhanced rate of formation argues that the electrostatic repulsion encountered by normally charged ssDNA is no longer rate limiting. This result, which agrees with experimental conditions that promote DNA condensation, argues that an important function of DNA strand-exchange proteins is to facilitate an increase in local DNA concentration by masking the strong electrostatic repulsion that occurs when DNA molecules are brought within 10 Å of one another.

CONCLUSIONS

The universal prevalence of proteins that can promote homologous pairing and the exchange of DNA strands argues for their definition as a new class of proteins. The ease with which these proteins locate DNA sequence homologies is both unique and remarkable. At present, these proteins comprise two classes that are distinguished by the need for or independence from ATP in their action; it is likely that most of the as-yet-uncharacterized

RecA-like proteins will prove to be members of the first class. The ATP-dependent proteins have, as a distinguishing characteristic, the ability to initiate pairing and exchange between ssDNA and dsDNA molecules, whereas the ATP-independent proteins are limited to the initiation of pairing between two ssDNA molecules. Both classes of protein promote a DNA heteroduplex extension phase, but only the ATP-dependent proteins have an absolute intrinsic polarity for this exchange. Interestingly, proteins that permit pairing between two fully intact and unperturbed duplex DNAs have not yet been described.

Although it is convenient from an organizational view to group these proteins into just two classes, it is perhaps more interesting to recognize that within each group, unique variations exist. For example, within the ATP-dependent class, in contrast to the *E. coli* RecA protein, we find a protein (T4 phage UvsX protein) that utilizes a third factor (UvsY protein) as an essential component of the complete functional apparatus and a protein that requires dATP instead of rATP (*B. subtilis* RecA protein). Within the ATP-independent class, we find some proteins (*S. cerevisiae* Sep1 and *S. pombe* p^{140}/exoII proteins) that possess an intrinsic nuclease activity that is essential for initiation via DNA annealing, and others (*E. coli* RecT and λ phage β proteins) that recruit a second protein (RecE protein and λ exonuclease, respectively) to provide nuclease function. The mechanistic bases and functional reasons for these intriguing differences are unknown.

Despite the extensive study of these proteins, a number of very significant questions regarding their function remain, including: What is the mechanism of the homology search? What is the precise structure of the homologously paired DNA molecules? What role does ATP hydrolysis play in DNA heteroduplex extension? What is the biological function of most of the eukaryotic ATP-independent proteins? Is the mechanism by which some of the ATP-independent proteins promote DNA heteroduplex extension active or passive? What are the biochemical activities of the apparently ubiquitous eukaryotic RecA-like proteins?

It is already clear, however, that these interesting proteins will be very useful to those wishing to locate and manipulate specific DNA sites in genomes. *E. coli* RecA protein has been used to enrich for homologous DNA sequences in a genomic pool by more than 10^4–10^5-fold (219), and has been used to target unique DNA sequences for enzymatic modification (220, 221) and identification (222–224). Applications for homologous pairing proteins will continue to evolve, bolstered by further appreciation of both their enzymatic characteristics and biological behavior, ultimately permitting their use in applications as far reaching as gene replacement strategies [see (225)]. These proteins do, indeed, represent a fascinating and important group.

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