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Studies of the Mechanism of DNA Pairing by the RecA Protein of *Escherichia coli*

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The RecA protein is indispensable for homologous recombination in *Escherichia coli* (Dressler and Potter 1982). Our aim is to discover its mechanism. A model reaction that has been particularly useful in our analysis has been the exchange of strands between a single-stranded (+) DNA (ssDNA) circle and its homologous duplex (Cox and Lehman 1981a). Strand exchange requires stoichiometric amounts of RecA protein (Cox and Lehman 1981a), is dependent upon ATP hydrolysis (Cox and Lehman 1981a), proceeds 5'→3' relative to the displaced (+) strand of the duplex (Cox and Lehman 1981b; Kahn et al. 1981; West et al. 1981), and is strongly stimulated (5-fold to 20-fold) by the single-stranded DNA binding protein (SSB) of *E. coli* (West et al. 1982; Cox et al. 1983).

This paper describes our investigation of one of the initial steps in strand exchange: the interaction between the recombining DNA molecules that produces the first heteroduplex base pairs. In particular, we have examined the role of SSB and ATP in the formation of paranemic and plectonemic joints.[1] These studies have led to a refinement and an expansion of our previous model (Cox and Lehman 1981a) (Fig. 1). We also summarize our efforts to determine the mechanism of the RecA protein-catalyzed renaturation of complementary DNA strands (Weinstock et al. 1979). This is the simplest of the RecA protein-promoted pairing reactions and consequently the most amenable to detailed kinetic analysis.

Initial Steps in Strand Exchange

An essential step in strand exchange is the binding of RecA protein to ssDNA to form a RecA protein-ssDNA complex (Shibata et al. 1979; Flory and Radding 1982; Cox et al. 1983; Howard-Flanders et al. 1984). One role of SSB is to facilitate formation of the complex by a mechanism that is not yet fully understood (Cohen et al. 1983; Cox et al. 1983; Muniyappa et al. 1984). We have observed that strand exchange proceeds most rapidly if SSB and ATP are added to the preformed RecA protein-ssDNA complex and the reaction is initiated by addition of the duplex DNA. Addition of SSB to the ssDNA before RecA protein results in a considerably reduced rate and a lag of 5 min or longer (P.W. Riddles and I.R. Lehman, in prep.).

Formation of Paranemic Joints

The RecA protein promotes the formation of a paranemic joint between a circular single strand and a homologous DNA duplex, and it has been suggested that such structures represent early intermediates in the exchange reaction (Bianchi et al. 1983). A similar observation has been reported for the *rec1* protein from *Ustilago maydis* (Kmiec and Holloman 1984). We have found that SSB enhances the rate of formation of paranemic joints by greater than 10-fold under conditions in which only paranemic joints can form (duplex DNA containing heterologous sequences at both ends). In addition, we have been able to resolve the initial steps of strand exchange between completely homologous DNA molecules into paranemic and plectonemic joint formation. In Figure 1, we have indicated the paranemic joint with a displaced strand; this is one of several possible structures (Bianchi et al. 1983; Wu et al. 1983). SSB might stabilize the paranemic joint by binding to the displaced strand, thereby preventing the reannealing of the duplex. Alternatively, SSB may exert its effect by stabilizing the RecA protein-ssDNA complex, ensuring that there is a high concentration of reactive ssDNA (Cox et al. 1983).

The paranemic joint must be a structure in which the DNAs are not paired by intertwining at either end of the duplex, since pairing occurs even when the duplex contains stretches of heterologous DNA at either end (Bianchi et al. 1983; P.W. Riddles and I.R. Lehman, in prep.). The exact nature of the pairing promoted by RecA protein is not known, although it has been suggested that Z-DNA is present in paranemic joints formed by the rec1 protein (Kmiec and Holloman 1984). A recent model for RecA protein-promoted recombination excludes intermediates with a displaced strand and instead proposes a triple-stranded helix at the initial pairing step (Howard-Flanders et al. 1984). In Figure 1a, we have indicated this step with a double set of arrows to suggest that other intermediates may lie on the pathway (e.g., a triple-stranded helix).

It has been reported that the search for homology that precedes strand exchange occurs during paranemic joint formation (synapsis) by a processive mech-

[1] Following the nomenclature of Bianchi et al. (1983), we use the term *plectonemic joint* to describe pairing of ssDNA and linear duplex DNA at an end such that intertwining of strands leads to Watson-Crick base pairing. The term *paranemic joint* indicates pairing in which the strands are not intertwined as, for example, when plectonemic joint formation is topologically restricted.

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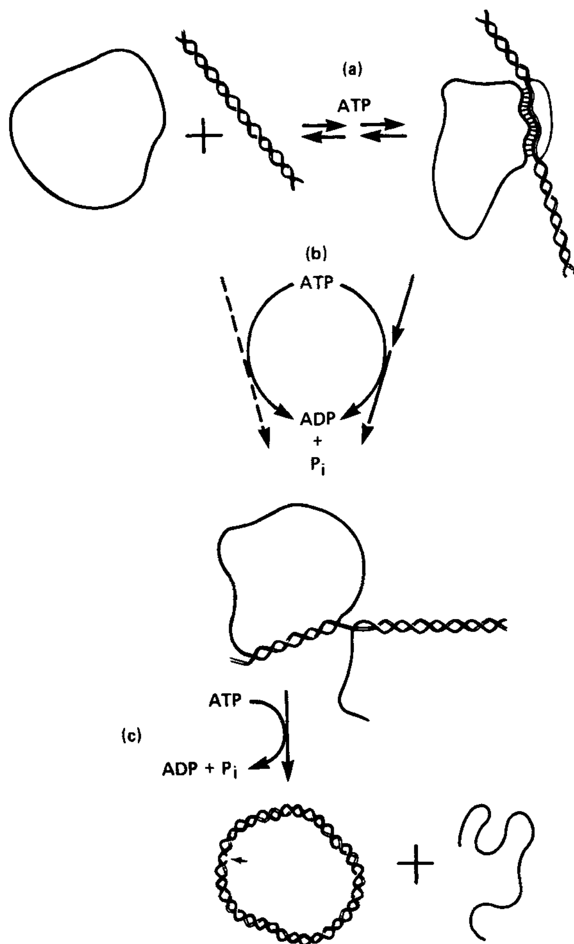

Figure 1. Reaction pathway for the transfer of a strand from a linear duplex DNA to a circular ssDNA, showing the formation of paranemic joints (*a*) and plectonemic joints (*b*). The requirement for ATP is shown at each step (see text for details). Double sets of arrows (→→) indicate that we have not excluded the likelihood that other intermediates exist. Branch migration (*c*) is shown as a separate step to plectonemic joint formation (*b*), but is most likely an extension of the same process (see text).

anism (Gonda and Radding 1983); however, we have found no evidence for processivity in the presence of SSB (P.W. Riddles and I.R. Lehman, unpubl.). Under these conditions the search for homology may not be rate limiting and hence not amenable to kinetic analysis.

Formation of Plectonemic Joints

Table 1 summarizes the differences between paranemic and plectonemic joint formation by the combined action of RecA protein and SSB. The last entries (items 6–9) are discussed at various points in the text; items 1–5 list the experimental conditions used to determine each structure. With these criteria, we have shown that paranemic joints are formed several-fold more rapidly than plectonemic joints. Such kinetics are consistent with the idea that plectonemic joint formation is rate limiting in the presence of SSB. Studies on the effect of SSB have in fact supported this proposition. Thus, when SSB was omitted, formation of plectonemic joints was reduced approximately 10-fold, concomitantly with paranemic joint formation. This decrease is similar to that observed when SSB was omitted from a reaction in which only paranemic joints could form (heterologous duplex DNA termini).

We have indicated two pathways in Figure 1 for the conversion of paranemic to plectonemic joints. Paranemic joints may dissociate and form plectonemic joints only if the critical pairing is at an end. Alternatively, paranemic joints could give rise directly to plectonemic joints by extension of the joint until an end (nick or gap) is reached, so that intertwining can take place. Although these mechanisms may not occur exclusively, the second pathway is supported by the observation that paranemic joints can contain up to several hundred base pairs (Bianchi et al. 1983). It would be of interest to know whether extension of paranemic joints is a directional process analogous to branch migration (Cox and Lehman 1981b; Kahn et al. 1981; West et al. 1981).

It might be expected that plectonemic joints as depicted in Figure 1 could dissociate by random branch migration. Indeed, we have found that at elevated temperatures (56°C) in guanidinium chloride there is a first-order decrease in plectonemic joints ($k_{obs} = 0.9$ min^{-1}), an observation that is consistent with this expectation.

Role of ATP in Paranemic and Plectonemic Joint Formation

Under conditions that specifically determine plectonemic joint formation, there is no detectable homology-dependent reaction in the presence of the nonhydrolyzable ATP analog adenosine-5′-*O*-(3-thiotriphosphate) (ATPγS, 200 μM) (P.W. Riddles and I.R. Lehman, in prep.). This finding suggests that plectonemic joint formation requires ATP hydrolysis. In an experiment designed to test this point, ATPase activity and plectonemic joint formation of an ongoing strand exchange reaction were measured simultaneously. After the reaction had proceeded for several minutes, ATPγS was added. ATPγS is a good inhibitor of the ssDNA-dependent ATPase activity of RecA protein (Weinstock et al. 1981); hence, the rate of ATP hydrolysis decreased sharply. This inhibition was accompanied by a parallel decrease in plectonemic joint formation. Branch migration has an absolute requirement for ATP hydrolysis (Cox and Lehman 1981a). Consequently, plectonemic joint formation and branch migration (Fig. 1c) are integrally related processes, possibly depending on ATP hydrolysis to unwind the duplex DNA.

When duplex DNA was added to a preformed RecA protein–ssDNA complex in which ATP was replaced by ATPγS, no homology-dependent pairing was observed under conditions that detect paranemic joints.

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Table 1. Comparison of Plectonemic and Paranemic Joints Formed by the Action of RecA and SSB Proteins of *E. coli*

Property	Plectonemic	Paranemic
Requirement for homology	yes	yes
Requirement for end of duplex	yes	no
Stable in 2 M NaCl at 0°C	yes	yes
Stable in 5.2 M GHCl[a] at 32°C	yes	no
Binding to nitrocellulose	ssDNA	protein and/or ssDNA
Type of pairing	Watson-Crick intertwined strands	unknown, but not required to be intertwined
Effect of omission of SSB	formation decreased ~10-fold	formation decreased ~10-fold
Order of appearance in reaction	2nd	1st
Requirement for ATP hydrolysis	yes	probably yes

[a](GHCl) Guanidium chloride.

However, when RecA protein was added following preincubation of ssDNA and duplex DNA with SSB and ATPγS, there was a significant homology-dependent pairing reaction, consistent with earlier observations (Cox and Lehman 1981a). We further observed that the paired structures were unstable upon deproteinization and had no requirement for homologous duplex termini. Thus, under these conditions only paranemic structures are formed.

Renaturation of Complementary Single Strands

The renaturation of complementary single strands was the first DNA pairing reaction of RecA protein to be discovered; it is also the simplest pairing activity associated with RecA protein (Weinstock et al. 1979). As such, it is an attractive model system for studying the mechanism of the RecA protein-dependent alignment of complementary DNA sequences. We have therefore undertaken a detailed study of this reaction.

To understand the RecA protein-promoted renaturation reaction, it is first necessary to understand how RecA protein interacts with ssDNA. The binding of RecA protein to ssDNA has been analyzed using a nitrocellulose filter assay (McEntee et al. 1981). However, this assay is of limited value because it is insensitive to the binding of RecA protein monomers in excess of that required for DNA retention. We have therefore developed a nuclease protection assay that allows direct quantitation of the binding RecA protein to ^{3}H-labeled φX174 ssDNA (+ strand). This assay has been used to examine (1) the stoichiometry of binding and (2) the kinetics of transfer of RecA protein between ssDNA molecules.

Stoichiometry of interaction of RecA protein with ssDNA. In the nuclease protection assay, increasing amounts of RecA protein are added to a fixed concentration of ^{3}H-labeled φX174 ssDNA to form RecA protein-ssDNA complexes. The complexes are then treated with DNase I and venom phosphodiesterase to digest ssDNA that is not bound to RecA protein. The remaining, protected fragments are measured either by acid precipitation or by nitrocellulose filter binding. Addition of RecA protein to ^{3}H-labeled φX174 ssDNA rendered a portion of the DNA resistant to nuclease digestion. The amount of DNA protected from digestion was proportional to the amount of RecA protein added, indicating that the mechanism of protection involves binding of RecA protein to the DNA. The stoichiometries of protection presented in Table 2 were determined by titration in the presence of various nucleotide cofactors.

In the absence of nucleotide, the stoichiometry was approximately 1 RecA protein monomer per 4 nucleotides. This value is in good agreement with the value of 1 RecA protein monomer per 4 nucleotides recently determined by S.W. Morrical and M.M. Cox (pers. comm.) using light scattering techniques. The same stoichiometry was found for complexes formed in the presence of ATP supplemented with an ATP regeneration system. In the presence of ATP or ADP, all the DNA remained susceptible to digestion, presumably because of the weaker binding of RecA protein to ssDNA under these conditions. In the presence of ATPγS, exactly twice as much DNA was protected from digestion (1 RecA monomer/8 nucleotides of ssDNA). Electron microscopic studies have shown that addition of ATPγS to φX174 ssDNA in the presence of subsaturating concentrations of RecA protein results in extensive crosslinking of DNA molecules, suggesting that the double-binding stoichiometry may be due to the activation of a second DNA binding site on the RecA protein monomer (F.R. Bryant et al., in prep.).

Table 2. Stoichiometry of Protection of φX174 ssDNA by RecA Protein

Nucleotide added	Nucleotides/RecA monomer
None	4
ATP (plus regeneration system)	4
ATPγS	8
ATP (no regeneration system)	0
ADP	0

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Transfer of RecA Protein between ssDNA Molecules

Transfer of RecA protein from one ssDNA molecule to another is measured in the following way. RecA protein is preincubated with ^{3}H-labeled ϕX174 ssDNA to form RecA protein–ssDNA complexes in the presence or absence of nucleotide. Transfer is initiated by the addition of a second equivalent of unlabeled ϕX174 ssDNA to the preformed complexes. The reaction is then quenched at various times by addition of ATPγS, which converts the complexes to a nondissociable form (Weinstock et al. 1981). The complexes are then subjected to nuclease digestion. Under these conditions, there was a first-order decay in the amount of ^{3}H-labeled ϕX174 ssDNA protected from nuclease digestion as a function of time, reflecting the equilibration of RecA protein between the labeled and unlabeled DNA populations.

The kinetics of transfer in the presence of various nucleotides is summarized in Table 3. The binding of RecA protein to ϕX174 ssDNA is rather stable in the absence of a nucleotide cofactor, with a half time of equilibration of greater than 30 min. Addition of ATP resulted in an approximate 10-fold increase in the rate of equilibration, and ADP produced more than a 100-fold increase. These results suggest that ADP converts RecA protein to a form with a reduced affinity for ssDNA. The lower degree of stimulation of transfer by ATP suggests that a slow step leading to the hydrolysis of ATP to ADP may precede a rapid ADP-induced transfer step. ATPγS, on the other hand, caused a substantial reduction in the rate of transfer of RecA protein. A qualitatively similar effect was seen with AMP-PNP, another nonhydrolyzable ATP analog. Thus, these ATP analogs may trap RecA protein in a form with high affinity for ssDNA.

Kinetics of the Renaturation Reaction

The separated + and − strands of *Pst*I-cleaved, ^{3}H-labeled ϕX174 DNA (form I) serve as substrates for the RecA protein–catalyzed renaturation of complementary single strands. Conversion of the S1 nuclease-susceptible single strands to an S1-resistant form is then used to measure renaturation (Weinstock et al. 1979). The results of a typical renaturation reaction are shown in Figure 2. In the absence of RecA protein, there was no detectable renaturation. In the presence of RecA protein and ATP, renaturation occurred rapidly. In the absence of ATP, there was some renaturation; however, it occurred at a reduced rate and to a lower extent.

Table 3. Effect of Various Nucleotides on the Rate of Transfer of RecA Protein between ssDNA Molecules

Nucleotide added	ATPase K_i (μM)	Relative rate of transfer
None	–	1
ATP	32 (K_m)	10
ADP	20	150
AMP-PNP	60	<0.2
ATPγS	0.6	<0.03
AMP		1

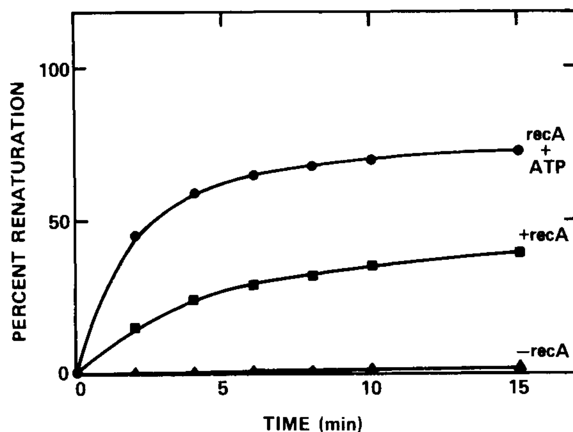

Figure 2. Renaturation of alkali-denatured, ^{3}H-labeled ϕX174 DNA (+ and − strands) by RecA protein. Reaction mixtures contained 25 mM Tris·HCl (pH 7.2), 10 mM $MgCl_2$, 30 μM ^{3}H-labeled ϕX174 DNA, 1 μM RecA protein, and 500 μM ATP (where indicated). The points represent the percentages of the DNA resistant to S1 nuclease (Christiansen and Baldwin 1977).

The rate of the ATP-dependent reaction increased with increasing RecA protein concentration until a maximum rate was reached at a ratio of 1 RecA monomer per 30 nucleotides of ssDNA. A similar optimal ratio was found for the ATP-independent reaction. Based on the binding stoichiometry, this optimal ratio corresponds to approximately 10–15% saturation of the DNA by RecA protein. Higher levels of RecA protein markedly reduced the rate of renaturation. Electron microscopic examination of RecA protein–ϕX174 ssDNA (+ strand) complexes prepared at the optimal ratio revealed that virtually all of the DNA molecules were partially covered with tracts of RecA protein (F.R. Bryant et al., in prep.). The requirement for subsaturating levels of RecA protein distinguishes this reaction from renaturation promoted by other ssDNA binding proteins (e.g., *E. coli* SSB and the T4 gene-*32* protein). These proteins are required in saturating amounts before any stimulation of renaturation is seen (Alberts and Frey 1970; Christiansen and Baldwin 1977).

SSB-promoted renaturation follows second-order reaction kinetics, as does nonenzymatic renaturation. These reactions proceed by a rate-limiting collision between homologous sequences followed by a rapid zippering of the strands to form a DNA duplex. Catalysis by the ssDNA-binding proteins is believed to involve unfolding of regions of secondary structure, resulting in increased rates of nucleation (Alberts and Frey 1970; Christiansen and Baldwin 1977).

ATP-dependent renaturation promoted by RecA protein shows very different kinetics. Thus, when the RecA protein:ssDNA ratio was kept at the optimal value of 1 RecA monomer per 30 nucleotides, the half time for renaturation was independent of the DNA

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concentration, characteristic of an overall first-order rather than second-order process. This finding strongly suggests that renaturation proceeds by the rapid formation of an intermediate prior to complete renaturation.

Another distinguishing feature of RecA protein-promoted renaturation is its requirement for ATP. As shown above, the reaction proceeds optimally in the presence of ATP (and 10 mM $MgCl_2$); however, there is a limited reaction in the absence of ATP. When the $MgCl_2$ concentration was increased to 30 mM, the ATP-independent renaturation proceeded at the same rate as the ATP-stimulated reaction. $MgCl_2$ concentrations higher than 40 mM resulted in the loss of ATP-independent renaturation.

The basis for the apparently similar effects of ATP (at 10 mM $MgCl_2$) and 30 mM $MgCl_2$ alone on RecA protein-promoted renaturation is not clear. It does not appear to be related simply to ionic strength since the inclusion of increasing concentrations of NaCl in addition to 10 mM $MgCl_2$ resulted in inhibition of the ATP-independent reaction. As described earlier, ATP stimulates the transfer of RecA protein between ssDNA molecules in the presence of 10 mM $MgCl_2$. An increase of the $MgCl_2$ concentration to 30 mM did not result in stimulation of transfer in the absence of ATP. The similar effect of 30 mM Mg^{++} and ATP (in the presence of 10 mM Mg^{++}) does not appear, therefore, to be at the level of RecA protein-ssDNA interactions. Possibly both affect RecA protein-RecA protein interactions. RecA protein has been shown to aggregate (in the absence of DNA) in the presence of low concentrations of Mg^{++} (10 mM), whereas higher levels of Mg^{++} (50 mM) cause complete disruption of aggregates (Cotterill and Fersht 1983). The midpoint of this transition is approximately 30 mM, close to the optimal level we find for the ATP-independent reaction. This range of Mg^{++} concentrations clearly affects RecA protein-RecA protein interactions. Aggregation of RecA monomers may therefore be a critical feature of the ATP-independent renaturation reaction.

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