

The Simultaneous Binding of Two Double-stranded DNA Molecules by *Escherichia coli* RecA Protein

Eugene N. Zaitsev and Stephen C. Kowalczykowski*

Division of Biological Sciences
Sections of Microbiology and of
Molecular and Cell Biology
University of California, Davis
CA 95616-8665, USA

We have characterized the double-stranded DNA (dsDNA) binding properties of RecA protein, using an assay based on changes in the fluorescence of 4',6-diamidino-2-phenylindole (DAPI)-dsDNA complexes. Here we use fluorescence, nitrocellulose filter-binding, and DNase I-sensitivity assays to demonstrate the binding of two duplex DNA molecules by the RecA protein filament. We previously established that the binding stoichiometry for the RecA protein-dsDNA complex is three base-pairs per RecA protein monomer, in the presence of ATP. In the presence of ATP γ S, however, the binding stoichiometry depends on the MgCl₂ concentration. The stoichiometry is 3 bp per monomer at low MgCl₂ concentrations, but changes to 6 bp per monomer at higher MgCl₂ concentrations, with the transition occurring at approximately 5 mM MgCl₂. Above this MgCl₂ concentration, the dsDNA within the RecA nucleoprotein complex becomes uncharacteristically sensitive to DNase I digestion. For these reasons we suggest that, at the elevated MgCl₂ conditions, the RecA-dsDNA nucleoprotein filament can bind a second equivalent of dsDNA. These results demonstrate that RecA protein has the capacity to bind two dsDNA molecules, and they suggest that RecA or RecA-like proteins may effect homologous recognition between intact DNA duplexes.

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*Corresponding author

Introduction

The RecA protein of *Escherichia coli* plays a central role in genetic recombination and is essential for the induction of the SOS pathway of DNA repair and mutagenesis (Kowalczykowski *et al.*, 1994). *In vitro*, RecA protein promotes efficient DNA strand exchange reactions involving either three or four strands of DNA (Clark & Sandler, 1994; Cox, 1994; Kowalczykowski *et al.*, 1994; Roca & Cox, 1997; Stasiak & Egelman, 1994; West, 1992). In the case of the four-stranded DNA exchange reaction, RecA protein promotes the pairing of two duplex DNA molecules and, therefore, it must simultaneously bind both molecules. Although initiation of DNA strand exchange between two fully duplexed DNA molecules has not been described, RecA protein can pair two duplexes if

one of them contains a short region of single-stranded DNA (ssDNA; Cassuto *et al.*, 1980; Cunningham *et al.*, 1980; DasGupta *et al.*, 1981; West *et al.*, 1981). The need for ssDNA in pairing reactions results, at least in part, from the limited ability of RecA protein to fully bind duplex DNA at physiological pH (Kowalczykowski *et al.*, 1987; McEntee *et al.*, 1981). In addition, this short region of ssDNA serves as the initiation point for DNA strand exchange (Conley & West, 1989, 1990). The apparent weak binding to duplex DNA at pH 7.5 represents a slow nucleation step in the association process rather than an unfavorable binding affinity (Kowalczykowski *et al.*, 1987; Pugh & Cox, 1987). This binding of RecA protein to dsDNA is greatly facilitated at lower pH (McEntee *et al.*, 1981), in the presence of ATP γ S, and at low Mg²⁺ concentrations (Kowalczykowski *et al.*, 1987; Pugh & Cox, 1988).

Despite the crucial importance of double-stranded DNA (dsDNA) binding in the DNA pairing process, the dsDNA binding properties of RecA protein and, especially, of the RecA nucleoprotein filament (i.e. that which assembles on

Abbreviations used: DAPI, 4',6-diamidino-2-phenylindole; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; EtBr, ethidium bromide.

E-mail address of the corresponding author: sckowalczykowski@ucdavis.edu

ss- or dsDNA) are relatively poorly characterized. In spite of numerous and extensive studies of RecA protein-DNA interactions, the binding of two molecules of intact dsDNA has never been detected (Muller *et al.*, 1990; Takahashi, 1989; Takahashi & Hagmar, 1991), though less direct, enzymatic evidence for RecA protein-mediated homologous contacts between duplex DNA regions is compelling (Chiu *et al.*, 1990; Chow *et al.*, 1991, 1992; Conley & West, 1989, 1990; Lindsley & Cox, 1990).

Recently, we described a new procedure to measure the binding of RecA protein to dsDNA (Zaitsev & Kowalczykowski, 1998). It is based on changes in the fluorescence of 4',6'-diamidino-2-phenyl-indole (DAPI)-dsDNA complexes that occur upon RecA protein binding. Using this DAPI displacement assay, we showed that: (1) the ATP-dependent binding of RecA protein to dsDNA results in displacement of DAPI from the minor groove of dsDNA; (2) the binding stoichiometry for the resultant complex is 3 bp per RecA protein monomer; and (3) RecA protein in the RecA-dsDNA filament is in the high-affinity DNA-binding state that is normally competent for DNA strand exchange (Zaitsev & Kowalczykowski, 1998).

Here, using this DAPI displacement and other supportive assays, we describe conditions where RecA protein can apparently bind two dsDNA molecules simultaneously. This finding, in turn, raises the possibility that RecA protein might possess the capacity to permit homologous recognition of intact DNA duplexes.

Results

Both the extent of DAPI displacement and the stoichiometry of RecA protein binding to dsDNA are unique in the presence of ATP γ S

Previously, we demonstrated that in the presence of either ATP or dATP, RecA protein could almost fully (90%) displace DAPI from dsDNA; the amount displaced was directly proportional to the amount of RecA protein added, until saturation was achieved at a stoichiometry of three base-pairs per RecA protein (Figure 1). Because ATP γ S is a non-hydrolyzable ATP analog that is bound by RecA protein with high affinity (Weinstock *et al.*, 1981) and can induce the high-affinity DNA-binding state of RecA protein (Kowalczykowski, 1991), we examined its effects on the binding of dsDNA by RecA protein. This ATP analog mimics nearly all of the ATP-dependent properties of the ATP-bound form of the protein, except those requiring ATP hydrolysis, and induces formation of RecA protein-DNA complexes with heightened stability. Therefore, we were surprised that in the DAPI-displacement assay, the ATP γ S-bound form of RecA protein displayed two unique characteristics. First, even at the highest RecA protein concentrations, only about 50% of the DAPI was displaced.

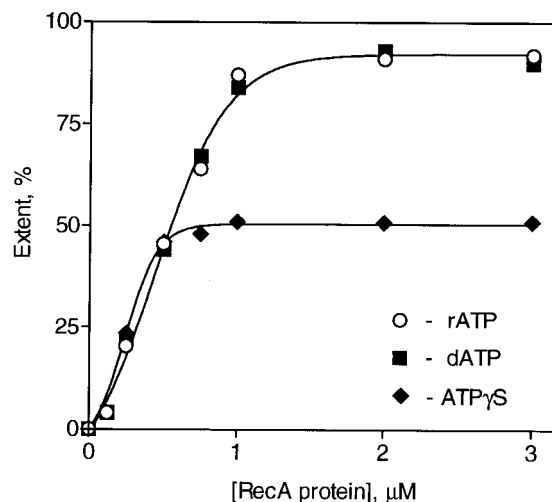


Figure 1. The binding of RecA protein to dsDNA reveals two distinct stoichiometric forms. The maximum extent of DAPI displacement was plotted as a function of RecA protein concentration using either (○) ATP, (■) dATP, or (◆) ATP γ S as cofactors. Titrations in the presence of 1 mM ATP γ S were performed as described in Materials and Methods; data for ATP and dATP are from Zaitsev & Kowalczykowski (1998). The DNA concentration in all experiments is 6 μ M (nucleotides).

Second, saturation of the observed DAPI displacement occurred at an apparent stoichiometry of approximately 6 bp/RecA protein monomer; this is twice the value that was obtained with ATP. This discrepancy suggests either that all of the DNA has bound to RecA protein, but only 50% of the DAPI is displaced from this bound dsDNA or, the less likely possibility, that there are two classes of DNA: half of the dsDNA is complexed with RecA protein and is accompanied by complete displacement of DAPI, whereas the other half of the dsDNA remains protein-free and fully complexed with DAPI.

To distinguish between these possibilities, the potentially depleted component was added (either half of an equivalent of DNA or of RecA protein) at the end of a standard DAPI-displacement reaction (the DAPI concentration is in excess). Figure 2 demonstrates the fluorescence changes in response to the sequential addition of a standard amount of DNA (6 μ M), followed by an additional one-half (3 μ M) and a one-quarter concentration (1.5 μ M) of the initial amount of DNA. Addition of the DNA component resulted in additional DAPI displacement, and the extent of this second reaction was 50% of the second, initial value. In all cases, the fluorescence change for each subsequent addition was about 50%. In contrast, sequential addition of RecA protein did not change the fluorescence signal (data not shown). These results show that RecA protein is not the limiting component and that the DNA is saturated by the indicated amount of RecA protein, but only 50% of the DAPI is displaced from the DNA.

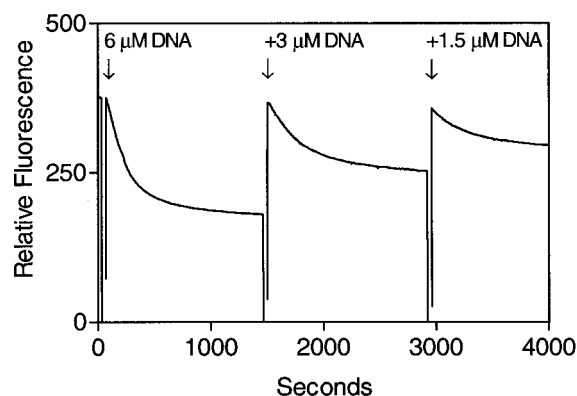


Figure 2. Response of DAPI fluorescence to sequential additions of DNA. DAPI displacement was performed as described in Materials and Methods in the presence of 1 mM ATP γ S and 10 mM MgCl $_2$. The concentration of RecA protein is 1 μ M. At time zero, 6 μ M DNA was added and the fluorescence monitored; subsequently at 1500 and 3000 seconds, DNA at concentrations equal to one-half and one-fourth of the initial amount of DNA (3 μ M and 1.5 μ M, respectively), was added.

Binding of dsDNA in the presence of ATP γ S is characterized by two distinct stoichiometric forms

The preceding experiments demonstrated that the apparent DNA binding stoichiometry and the final extent of DAPI displacement are different when ATP γ S is the cofactor, rather than ATP or dATP. To further characterize this behavior, titrations with RecA protein were performed in the presence of ATP γ S at different binding conditions.

At 10 mM MgCl $_2$, the concentration used in Figures 1 and 2, the fluorescence signal saturates at one-half of the RecA protein concentration that was required for saturation in the presence of ATP (Zaitsev & Kowalczykowski, 1998), and it plateaus at one-half of the fluorescence change (Figure 3(a); compare with Figure 1). This effect is unexpectedly dependent on the MgCl $_2$ concentration. At 1 mM MgCl $_2$ (Figure 3(a)), both the apparent binding stoichiometry and the extent of DAPI displacement revert to the values observed for ATP. In the presence ATP γ S, RecA protein apparently binds 6 bp of DNA per protein monomer at 10 mM MgCl $_2$, but only 3 bp per monomer at 1 mM MgCl $_2$. This unusual dependence of the extent of DAPI displacement on MgCl $_2$ concentration in the presence of ATP γ S displays a transition midpoint at about 5 mM MgCl $_2$ (Figure 3(b)). In contrast, in the presence of ATP the binding stoichiometry is 3 bp per RecA protein monomer and is independent of MgCl $_2$ concentrations in the range of 1-10 mM (data not shown). Thus, in the presence of ATP γ S and elevated MgCl $_2$ concentrations, RecA protein binds two 3 bp-equivalents of dsDNA; this is one equivalent (3 bp) more than the amount bound in the presence of ATP.

To determine whether the stoichiometric complex that is formed at the higher MgCl $_2$ concentrations could be converted to the complex that is formed at the lower MgCl $_2$ concentrations, complexes formed at 10 mM MgCl $_2$ were treated with 10 mM EDTA after their formation (Figure 3(a)), open triangles). The results show that treatment of these existing complexes with EDTA converts them into complexes with characteristics typical for those which form at the low MgCl $_2$ concentration:

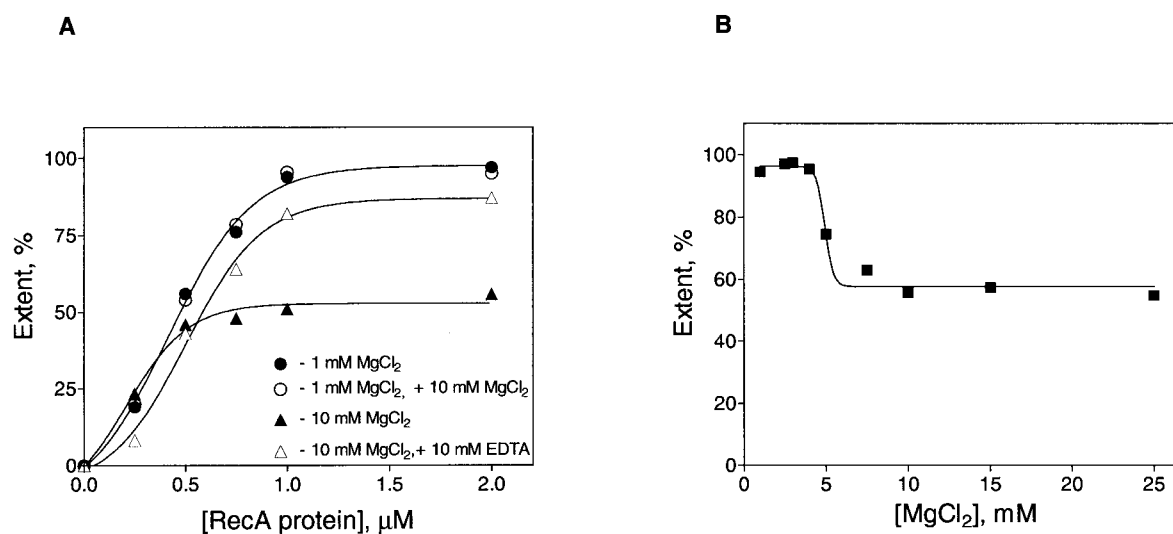


Figure 3. The two stoichiometric forms of the ATP γ S-RecA protein-dsDNA complexes are not fully inter-convertible. (a) Titrations were performed as described in Materials and Methods in the presence 1 mM ATP γ S and either (●) 1 mM MgCl $_2$ or (▲) 10 mM MgCl $_2$. At the end of each DAPI-displacement reaction, conducted at either 1 mM MgCl $_2$ or 10 mM MgCl $_2$, either an additional (○) 10 mM MgCl $_2$ or (△) 10 mM EDTA was added, respectively, to the final concentration indicated. The DNA concentration in all experiments was 6 μ M (nucleotides). (b) The extent of DAPI displacement was monitored as a function of MgCl $_2$ concentrations: the RecA protein concentration was 1 μ M and the DNA concentration was 6 μ M (nucleotides); each data point represents separate experiments.

i.e. a stoichiometry of 3 bp per monomer and high extent of DAPI displacement. However, the converse is not true: i.e. complexes formed at 1 mM $MgCl_2$ remain unchanged by the addition of 10 mM $MgCl_2$ (Figure 3(a), open circles). This behavior suggests that the complexes formed at 1 mM $MgCl_2$ in the presence of $ATP\gamma S$ are quite stable and resist rearrangement, whereas the additional DNA that is bound at 10 mM $MgCl_2$ is bound much more weakly by the 6 bp per RecA protein complex, and that this additional DNA can be dissociated by EDTA to produce the fully DAPI-displaced 3 bp per monomer complex (this low $[MgCl_2]$ -complex is, however, stable to further addition of EDTA; data not shown).

Displacement of ethidium bromide from dsDNA confirms the existence of two distinct stoichiometric forms

Ethidium bromide (EtBr), a DNA intercalator, is displaced from its intercalating site upon RecA protein binding in the presence of $ATP\gamma S$ (Kim *et al.*, 1993). This displacement of EtBr from dsDNA provides an alternative means to independently measure the binding stoichiometry of the RecA protein-dsDNA complexes. Figure 4 shows the results of such a titration in the presence of $ATP\gamma S$, and either 1 mM or 10 mM $MgCl_2$. The binding stoichiometries are identical with those seen in the DAPI assay: one RecA protein monomer per 3 bp at 1 mM $MgCl_2$ and one RecA protein monomer per 6 bp at 10 mM $MgCl_2$. However, in contrast to DAPI displacement, the maximum fluorescence change for the EtBr experiments is not significantly different at

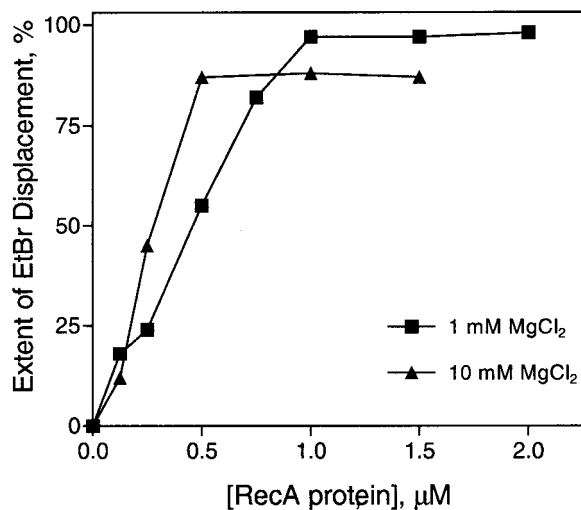


Figure 4. Ethidium bromide displacement also reveals two stoichiometric forms of RecA protein-dsDNA complex. EtBr displacement was performed as described in Materials and Methods in the presence of 1 mM $ATP\gamma S$ and either (■) 1 mM $MgCl_2$ or (▲) 10 mM $MgCl_2$. The DNA concentration was 6 μM (nucleotides).

each $MgCl_2$ concentration, indicating that there is nearly complete displacement of EtBr at both sets of conditions. These results agree with the DAPI displacement data which demonstrate a change in the RecA protein dsDNA-binding stoichiometry but, the different extents of displacement also show that DAPI and EtBr sense different features of these distinct RecA protein-dsDNA complexes.

Nitrocellulose filter-binding, a non-spectroscopic method, confirms the fluorescence assays

Nitrocellulose filter-binding is an established means of quantifying DNA binding (Kowalczykowski, 1990). This method is a direct measure of the interaction between protein and DNA, and is independent of spectroscopic assumptions. Because of the highly cooperative nature of RecA protein binding to DNA, filter-binding can be used to determine DNA binding stoichiometries (see Kowalczykowski, 1990). Figure 5 shows the results of filter-binding experiments performed in the presence of $ATP\gamma S$, at either 1 mM or 10 mM $MgCl_2$ (at two different DNA concentrations, for the latter). Again, as for both the DAPI and EtBr displacement assays, the stoichiometry of RecA protein-dsDNA complex formation is 3 bp per RecA protein monomer at 1 mM $MgCl_2$, and 6 bp per RecA protein monomer at 10 mM $MgCl_2$. Thus, three independent assays corroborate this unexpected dichotomy for the stoichiometry of RecA protein binding to dsDNA.

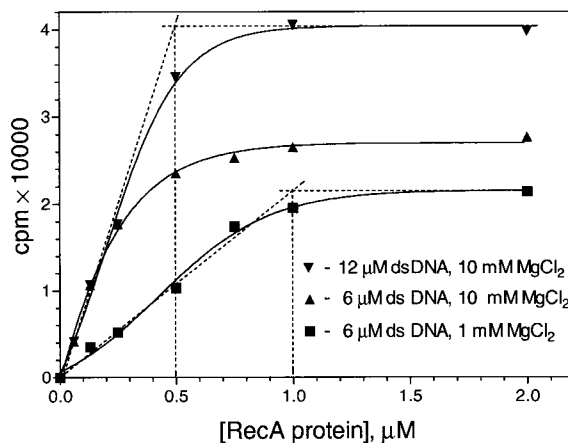


Figure 5. Filter-binding assays confirm the stoichiometries obtained from DAPI and EtBr displacement assays. Filter-binding assays were performed as described in Materials and Methods. Reactions contained the indicated concentrations of RecA protein, 1 mM $ATP\gamma S$, 6 μM (nucleotides) ^{32}P -labeled pBR322 dsDNA, and either (■) 1 mM or (▲) 10 mM $MgCl_2$. Assays were also performed using 12 μM (nucleotides) dsDNA at (▼) 10 mM $MgCl_2$. The amount of DNA retained on the filters at saturation was (■) 80%, (▲) 98%, (▼) 82%. The broken lines illustrate the experimental binding stoichiometries.

Sensitivity to DNase I reveals a structural difference in the two stoichiometric forms

DNase I is a non-specific ssDNA and dsDNA endonuclease. Earlier observations showed that the binding of RecA protein to duplex DNA under appropriate conditions conferred protection against DNase I (Chow *et al.*, 1986, 1988; Pugh & Cox, 1987). We utilized this nuclease protection assay to compare the RecA protein-dsDNA complexes formed at the low and high $MgCl_2$ concentrations. As shown in Figure 6, RecA protein-dsDNA complexes formed at 10 mM $MgCl_2$ are either slightly resistant or just as sensitive to DNase I as free dsDNA. On the other hand, the RecA protein-dsDNA complexes formed at 1 mM $MgCl_2$ are at least two to three orders of magnitude more resistant than free dsDNA. These results demonstrate a significant structural difference in the RecA protein-dsDNA complexes formed at these reaction conditions.

Using DNase I protection, we also repeated the experiments involving addition of EDTA to complexes pre-formed at the high $MgCl_2$ concentration (see Figure 3(a)). Because EDTA inhibits DNase I activity, this experiment was designed so that the $MgCl_2$ and EDTA concentrations were the same for both the experimental and control reactions. The RecA protein-dsDNA complexes that were formed at the high $MgCl_2$ concentration were processed in one of two ways: they were treated sequentially either with 10 mM EDTA for 20 minutes and then followed by the addition of 10 mM $MgCl_2$, or they were treated with 10 mM $MgCl_2$

for 20 minutes and then followed by the addition of 10 mM EDTA (designated on Figure 6 as EDTA first and Mg^{2+} first, respectively). The resultant complexes were then treated with DNase I. It was expected (based on Figure 3(a)) that, for the EDTA first procedure, addition of EDTA to the high Mg^{2+} complexes would convert them to the low-binding stoichiometry form which is present at the low $MgCl_2$ conditions and which is resistant to DNase I (see Figure 6); in contrast, for the Mg^{2+} first procedure, the high-binding stoichiometry complexes remain at a high Mg^{2+} concentration throughout and should be more sensitive to DNase I (see Figure 6). Indeed, as shown in Figure 7, the EDTA first procedure produces RecA protein-dsDNA complexes that are more resistant to DNase I than the Mg^{2+} first procedure (due to the presence of nearly an equivalent of EDTA, the absolute rates of DNase I digestion in these assays are lower than those shown in Figure 6; however, the relative rate differences in Figure 7 are significant). Thus, the nuclease sensitivity results parallel those of the binding experiments and, furthermore, they demonstrate an unexpectedly high sensitivity of the high-stoichiometry complexes to DNase I.

Discussion

Here, we uncover an unexpected characteristic of RecA protein binding to dsDNA. When taken together, the data from fluorescent spectroscopic assays employing DAPI (a minor groove binder; Trotta *et al.*, 1993) or EtBr (a DNA intercalator;

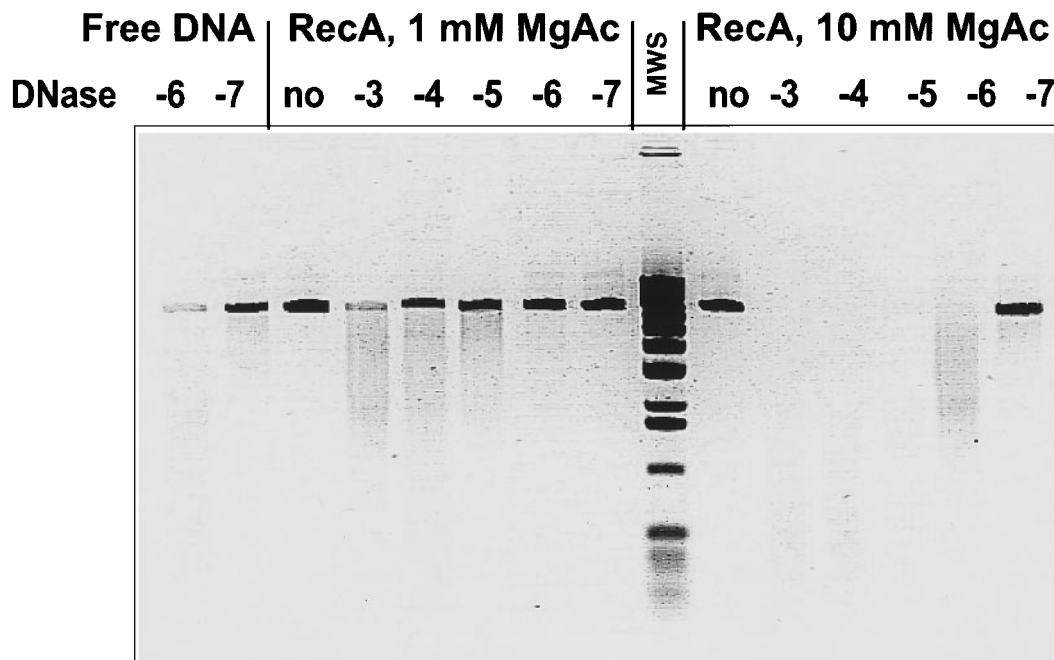


Figure 6. Differential sensitivity of RecA protein-dsDNA complexes to DNase I. Experiments were performed as described in the Materials and Methods. The numbers ranging from -7 to -3 designate tenfold serial dilutions of DNase I from 10^{-7} to 10^{-3} . The lane marked MWS is a 1 kb molecular mass standard ladder (Life Science).

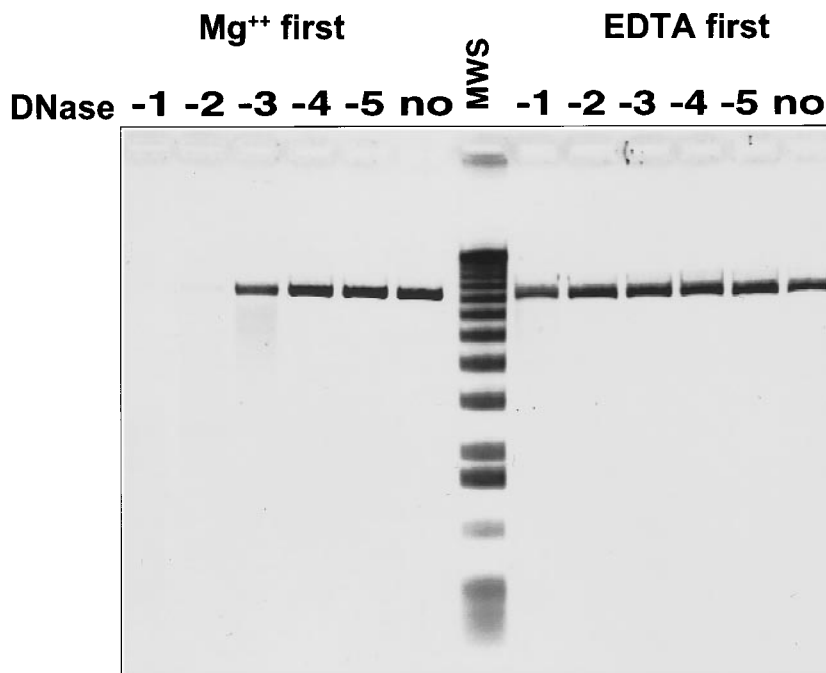


Figure 7. DNase I sensitivity reveals that EDTA dissociates the second bound-dsDNA molecule from the high-Mg²⁺ RecA nucleoprotein complex. The RecA protein-dsDNA complexes, initially formed in 10 mM MgCl₂ with an excess of RecA protein (one RecA protein per three nucleotides), were processed in one of two ways: EDTA first (subsequent incubation with 10 mM of EDTA for 20 minutes followed by the addition of 10 mM MgCl₂) or MgCl₂ first (subsequent incubation with an additional 10 mM of MgCl₂ for 20 minutes followed by the addition of 10 mM EDTA). The resulting complexes were digested using varying dilutions of DNase I, as described in the legend to Figure 6.

Baguley & Falkenhaus, 1978; Morgan *et al.*, 1979), nitrocellulose filter-binding assays, and DNase I sensitivity assays argue that RecA protein can bind one or two equivalents of dsDNA, depending on reaction conditions. This means that the RecA protein-dsDNA filament can bind a second molecule of dsDNA.

In the presence of ATP γ S, the binding stoichiometry differs by twofold, depending on the MgCl₂ concentration. This altered stoichiometry, 3 bp per monomer of RecA protein at low MgCl₂ concentration *versus* 6 bp at high MgCl₂ concentration, suggests that the same RecA protein filament binds twice as much dsDNA at high MgCl₂ concentrations, and that two dsDNA equivalents can be taken up in the final RecA protein-dsDNA complex. This unanticipated dependence of the DNA binding stoichiometry on MgCl₂ concentration provides an explanation as to why previous studies, employing a diverse range of experimental procedures, had failed to observe binding of a second dsDNA molecule by the RecA-dsDNA nucleoprotein filament (Dombroski *et al.*, 1983; Müller *et al.*, 1990; Takahashi & Hagmar, 1991): all of these experiments employed ATP γ S and low Mg²⁺ concentrations (where RecA protein shows the low 3 bp per monomer binding stoichiometry). Hence, the binding of two molecules of dsDNA by RecA protein could not have been observed. However, we do not believe that this binding is peculiar to the use of ATP γ S; rather, ATP γ S substantially increases the affinity of DNA to both the primary and secondary DNA binding sites of RecA protein and, by amplifying these affinities, permits observation of the normally weak binding of dsDNA to the secondary DNA binding site.

The binding of two dsDNA equivalents by the RecA protein filament requires a high MgCl₂ concentration, with the transition midpoint occurring at about 5 mM MgCl₂ (see Figures 3(b) and 8). This transition between stoichiometric forms is observed within a relatively small range of MgCl₂ concentration, demonstrating that it occurs in a highly cooperative manner. Similar cooperative behavior was observed by electron microscopy for bundle formation by ATP γ S-RecA protein-dsDNA filaments (Egelman & Stasiak, 1988; Yu & Egelman, 1992), and by light-scattering for free RecA protein (Cotterill & Fersht, 1983). Finally, the binding of a second dsDNA molecule by the RecA-dsDNA nucleoprotein filament observed here, formally resembles the coaggregation of dsDNA by the RecA-ssDNA nucleoprotein filaments, which is another cooperative Mg²⁺-dependent process that also has a transition midpoint at about 5 mM MgCl₂ (Tsang *et al.*, 1985).

The two dsDNA binding sites of the RecA protein filament cannot be equivalent, because DAPI displacement occurs only upon binding to the first site but not to the second site, as inferred from the change in fluorescence of only 50% (see Figures 3(a) and 8); in contrast, EtBr is displaced from both DNA binding sites equally (see Figure 4). The transition between these two types of complexes displays hysteresis in that conversion of the complex formed at high Mg²⁺ concentrations to the one formed at low Mg²⁺ concentration (see Figure 8) occurs upon addition of 10 mM EDTA but not *vice versa*, since the addition of MgCl₂ does not change the observed fluorescence (see Figure 3(a)). This non-equivalence of dsDNA binding may be analogous to the asymmetric binding

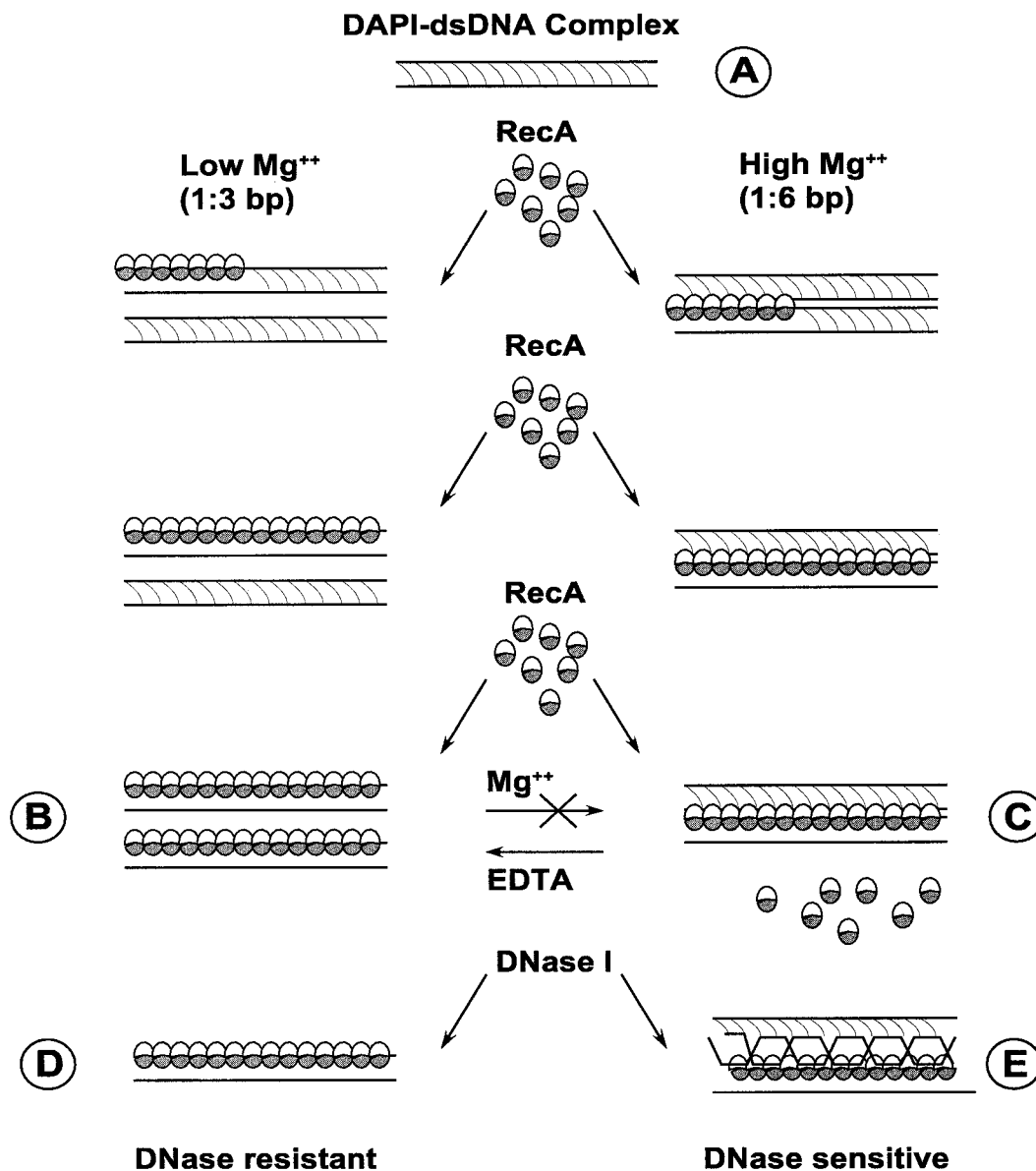


Figure 8. A model for the binding of RecA protein to dsDNA in the presence of ATP_γS. (a) DAPI-dsDNA complexes: the curved thin lines between the DNA strands represent bound DAPI, and not the DNA bases. RecA protein binding is depicted both at the low MgCl₂ conditions (1 mM) that give rise to the 1 RecA protein:3 bp stoichiometric complex (left-hand side), and at the high MgCl₂ conditions (10 mM) that give rise to the 1 RecA protein:6 bp stoichiometric complex (right-hand side). (b) Final form of the complexes formed at 1 mM MgCl₂: in this complex, all of the bound DAPI is displaced from DNA, and no change in fluorescence is observed upon raising the MgCl₂ concentration; this complex is DNase I resistant. (c) Final form of the complex formed at 10 mM MgCl₂: in this complex, only 50% of the bound DAPI is displaced and the remainder is bound to one of the DNA molecules in the complex; this complex is DNase I sensitive. Addition of EDTA (10 mM) results in dissociation of the second DNA molecule with the bound DAPI from the RecA nucleoprotein complex; free RecA protein then binds this dsDNA and displaces the bound DAPI. (d), (e) The imagined forms of the DNase I resistant and sensitive forms, respectively; the sensitive form has both DNA duplexes either unwound or poised for exchange.

of ssDNA reported earlier (Lauder & Kowalczykowski, 1991; Mazin & Kowalczykowski, 1996). Furthermore, the data argue that both dsDNA binding sites are occupied simultaneously.

Previously, we argued that DAPI displacement was due to a direct consequence of occlusion by RecA protein, rather than to an indirect effect mediated by protein-dependent DNA unwinding or extension (Zaitsev & Kowalczykowski, 1998).

Therefore, this interpretation suggests that binding of the first dsDNA molecule to RecA protein occurs *via* the minor groove of the dsDNA, a conclusion which is in accordance with that of several other studies (DiCapua & Müller, 1987; Dombroski *et al.*, 1983; Kumar & Muniyappa, 1992; Leahy & Radding, 1986; Tuite *et al.*, 1997). This conclusion, in turn, implies that the major groove of this first dsDNA molecule may be free and potentially

available for recognition events. However, binding of the second dsDNA molecule to the RecA protein-dsDNA filament must be different. This follows from the fact that binding of the second dsDNA molecule does not result in DAPI displacement, but does result in EtBr displacement; these results suggest that interaction of the RecA protein-dsDNA complex with the second dsDNA molecule does not involve the minor groove of this duplex. Instead, it must be occurring *via* either the sugar-phosphate backbone or the major groove. Thus, the two dsDNA molecules within the RecA nucleoprotein filament are likely to oppose one another along their major grooves. Our conclusion that the second dsDNA molecule does not interact with RecA-dsDNA nucleoprotein filament through its minor groove but rather probably through its major groove, seems to conflict with published reports which conclude homologous pairing of dsDNA with ssDNA occurred through interaction with the minor groove (Baliga *et al.*, 1995; Podyminogin *et al.*, 1995; Zhou & Adzuma, 1997). However, since the experimental approaches that were used to arrive at this conclusion involved the use of crosslinking agents with long flexible arms, these experiments cannot be regarded as unequivocal.

The DNase I sensitivity results clearly demonstrate a physical difference between the RecA protein-DNA complexes that bind only one dsDNA molecule *versus* those that bind two. The nuclease sensitivity of the RecA protein-DNA complexes containing two dsDNA molecules is nearly that of free DNA. This result was entirely unexpected; we anticipated that the dsDNA bound to the primary site would be protected by RecA protein and that it would remain resistant to DNase I (because both RecA protein and DNase I interact with dsDNA through its minor groove). However, we expected that the dsDNA bound to the secondary binding site would be sensitive to DNase I, because its minor groove is not occupied by RecA protein (because DAPI is not displaced from the dsDNA at the secondary DNA binding site of RecA filament). How and why does the dsDNA which is bound to the primary site of the high-Mg²⁺ form of the RecA protein-dsDNA complex become sensitive to DNase I? It seems unlikely that the structure of the primary site-dsDNA nucleoprotein complex is fundamentally different in the two stoichiometric forms, because the high-Mg²⁺ complex can be converted to the low-Mg²⁺ form, presumably without dissociation of the primary site complex (due to the high stability of the ATP γ S complex; Mazin & Kowalczykowski, 1996; Menetski & Kowalczykowski, 1985). An alternative explanation is that both dsDNA species within this high-Mg²⁺ form, "sense" each other's presence, and they "test" for homology by initiating local unwinding or melting of both dsDNA duplexes (Chiu *et al.*, 1990; Conley & West, 1990; Lindsley & Cox, 1990; Wong *et al.*, 1998), or possibly by participating in a limited amount of local DNA strand exchange.

Such complexes would be expected to form as a preliminary but necessary step in the homology search: the partial melting and transient exchange of strands between two dsDNA molecules within the two different DNA binding sites of RecA protein could occur as the prelude to homologous recognition and, hence, should occur even if the DNA molecules are not homologously paired. We suggest that such unwound or transiently "exchanged" structures are susceptible to nicking by DNase I, explaining the complete sensitivity of the complexes with two molecules of bound dsDNA compared to those with only one molecule of bound DNA. We suppose that this conformation of the duplex DNA molecules within RecA nucleoprotein filament is the same as that responsible for the increased efficiency of joint molecule formation that is mediated by homologous duplex-duplex DNA contacts (Chow *et al.*, 1991; Conley & West, 1989).

The finding that the RecA protein filament is able to bind two dsDNA molecules raises the important question as to whether the two DNA duplexes within this complex are homologously aligned, and whether they are capable of exchanging DNA strands. Despite numerous attempts, we were unable to obtain an unambiguous answer because of difficulties associated with the hysteresis that exists for conversion between the low and high-Mg²⁺ complexes (see Figure 8): though we could load RecA protein onto one dsDNA partner of a pairing reaction that could measure pairing with a second, distinct dsDNA partner, conversion to the form that could bind the two different duplexes at the higher Mg²⁺ concentration is not possible; conversely, if RecA protein was loaded onto dsDNA at the high Mg²⁺ concentration, then we believe that the "self-pairing" of the dsDNA occurred, precluding detection of any homologous pairing or DNA strand exchange that might have resulted with a second DNA duplex. Presently, we favor the idea that this "pairing" is mostly non-homologous, based on the observation that an attempted DNA strand exchange reaction between two homologous dsDNA fragments with lengths of 80 bp and 123 bp, respectively, produced about 1.5% of the 80-123 tailed duplex heteroduplex product (data not shown), a yield that is expected for random pairing events. Interestingly, visualization of RecA protein-dsDNA complexes by electron microscopy produced the same conclusion: dsDNA molecules are paired non-homologously for several thousand nucleotides under conditions that favor the binding of RecA protein (McEntee *et al.*, 1981). It just may be that the very nature of this ATP γ S-dependent reaction, which permits detection of two stably bound dsDNA molecules, also prevents any extensive homologous re-alignment: the intrinsic stability of these nucleoprotein complexes is too high to permit the rapid dissociation needed for the homology search process; in the presence of ATP γ S, the enhanced affinity of the secondary DNA binding site restricts the dissociation and

rebinding events that comprise the homologous alignment process.

In conclusion, this DAPI displacement assay provides a convenient means to monitor the binding of RecA protein to dsDNA, and provides evidence for a $MgCl_2$ -induced structural change that allows binding of two dsDNA molecules by RecA protein. The existence of this latter complex demonstrates that RecA-dsDNA nucleoprotein filament can bind a second molecule of dsDNA. Furthermore, as established quite some time ago, RecA protein-mediated DNA strand exchange occurs not only in the presence of ATP, but also in the presence of either $ATP\gamma S$ or $ADP\text{-}AlF_4^-$ (Kowalczykowski & Krupp, 1995; Menetski *et al.*, 1990); thus, we infer that the properties of RecA protein reported here in the presence of $ATP\gamma S$, also apply qualitatively to the ATP-dependent situation. We suggest that this complex is likely to be the active species in four-stranded DNA exchange reactions, and that the RecA protein-dsDNA filament, like the RecA protein-ssDNA filament, may possess homologous pairing activity (E.N.Z. & S.C.K., unpublished results). Though the amount of net DNA strand exchange in this duplex-by-duplex paired structure may be quite limited, it may be sufficient to contribute to the weaker, but multiple, homologous pairing interactions that are needed to homologously pair chromosomes in processes such as eukaryotic meiosis: it would be interesting to learn whether the eukaryotic RecA-like proteins, the Rad51 and Dmc1 proteins, possess such an ability.

Materials and Methods

Reagents

Chemicals were reagent grade, and solutions were prepared using Barnstead NANOpure water. ATP and dATP were purchased from Sigma; $ATP\gamma S$ was purchased from Boehringer Mannheim. Dyes were obtained from Molecular Probes, and their concentrations were determined using the following extinction coefficients: ethidium bromide (EtBr), $5.5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 546 nm; DAPI, $33 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 345 nm (Eggleston *et al.*, 1996).

RecA protein

RecA protein was purified from *Escherichia coli* strain JC12772 (Uhlen & Clark, 1981) using a preparative protocol based on spermidine acetate precipitation (Griffith & Shores, 1985).

DNA substrates

Double-stranded replicative form DNA from bacteriophage M13mp7 was prepared using the procedure described by Messing (1983). The duplex DNA was linearized by digestion with *EcoRI* restriction endonuclease. The plasmid pBR322 was purified from strain S819 by CsCl density gradient centrifugation followed by chromatography on Sephacryl S-500 (Pharmacia). The DNA was linearized with *HindIII* restriction endonuclease (NEB) and end-labeled at the 5' termini by sequential

reaction with shrimp alkaline phosphatase (USB) and T4 polynucleotide kinase (NEB) in the presence of [γ - ^{32}P]ATP (ICN).

DAPI-displacement assay

The standard reaction buffer (Buffer A) consisted of 25 mM sodium Mes (pH 6.2), from 1 to 10 mM $MgCl_2$ (as indicated), 1 mM dithiothreitol, and 1 mM $ATP\gamma S$. Each reaction contained standard buffer, 400 nM DAPI, 6 μM (nucleotides) linear M13 dsDNA, and the indicated concentration of RecA protein.

Fluorescence measurements were carried out on a Shimadzu RF5000U spectrofluorophotometer. The excitation/emission wavelengths and the slit widths were 345/467 nm and 5/10 nm, respectively. Reactions were performed as described (Zaitsev & Kowalczykowski, 1998). Unless otherwise noted, assays were conducted at 37°C.

Ethidium bromide displacement assay

Reaction conditions were the same as for the DAPI-displacement assay except that ethidium bromide (2 μM) was substituted for DAPI. The excitation/emission wavelengths and the slit widths were 535/600 nm and 5/10 nm, respectively.

Filter-binding assay

Formation of RecA protein-dsDNA complexes was measured as described (McEntee *et al.*, 1981). Alkali-treated filters (HAWP 025; Millipore Corp.) were soaked in buffer A for at least 30 minutes prior to use. Reaction mixtures (20 μl) containing reaction buffer, 6 μM ^{32}P -labeled pBR322 dsDNA, and RecA protein, as indicated, were incubated at 37°C for 30 minutes. Samples were applied to the filter under suction and washed with 3 ml of buffer A. After drying, the amount of radioactivity on the filters was measured using a Betascope 603 radioisotope analyzer (Betagen).

DNase I assay

Chromatographically purified bovine deoxyribonuclease I (Sigma) was reconstituted from lyophilized powder at a concentration of 10 kU/ml in dilution buffer (sodium Mes (pH 6.4), 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 40% (v/v) glycerol) and stored at -80°C. To form complexes of RecA protein and dsDNA, M13 dsDNA (10 μM , nucleotides) and RecA protein (3.3 μM) were incubated for 20 minutes at 37°C in buffer A with the $MgCl_2$ concentration indicated in the legend to Figure 5. To determine sensitivity to DNase I, 1 μl of a tenfold dilution of DNase I in dilution buffer was added to these pre-formed RecA protein-dsDNA complexes in a final volume of 15 μl . To avoid any [Mg^{2+}]-dependent difference in DNase I activity, all samples were adjusted to the same final $MgCl_2$ concentration before nuclease digestion. Nuclease digests were performed at 37°C for one hour, and were stopped by adding EDTA and SDS to final concentrations of 30 mM and 0.5% (w/v), respectively. Reaction products were analyzed by agarose gel electrophoresis in Tris acetate-EDTA buffer. DNA was visualized under UV light after EtBr staining. Digital photoimages were inverted for viewing convenience.

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