Direct Visualization of RecA Protein Binding to and Unwinding Duplex DNA following the D-loop Cycle*

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The RecA protein of Escherichia coli will promote the plecotenemic joining of a linear single-stranded DNA molecule with a homologous supertwisted double-stranded (ds) DNA molecule. As shown by others, this reaction is characterized by a single cycle of joint formation and dissociation, termed the D-loop cycle. The released DNA products appear by electron microscopy. This result provides direct evidence that the block to a second cycle of joining is due to the presence of RecA protein remaining bound to the released dsDNA.

Homologous recombination in Escherichia coli is mediated by the RecA protein, and in vitro studies of model reactions utilizing simple DNA substrates have begun to describe how, at the molecular level, this protein promotes the exchange of DNA strands (reviewed in Ref. 1). At a biochemical level, strand exchange reactions can be experimentally separated into three stages. In the first stage, RecA protein binds to ssDNA forming a presynaptic filament. In the second stage, synapsis with a dsDNA at a site of homology produces a plecotenemically jointed joint. In the third stage, branch migration of the joint results in a net exchange of strands.

The ability to experimentally divide strand exchange reactions into partial reactions has resulted in each stage being examined as a distinct reaction. One very useful reaction has been the pairing of linear ssDNA with homologous supertwisted dsDNA. In this reaction, DNA pairing (accompanied by the exact relaxation of the dsDNA supertwists) occurs readily (2–5); however, the lack of a free end in the dsDNA blocks any net strand transfer. Despite this, plecotenemically jointed joints can be driven around the length of the dsDNA circles by RecA protein in a reaction we have termed a “rolling D-loop reaction,” apparently being released when the 3′ end of the ssDNA is reached (6) (Fig. 1). Thus at the completion of the D-loop cycle, the reactant DNA molecules are restored (4, 7, 8).

The ss and dsDNA products of this reaction are found to be intact (7, 8) and, as judged by EM, the RecA protein filaments which contain the ssDNA are also identical to the filaments observed at the initiation of the D-loop cycle (6). Nonetheless, only a single cycle of D-loop formation and dissociation is observed under the usual conditions used for strand exchange reactions (7, 8). This inability to undergo multiple rounds of pairing indicates that a change in reaction components must have occurred during the D-loop cycle. The observation that the kinetics of the D-loop cycle can be altered by several changes in reaction conditions which also affect the formation of RecA protein-ssDNA filaments (such as MgCl₂, ATP, and RecA protein concentrations (4, 7, 8)) points to changes in the filaments. Conversely, the changes in kinetics observed following variation of dsDNA concentration (7) and the appearance during D-loop dissociation of an ATPase activity that is dependent upon both ssDNA and homologous dsDNA (9) argue that the dsDNA may have been inactivated.

Shibata and colleagues (4, 7, 10) concluded that the dsDNA is inactivated during the D-loop cycle due to the loss of superhelical turns caused by the binding of RecA protein to the dsDNA following dissociation of the D-loop complex. These conclusions were based on several findings: (i) the D-loop cycle was observed only when the dsDNA was super-twisted (4); (ii) excess RecA protein inhibited the D-loop cycle, unless the concentration of dsDNA was increased proportionally (7); (iii) a restimulation of the joint cycle was observed when fresh dsDNA, but not fresh ssDNA or RecA protein, was added to the reaction mixture following dissociation of a majority of the joints (7). In addition, when aliquots of dsDNA from both before and after a D-loop cycle were treated with topoisoamerase and then electrophoresed on agarose gels, the two DNAs migrated differently, indicating that they had different superhelical densities (10). While it could be concluded from these studies that RecA protein had bound to post-D-loop cycle dsDNA, the approaches used did not allow for the characterization of the structure of these complexes. In addition, we have not visualized tracts of RecA

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1 The abbreviations used are: ss, single-stranded; ds, double-stranded; EM, electron microscopy; SSB protein, single-stranded DNA binding protein of E. coli; kbp, kilobase pair; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
protein bound to dsDNA following dissociation of either paranolinic or pleiotropic joints (3, 6). Therefore, we felt it was important to re-examine these reactions more closely.

RESULTS AND DISCUSSION

In this study, two significant changes were made from the conditions described by Shibata et al. (4, 7, 8, 10). First, SSB protein was included in these reactions, both because it has been genetically implicated in recombination in vivo (11-13) and because it is required for the efficient assembly of presynaptic filaments under the ionic conditions which are optimal for the strand exchange steps (14). Second, an ATP-regenerating system was used to eliminate ADP buildup in the reaction mixtures.

As noted above, Shibata et al. (7) demonstrated that multiple rounds of pairing are not observed even if fresh RecA protein or ssDNA is added to the reaction mixtures following the cycle of joint formation and dissociation, and we have verified this result (data not shown). However, it seemed possible that these results only reflected the inability of fresh RecA protein-ssDNA filaments to assemble in the reaction mixtures rather than an inability of the newly formed filaments to reinitiate the D-loop cycle. To exclude this possibility, presynaptic filaments were assembled in the absence of dsDNA and their formation verified by EM. These newly formed presynaptic filaments were then added to a D-loop reaction mixture following completion of a cycle. This resulted in no increase in the number of dsDNA molecules contained in joined complexes (data not shown), indicating that presynaptic filaments which have not participated in a D-loop cycle are unable to pair with dsDNA which has undergone a round of pairing and dissociation.

It was previously shown (7) that addition of fresh dsDNA to a post-joint cycle reaction mixture resulted in formation of joined molecules with the fresh dsDNA. In that study, however, it was not shown whether the addition of fresh dsDNA stimulated joint formation with only the new dsDNA or with both the new and "used" dsDNA. If dsDNA is inactivated during the D-loop cycle, then addition of fresh dsDNA should result in formation of new joints with only the new dsDNA. To test this hypothesis, linear M13mp7 ssDNA and super-twisted M13mp7 dsDNA were incubated together under strand exchange conditions and the reaction was allowed to proceed through the D-loop cycle. Following dissociation of a majority of the joints, a different supercoiled dsDNA was added to the reaction mixture. This dsDNA was an M13mp19 construct containing 4.3 kbp of E. coli DNA that could be visually distinguished from the M13mp7 dsDNA by virtue of its larger (11.6 kbp) size. As shown in Fig. 2A, addition of this dsDNA resulted in formation of joints with only the larger DNA; dissociation of joined complexes containing the original dsDNA proceeded unaltered. When the order of dsDNA ad-

FIG. 1. The rolling D-loop cycle. Under strand exchange conditions, homologous linear ssDNA and super-twisted dsDNA (A) can pair. Pairing is accompanied by the removal of all the superhelix in the dsDNA. We have postulated (6) that if this pairing occurs at the 5' end of the ssDNA a pleiotropic joint forms (B) which can move around the dsDNA (C) until the 3' end of the ssDNA is reached and the starting DNAs are released as products (D).

FIG. 2. Addition of fresh dsDNA to a post-D-loop cycle reaction mixture stimulates joint formation with only the freshly added dsDNA. Linear M13mp7 ssDNA (3 μM; cut by BamHI) was incubated with RecA protein at a molar ratio of one RecA protein monomer/18 nucleotides of ssDNA in a buffer containing 30 mM NaCl, 3 mM ATP, 12 mM MgCl2, and 20 mM Hepes (pH 7.5) at 37 °C for 5 min. An ATP-regenerating system consisting of creatine phosphokinase (4 μg/ml; Sigma) and phosphocreatine (20 mM; Sigma) was included in all reactions. SSB protein was then added at a molar ratio of one SSB protein monomer/18 nucleotides of ssDNA, and incubation at 37 °C was continued for 10 min. To form joints, either super-twisted M13mp7 dsDNA (3 μM, A) or an 11.6-kbp chimeric dsDNA (3, consisting of M13mp19 with a 4.3-kbp insert of E. coli DNA (3 μM; B) was then added. Following 45-min incubations (to allow for completion of the D-loop cycle, either the M13mp19 chimeric dsDNA (3 μM; A) or M13mp7 dsDNA (3 μM; B) was added to the reaction mixtures (arrows), and the incubations continued as shown. At the indicated times, complexes were fixed sequentially by addition of formaldehyde to 1% for 10 min at 21°C followed by the addition of glutaraldehyde to 0.6% for 10 min more. The samples were chromatographed over Sepharose 4B (Pharmacia LKB Biotechnology Inc.) and mounted onto thin carbon films supported by copper mesh grids (15, 16). Greater than 50 joined complexes containing either M13mp7 dsDNA (⊙; A; ○; B) or the M13mp19 chimeric dsDNA (△; A; ●; B) were scored for each time.

These results confirm the conclusions of Shibata et al. (7) that the super-twisted dsDNA is inactivated during the D-loop cycle. However, during these experiments no obvious alteration of the dsDNA following a cycle of joining was observed by EM. Thus, a more careful comparison of the pre- and post-D-loop cycle DNA was carried out, examining the number of superhelix in the two DNA populations.

We have shown that the EM methods used in this study (15, 16) can be used to count the superhelical turns in dsDNA circles of low to moderate superhelical density (17), one crossover in the two-dimensional projection of the DNA in the micrograph being equal to one superhelical turn. To quantitate the superwining of the dsDNA not bound to presynaptic filaments in these reactions, linear M13mp7 ssDNA was incubated with super-twisted M13mp7 dsDNA under strand exchange conditions (see legend, Fig. 2), and the reaction was allowed to proceed through the D-loop cycle. Complexes were then fixed sequentially with formaldehyde and glutaraldehyde, chromatographed over Sepharose 4B to remove unbound protein, and mounted directly for EM visualization. (16). Free dsDNA molecules were scored, and the number of crossovers in each molecule was determined. As a control, super-twisted M13mp7 dsDNA (in strand exchange buffer) which had not gone through a D-loop cycle was fixed and mounted for EM visualization in an identical manner. As can be seen in Fig. 3A, almost 90% of the dsDNA which had not been in a pairing reaction (n = 30) contained >20 crossovers (it was not possible to reliably count crossovers in most molecules with >20 crossovers; therefore, these molecules were combined into single groups in Fig. 3). However, only
described in Fig. 3C). The number of crossovers present in at least 30 sequentially observed supertwisted dsDNA molecules was scored for each sample. As a control, M13mp7 dsDNA in strand exchange buffer was prepared for EM visualization in the same manner including fixation with both fixatives (A).

FIG. 3. Distribution of superhelical turns in dsDNA before and after a D-loop cycle. Linear M13mp7 ssDNA and supertwisted M13mp7 dsDNA were allowed to proceed through a D-loop cycle as described in Fig. 2 for 45 min. Aliquots were then prepared for EM visualization (Fig. 2) following sequential fixation with formaldehyde and glutaraldehyde (B) or fixation with glutaraldehyde alone (C). The number of crossovers present in at least 30 sequentially observed supertwisted dsDNA molecules was scored for each sample. As a control, M13mp7 dsDNA in strand exchange buffer was prepared for EM visualization in the same manner including fixation with both fixatives (A).

FIG. 4. Visualization of RecA protein binding to and unwinding dsDNA following the D-loop cycle. Linear M13mp7 ssDNA was paired with supertwisted M13mp7 dsDNA under strand exchange conditions for 45 min and then prepared for EM as described in Fig. 3C. The micrographs were taken at a 45° tilt on a Philips EM400 TLG instrument. Bar = 0.5 μm.

45% of the dsDNA molecules which had undergone pairing (n = 30) contained >20 crossovers (Fig. 3B). The remainder contained 16 ± 3 crossovers, indicating that this DNA was partially unwound. Although no protein tracts were visible on the partially unwound dsDNA molecules following dissociation from the RecA protein-ssDNA filaments, the unwinding did appear to be protein-mediated; brief incubation of post-D-loop cycle reaction mixtures in 0.1% sodium dodecyl sulfate resulted in restoration of the distribution present in the starting population of dsDNA (data not shown).

To better visualize RecA protein bound to post-D-loop cycle dsDNA, numerous modifications of the two-step fixation procedure were evaluated. Good results were obtained when the formaldehyde step was eliminated and glutaraldehyde fixation performed as described (16). When such fixation was used following a 45-min D-loop reaction, much of the dsDNA not associated with presynaptic filaments contained short tracts of protein bound along the dsDNA (Fig. 4). The appearance of these tracts was very similar to that of the presynaptic filaments observed in the same fields. Both the length of these tracts and the amount of unwinding varied from molecule to molecule (compare Fig. 4A to 4B). When the number of crossovers was determined in those molecules (e.g. Fig. 4B) in which the twisting of the DNA strands could be followed, only about 20% of the molecules had >20 crossovers; the remainder contained an average of 12 ± 4 crossovers (Fig. 3C).

Thus following glutaraldehyde fixation alone, RecA protein was detected bound to the post-D-loop cycle dsDNA, and this DNA contained fewer supertwists (on the average) than after fixation with formaldehyde and glutaraldehyde, roughly one-third the number of supertwists of the protein-free DNA (17).

The presence of protein on the dsDNA following the use of one fixation procedure but not another could be explained if RecA protein binding to the dsDNA was very labile. Indeed, biochemical results have been presented which support this proposal. As discussed above, Shibata et al. (10) used high concentrations of topoisomerase for short (30 s) times to demonstrate that the superhelical density of pre- and post-D-loop cycle DNA was different. We have repeated this experiment and obtained similar results. However, if the topoisomerase concentration was decreased so that 20–30 min was required for complete unwinding of the starting dsDNA, then the pre- and post-D-loop cycle DNAs had the same superhelical densities (data not shown). These results are most easily explained if the unwinding effect was due to a labile RecA protein-dsDNA interaction which dissociated during the longer reactions.

While the single step fixation used here revealed some RecA protein bound to post-D-loop cycle dsDNA, this may still be a low estimate. Indeed, the great heterogeneity in the appearance of the complexes argues for this point, and Shibata et al. (8, 10) have proposed that enough RecA protein is present in these complexes to completely unwind the dsDNA.

Recently Shaner and Radding (18) and Shaner et al. (19) reported that RecA protein can bind to duplex DNA in molecules containing both duplex and single-stranded regions under strand exchange conditions. Similar to the results reported here, they found that a single-step glutaraldehyde fixation protocol was required to observe the RecA protein-dsDNA complexes; sequential formaldehyde-glutaraldehyde fixation did not preserve these structures (19). While the experiments detailed here describe RecA protein-dsDNA complexes which form by a different process (i.e. as a result of a rolling D-loop reaction), both phenomena are important as they show that RecA protein-dsDNA-ATP complexes can form under a variety of conditions. In addition, these reports further emphasize the importance of optimizing the fixation methods used for EM with regard to each protein-nucleic acid complex studied (15).

While the results presented here show directly that RecA protein binds to and unwinds dsDNA following the D-loop cycle, two major questions about this binding remain unanswered: (i) what is the origin of the RecA protein which binds to the dsDNA during the D-loop cycle; and (ii) is there a function for this binding? There are two potential sources for the RecA protein which is bound to dsDNA following the D-loop cycle. During movement of the D-loop, either free RecA protein could be sequestered by the unwound dsDNA or some of the RecA protein originally bound to the ssDNA could be transferred to the dsDNA partner. Both of these possibilities would most likely occur in joint regions. Shibata et al. (7) have argued that free RecA protein binds to the dsDNA during pairing. While both their data and the data presented here are consistent with that model, they also observed that the RecA protein to ssDNA stoichiometry required for the D-loop cycle was lower than that required for strand exchange involving a linear dsDNA (7) and that shown to be optimal for formation of RecA protein-ssDNA complexes under strand exchange conditions (20, 21). Thus, there was probably minimal free RecA protein in solution available to bind to dsDNA during their reactions. For this and other reasons, we favor a model in which RecA protein is transferred from the RecA...
protein-ssDNA filament to the dsDNA during pairing.

In vivo, the binding of RecA protein to post-D-loop cycle dsDNA could mark the segments of dsDNA which had recently undergone recombination. Precisely how this would intervene in the normal flux of DNA metabolism is difficult to predict. The transient nature of the RecA protein bound to the dsDNA might serve to inhibit multiple rounds of recombination at a single site over a short period of time.

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