The binding of RecA protein to duplex DNA molecules is directional and is promoted by a single stranded region

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ABSTRACT

RecA protein from *E. coli* binds more strongly to single stranded DNA than to duplex molecules. Using duplex DNA that contains single stranded gaps, we have studied the protection by RecA protein at various concentrations, of restriction sites as a function of their distance from the single stranded region. We show that the binding of RecA protein, initiated in the single stranded region, extends progressively along the adjoining duplex in the 5' to 3' direction with respect to the single stranded region. The strand exchange reaction is known to proceed in the same direction.

INTRODUCTION

RecA protein from *E. coli* promotes homologous pairing and strand exchange reactions between DNA molecules. It binds to single strand DNA (ssDNA) to form a complex which pairs with homologous double stranded DNA (dsDNA) (1-4). It also binds to circular duplex DNA that contains a single stranded gap (gapped DNA) (5) and pairs this DNA with duplex homologs (6,7). If the duplex has a 3' end complementary to the single strand in the gap, the enzyme mediates a strand exchange reaction which advances 5' to 3' along the ssDNA and continues in the same direction along the adjoining duplex (8). Reactions involving gapped and intact duplex molecules are of interest because genetic recombination in vivo is often between duplex molecules.

Information on the structure and properties of RecA-DNA complexes may help us to understand the mechanisms of genetic recombination. In the presence of the non-hydrolyzable analog ATP(γ -S), RecA protein polymerizes on dsDNA as a right handed spiral filament with deep grooves between turns. The DNA is stretched by about 50% in comparison with normal B form, and is bound with a stoichiometry of three base-pairs per RecA monomer (9,10). RecA spiral filaments also form on ssDNA in the presence of ATP or ATP(γ -S). They stretch DNA to the same extent and are generally similar in appearance to those formed on dsDNA with ATP(γ -S) (11). In contrast, the complexes formed on ssDNA in the

absence of nucleotide cofactor are smooth in appearance rather than deeply grooved, and the axial rise per base is only 2/3 of that in normal B form DNA (12). RecA protein is a ssDNA-dependent ATPase and the resulting ADP tends to dissociate RecA-DNA complexes and block reactions, unless an ATP regenerating system is present (2,13). RecA is not known to form stable complexes with dsDNA without a nucleotide cofactor.

In the presence of ATP(γ -S), RecA binds more extensively to gapped than to intact circular DNA (5,6). In this paper we report measurements of the protection by RecA protein of restriction sites in a gapped duplex. The results show that RecA binding, initially on the ssDNA of the gap, extends along the adjoining duplex in the 5' to 3' direction of the single stranded region.

MATERIALS AND METHODS

Enzymes and proteins. RecA protein was purified by a procedure which includes elution from a ssDNA agarose column by ATP (14,15). All restriction enzymes, exonuclease III and lambda exonuclease were from Bethesda Research Labs.

Preparation of DNA substrates. 32 P ϕ x174 form I DNA was purified by sedimentation through neutral sucrose (16) and viral ϕ x174 DNA was prepared by phenol extraction of purified ϕ x174 (17). 32 P form III DNA was obtained by treatment of 32 P ϕ x form I with SstII or PstI. For circular gapped DNA, 32 P ϕ x form I (200 μ g) was digested with an excess of AccI or HpaII for 60' at 37 C. The reactions were stopped with an excess EDTA and aliquots of the reaction mixtures were analyzed by agarose gel electrophoresis. After phenol extraction and dialysis, the digests were applied to vertical preparative 5% or 7.5% polyacrylamide gels, electrophoresed and stained with ethidium bromide. The bands corresponding to fragment 1 (2748 base pairs (bp)) for the HpaII digest and fragment 2 (2352 bp) (18) for the AccI digest were cut out and the DNA electroeluted for 3 hours at 4 C, phenol extracted and dialyzed. Annealing of the (-) strand of the purified fragments to circular ϕ x (+) strand was achieved by dialysis at room temperature. The mixture of both DNA species in 50% formamide, was successively dialyzed against 95% formamide, 10 mM EDTA (2 hrs); 50% formamide 200 mM Tris-HCl(pH 8), 10 mM EDTA (3 hrs); 100 mM Tris-HCl(pH 8), 10 mM EDTA, 100 mM NaCl (15 hrs); and finally 10 mM Tris-HCl(pH 8), 1 mM EDTA (2 hrs) (17). The gapped DNA was separated from the other DNA species by preparative electrophoresis in 1% agarose gels, electroeluted as above, phenol extracted and dialyzed. For the preparation of linear DNA with single stranded termini, 32 P ϕ x form I was cut with PstI and treated with saturating amounts of exonuclease III or lambda exonuclease. Conditions were adjusted to obtain

single stranded ends of 800 to 900 nucleotides on the average, as measured by the release of acid soluble radioactivity.

Binding of RecA protein to duplex DNA. Mixtures contained, in 70 μ l, 1.6 nmole of DNA, 20 mM Tris-HCl(pH 7.5), 25 mM $MgCl_2$, 2 mM dithiothreitol, 100 μ g/ml bovine serum albumin, 1 mM ATP(γ -S) and RecA protein as indicated. After 45 minutes at 37 C, an excess of restriction enzyme (HhaI or HindII, depending on the nature of the substrate) was added to the mixture and incubation continued for 2 hours. The reaction was terminated by the addition of EDTA and the products were separated by electrophoresis on a 7.5% polyacrylamide gel. In each lane the two top bands were cut out and counted in a liquid scintillation fluor (1 ml Protosol and 5 ml Formula 963, New England Nuclear).

RESULTS

The following experiments were designed to study the effect of single-strand regions on the extent and directionality of binding of RecA protein to duplex DNA. We assumed that in the same way as RecA protein can protect single-strand DNA from digestion with S1 nuclease, binding to duplex DNA would result in the protection of restriction sites. We compared the yields of selected restriction fragments observed with increasing amounts of RecA protein for circular and linear duplex and gapped DNA.

Binding of RecA protein to form I and form II DNA. As seen in Fig 1, ϕ x form I DNA has a large number of restriction sites for HhaI. Full length ϕ x DNA (form I, form III or gapped) does not enter a 5% or 7.5% polyacrylamide gel and only the digestion products can be observed. For form I DNA, the largest products are 4 fragments of 1553, 640, 614 and 532 bp (17) (Fig 1b). Fragments 2 and 3 comigrate, but because they are juxtaposed on the ϕ x map, they can be viewed as a single fragment.

Form III DNA obtained by treatment of form I with SstII has staggered ends of two nucleotides, and can therefore be considered as fully duplex DNA in this context. The largest products of digestion with HhaI are fragments of 1553, 640, 532 and 497 bp, since the SstII cut at position 2862 removes 117 nucleotides from the 614 bp fragment.

32 P form I and 32 P form III ϕ x DNA were incubated with increasing amounts of RecA protein, then with HhaI as described in methods. After gel electrophoresis the 2 top bands (1553 and 640/614 bp for form I, 1553 and 640 bp for form III) were cut out and counted for 32 P radioactivity. In both cases, the extent of digestion was unaffected by increasing concentrations of RecA protein up to 170 μ g/ml. The decrease seen above 170 μ g/ml indicates that at high

Figure 1: Binding of RecA protein to circular and linear duplex DNA.

- HhaI restriction map of ϕ x174 form I. The shaded regions correspond to the fragments analyzed.
- gel electrophoresis of the HhaI digests of form I DNA (lanes 1-5) and form III DNA (lanes 6-10) after incubation with increasing amounts of RecA protein. The band second from top corresponds to fragments 2 + 3 in lanes 1 to 5, to fragments 2 in lanes 6 to 10. The concentrations of RecA protein in reactions 1 through 5, and 6 through 10, are respectively 0, 57, 114, 171, and 228 μ g/ml. (Lane 7 shows partial digestion due, presumably, to contamination.)
- the 2 top bands from each lane were excised, counted as described in the Methods, and the radioactivity is plotted against the concentration of RecA.
 - form I DNA, fragment 1; ○— form I DNA, fragments 2 + 3; ▼—, form III DNA, fragment 1; ✕—, form III DNA fragment 2.

concentrations of RecA, binding occurs in the absence of any single stranded region. This is consistent with electron microscopic data which show that fully covered nicked circular molecules can be observed at a concentration of 200 μ g/ml of RecA in the presence of ATP(γ -S) (9).

Binding of RecA protein to circular gapped DNA

Two different substrates were constructed for this experiment. Substrate 1 was obtained by annealing of the (-) strand of the 2748 bp fragment of a Hpa II digest of 32 P form I DNA to unlabeled viral ϕ x DNA. For substrate 2, we used the 2352 bp of an AccI digest (Fig 2a). The largest fragments resulting from restriction of substrate 1 with HhaI are 1553 and 532 bp, whereas

Figure 2: Binding of RecA protein to circular gapped DNA.

- a) HpaI restriction map of substrate 1 and HindII restriction map of substrate 2. The shaded regions correspond to the fragments analyzed. For each fragment the distance from the first restriction site to the 3' end of the gapped strand is indicated. $\longrightarrow$, 3' end; $\longleftarrow$, 5' end.
- b) gel electrophoresis of the HpaI digests of substrate 1 (left) and the HindII digests of substrate 2 (right) after incubation with increasing amounts of RecA protein. The concentrations of RecA protein in the reaction mixtures are respectively: - Left 1-9; 0, 28.5, 57, 86, 114, 143, 171, 200, 228 $\mu\text{g/ml}$. Right 1-9; 28.5, 57, 86, 114, 143, 171, 200, 228, 257 $\mu\text{g/ml}$.
- c) The 2 top bands from each lane, 1553 and 532 bp for substrate 1, 1057 and 770 bp for substrate 2, were excised and counted.

treatment of substrate 2 with Hind II yields significant fragments of 1057 and 770 bp. Each substrate was incubated with increasing amounts of RecA protein, followed by treatment with either HpaI or Hind II. After gel electrophoresis of the products, the 2 top bands from each lane (Fig 2b) were cut out

Figure 3: RecA protein required for the protection of restriction sites. The data were taken from Figure 2, a and c. The concentration of RecA protein (about 100 $\mu\text{g/ml}$) needed for protection of proximal restriction sites is comparable to that required for an efficient strand exchange reaction with gapped DNA (8).

and counted for ^{32}p radioactivity. For each substrate, the radioactivity in both bands was affected by increasing concentration of RecA protein, the slower migrating band more so than the faster band (Fig 2c). The effect was detectable above 30 $\mu\text{g/ml}$ of RecA protein for the top band, 90 $\mu\text{g/ml}$ for the lower band. Since in both substrates the larger fragment was closer to the 3' end of the gapped strand, these results seemed to indicate a directionality in the binding of RecA protein to the duplex regions of the gapped DNA. Figure 3 shows the RecA concentration needed for 50% protection at various distances from the 3' end of the gapped strand.

Binding of RecA protein to linear DNA with terminal gaps

In order to confirm these results, linear duplex DNA obtained by treatment of ^{32}p ϕx form I with PstI was digested with either exonuclease III or lambda exonuclease. The resulting products (Fig 4a) had terminal gaps of approximately 850 nucleotides. Each substrate was incubated with increasing amounts of RecA protein, followed by digestion with HhaI. As the result of exonucleolytic digestion, fragments 1 and 4 (1553 and 532 bp) of the HhaI digest, which are very close to the PstI site, became largely single-stranded and stained very poorly (Fig 4b). The band corresponding to fragments 2 and 3 (640 and 614 bp) was cut out from each lane and counted. As seen in Fig 4c, protection from restriction occurred preferentially in the DNA digested with exonuclease III which carried overhanging 5' ends.

Figure 4: Binding of RecA protein to linear duplex DNA with single strand termini.

- a) HhaI restriction map of linear duplex DNA with terminal gaps obtained by cutting of ϕ x form I with PstI, followed by limited digestion with exonuclease III or lambda exonuclease. The digested regions are indicated by broken lines. The shaded regions correspond to the fragments analyzed. $\circ$ —, 3' end; $\leftarrow$, 5' end.
- b) gel electrophoresis analysis of the HhaI digests of the linear gapped DNA after incubation with increasing amounts of RecA protein.
- c) The top band in each lane corresponding to 2 comigrating fragments of 640 and 614 bp was excised and counted. $\bullet$ —, exonuclease III digest; $\times$ —, lambda exonuclease digest.
- The concentrations of RecA protein in the reaction mixtures for both b) and c) were respectively; for lanes 1 - 5; 0, 57, 114, 171, 228.

DISCUSSION

RecA protein monomers appear to be oriented head to tail when polymerized within the helical filaments, as has been shown both by X-ray diffraction studies on crystals of pure RecA (19) and analysis of electron micrographs of the spiral filaments by image reconstruction methods (20, Stasiak, A and Egelman, EH, personal communication). At concentrations of 20 μ g/ml, RecA binds preferentially to the single stranded region of partially single stranded substrates (21,22). The orientation of RecA monomers on single stranded DNA is

easy to understand because the strand itself has a 5' to 3' orientation in the sugar phosphate backbone.

The processed images mentioned above show that RecA protein is also oriented on duplex DNA. The present results extend these observations and show that at the appropriate concentration, RecA binds progressively to duplex DNA adjoining a single stranded region. Our data are not easily reconciled with the idea that RecA binding extends equally in both directions. We found that RecA binding interferes with restriction enzymes more strongly at sites 100 to 300 than at sites 800 to 1500 base pairs along the duplex from the 3' terminus at the gap. Therefore the binding appears to extend in the 5' to 3' direction from the single stranded region onto the adjoining duplex. These results show that the direction in which RecA polymerizes along gapped duplex DNA is the same as the direction of advance of strand transfer (23,24) and reciprocal strand exchange (8,25), as has been assumed in the construction of a molecular model for RecA mediated recombination (26).

These results explain previous observations on the effects of two strand breaks on reciprocal strand exchanges between gapped circular and linear duplex molecules mediated by RecA. In this reaction, the strand exchange was found to pass two strand breaks in the linear duplex, but not in the gapped DNA (27). Progressive polymerization along the entire length of the gapped molecule provides for an exchange involving the whole linear duplex regardless of interruptions, but a two strand break in the gapped DNA interferes with the polymerization and so prevents further advance.

During the preparation of this manuscript, Register and Griffith (In press, 1985) communicated to us the results of their electron microscopic study which shows the direction of assembly of RecA protein monomers on ssDNA to be 5' to 3', which is the same as that reported here for assembly on an adjoining duplex.

In summary, we have found that in the presence of ATP(γ -S), RecA protein binds to circular duplex DNA carrying a single stranded region, and that the binding extends from the single strand onto an adjoining duplex at high RecA concentrations and in the same direction as branch migration, that is 5' to 3' along the single strand.

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