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Homologous Pairing In Vitro Stimulated by the Recombination Hotspot, Chi

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Summary

Genetic recombination in Escherichia coli is stimulated at DNA sequences known as Chi sites, 5′-GCTGGTGG-3′. We describe the in vitro formation of homologously paired joint molecules that is dependent upon this recombination hotspot. Chi-dependent joint molecule formation requires RecA, RecBCD, and SSB proteins and a Chi site in the donor linear dsDNA. The donor dsDNA is unwound by RecBCD enzyme, and the invasive strand is generated by nicking at Chi. This Chi-dependent invading strand must contain homology to the recipient supercoiled DNA substrate at its newly formed 3′ end for efficient joint molecule formation. Action at Chi generates invasive ssDNA from the 5′ but not the 3′ side of Chi, suggesting that the nuclease activity of RecBCD enzyme is attenuated upon encountering a Chi site. These results support the view that RecBCD enzyme action can precede RecA protein action and reconcile the seemingly opposing degradative and recombination functions of RecBCD enzyme.

Introduction

Homologous genetic recombination in wild-type Escherichia coli occurs principally through the RecBCD pathway of generalized recombination (for review see Smith, 1988). Genetic analysis shows that this pathway is dependent on the RecA, RecBCD, and E. coli single-stranded DNA-binding (SSB) proteins (Clark and Margulies, 1965; Glassberg et al., 1979; Smith, 1989). In addition to these protein components, recombination in the RecBCD pathway is enhanced by specific DNA sequences. These recombination hotspots, called Chi sites (5′-GCTGGTGG-3′), increase the frequency of genetic exchange in their vicinity (Smith, 1983). While the genetic studies have established the involvement of these components in the RecBCD pathway of recombination, the biochemical mechanism of this process is yet to be elucidated.

The biochemical activities of RecA and SSB proteins are well characterized (for reviews, see Cox and Lehman, 1987; Kowalczykowski, 1987, 1991a, 1991b; West, 1988; Radding, 1989). RecA protein promotes both strand exchange between complementary single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) molecules and renaturation of homologous ssDNA molecules. The DNA strand exchange activity is stimulated by SSB protein, and biochemical studies with mutant RecA proteins suggest that the process of DNA strand exchange is likely to represent recombinational events occurring in vivo, although the participation of DNA renaturation activity cannot be completely excluded (Roman and Kowalczykowski, 1989b; Kowalczykowski and Roman, 1990; Roman et al., 1991; Kowalczykowski, 1991a).

The RecBCD enzyme (exonuclease V) is a multifunctional enzyme consisting of three nonidentical subunits, the RecB, RecC, and RecD polypeptides. In vitro, it has the following biochemical activities: DNA-dependent ATPase, ssDNA and dsDNA exonuclease, ssDNA endonuclease, and ATP-dependent DNA helicase (for reviews, see Telander-Muskavitch and Linn, 1981; Taylor, 1988; Smith, 1990). In addition to its degradative nonspecific nuclease activities, RecBCD enzyme has a site-specific nuclease activity that is specific for the Chi sequence (Ponticelli et al., 1985).

Chi sites are cis-acting DNA sequence elements that enhance recombination promoted by the RecBCD pathway in E. coli (for review see Smith, 1988). First identified in bacteriophage λ, Chi sites amplify the growth of mutant λ phage that are dependent on E. coli recombination functions for growth (Lam et al., 1974; Stahl et al., 1974). Chi stimulation of recombination requires functional RecBCD enzyme and occurs primarily to the left of a Chi site, when the λ genome is depicted in the conventional manner. Genetic and nucleotide sequence analyses have identified the Chi site as 5′-GCTGGTGG-3′ (Smith et al., 1981a). The RecBCD enzyme interacts with a Chi site by nicking the DNA 4 to 6 nucleotides to the 3′ side of the Chi sequence on the DNA strand containing the Chi sequence (Taylor et al., 1985). This specific interaction occurs during the unwinding of dsDNA and is orientation dependent; RecBCD enzyme must approach from the 3′ side of the Chi sequence for specific nicking to occur (Taylor et al., 1985).

Although the biochemical activities of RecBCD enzyme have been characterized, the function of this protein in genetic recombination is not as well understood. Models for recombination by the RecBCD pathway propose that the interaction between Chi and the RecBCD enzyme can either act early, in the initiation of DNA strand exchange (Smith et al., 1981b, 1984), or late, in the resolution of recombination intermediates (Stahl, 1979; Thaler et al., 1988). It is proposed that the nuclease activity is involved in the creation of either ssDNA, which is necessary to initiate homologous DNA exchange (Smith et al., 1984), or dsDNA breaks, which are involved in the resolution of Holliday junctions (Stahl, 1979). The DNA helicase activity creates ssDNA that is used as a substrate for RecA protein-catalyzed heteroduplex DNA formation (Roman and Kowalczykowski, 1989b; Wang and Smith, 1989; Kowalczykowski and Roman, 1990; Roman et al., 1991). Despite these ideas and observations, the biochemical mechanism by which Chi sites stimulate recombination and, perhaps, regulate the various biochemical activities of the

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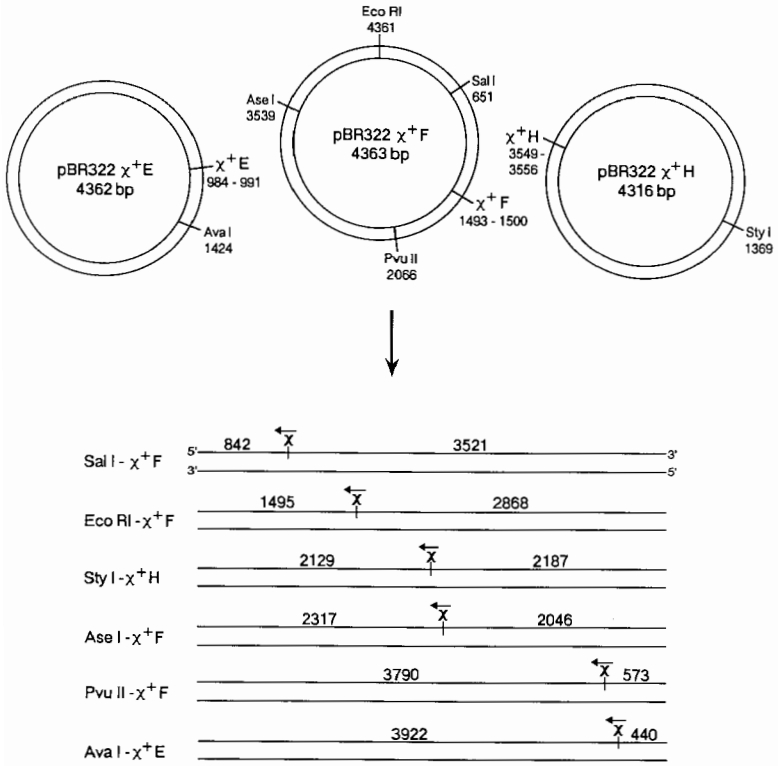

Figure 1. Location of the Chi Sites χ^+E, χ^+F, and χ^+H in Circular and Linear dsDNA

Cleavage of the plasmid pBR322 derivatives, pBR322 χ^+E, pBR322 χ^+F, and pBR322 χ^+H containing Chi sequences by the indicated restriction endonucleases generates the linear Chi-containing dsDNA substrates illustrated. Locations and nucleotide numbering of the Chi sites and restriction endonuclease sites are from Smith et al. (1981a) and Sutcliffe (1978). The distances listed for Chi-containing linear dsDNA indicate the distance, in nucleotides, from the 5′ base in the restriction endonuclease recognition sequence to the 5′ base of the Chi sequence 5′-GCTGGTGG-3′ on the Chi-containing strand of DNA. The arrow above a χ indicates the direction from which RecBCD enzyme must bind and unwind the DNA in order to recognize the Chi sequence (Taylor et al., 1985).

enzymes involved in the RecBCD pathway remains uncertain.

In this paper, we use an in vitro reaction to demonstrate that Chi sites promote enhanced homologous pairing in their vicinity. To understand how DNA strand cleavage in the region of a Chi site may stimulate recombination, DNA substrates (i.e., homologous linear dsDNA and supercoiled DNA) were used that require the coordinated actions of the RecA, RecBCD, and SSB proteins for joint molecule formation (Roman et al., 1991) and that are representative of the cellular DNA substrates (see Smith, 1991). This reaction is dependent upon RecBCD enzyme to initiate the process by unwinding Chi-containing linear dsDNA. Upon reaching a Chi site, RecBCD enzyme nicks the DNA strand and continues to unwind the DNA, thus producing a Chi-dependent ssDNA fragment that is used by RecA and SSB proteins to promote strand invasion of a homologous supercoiled DNA molecule. The size of the resulting plectonemic joint molecules is dependent upon the location of the Chi site in the linear dsDNA, pairing is stimulated to only one side of Chi, and invasion is initiated at the 3′ end generated by nicking at Chi. Finally, we propose that the nuclease activity of RecBCD enzyme is attenuated by the interaction with a Chi site, resulting in the observed stimulation of recombination and providing a molecular explanation for the proposal that interaction with Chi changes RecBCD enzyme from an antirecombinogenic activity to a recombinogenic one as suggested by Thaler et al. (1988, 1989).

Results

Experimental Design

Joint molecule formation dependent upon the major components of the RecBCD pathway of recombination, RecA, RecBCD, and SSB proteins (RecABCD-dependent reaction) was demonstrated previously (Roman et al., 1991). The formation of joint molecules required the creation of ssDNA through the unwinding of the linear dsDNA by RecBCD enzyme, trapping of the ssDNA strands by RecA and SSB proteins, and RecA protein–promoted strand invasion of a homologous supercoiled DNA molecule. The resultant joint molecules comprised a heterogeneous distribution upon which two distinct species were superimposed (Roman et al., 1991). Preliminary analysis suggested that the discrete joint molecule species differed only in the size of the ssDNA strand of M13 DNA that invaded a supercoiled DNA molecule (data not shown); one species appeared to assimilate a full-length ssDNA molecule, while the size of the other was consistent with invasion by a DNA strand resulting from cleavage at a Chi site (position 4943 in M13mp7 DNA [Messing et al., 1981]).

To verify and expand on these findings, linear dsDNA substrates containing Chi (χ^+) in various positions were constructed (Figure 1). The substrates are derivatives of the plasmid pBR322 containing a Chi sequence; the plasmids pBR322 χ^+E and pBR322 χ^+F contain single base pair mutations, creating a Chi sequence octamer at positions 984–991 and 1493–1500, respectively (Smith et al.,

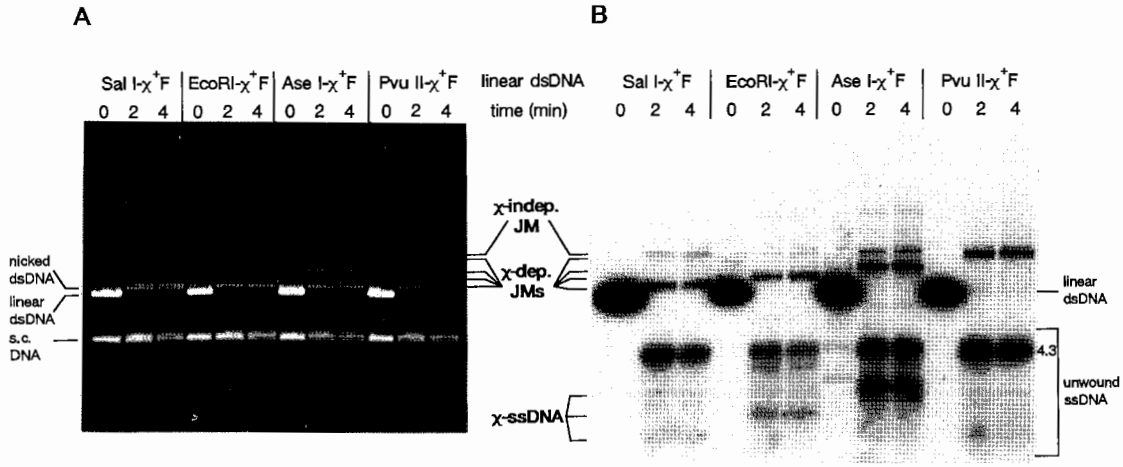

Figure 2. Chi-Dependent Joint Molecule Formation Using 5′ End–Labeled Chi-Containing Linear dsDNA

The linear dsDNA substrates shown in Figure 1 were labeled at the 5′ termini with ^{32}P. Each linear dsDNA was used in a RecABCD reaction with homologous supercoiled DNA (s.c. DNA) under standard reaction conditions. Aliquots of each reaction were taken at the times indicated. For clarity, all zero time points contained one-half the volume of the remaining time points. The reaction products were analyzed on a 0.75% agarose gel stained with ethidium bromide (A). The gel was dried and visualized by autoradiography (B). The bands corresponding to discrete-sized Chi-independent joint molecules (χ-indep. JM), Chi-dependent joint molecules (χ-dep. JMs), and Chi-dependent ssDNA fragments (χ-ssDNA) are indicated. The mobility of the Chi-dependent joint molecules is dependent on the location of the Chi site in the linear dsDNA used (see Figure 1). The discrete bands (unlabeled) appearing above the Chi-independent joint molecules are presumed to be joint molecules containing both a Chi-dependent ssDNA fragment and a full-length ssDNA strand based on their mobility in the gel, but the actual composition remains to be determined. The appearance of a ladder of bands seen in the autoradiogram is an artifact of the agarose gel drying process. Full-length unwound ssDNA (4.3 kb) and contaminating nicked circular dsDNA (nicked dsDNA) are indicated.

1981a), and plasmid pBR322 χ^+H (this work) contains a Chi site inserted at position 3549–3556. These plasmids were linearized at unique restriction endonuclease sites to generate a series of molecules containing a Chi site at different locations in the linear dsDNA (Figure 1). For each substrate, Chi is properly oriented only for a RecBCD enzyme molecule that enters from the right as depicted (Ponticelli et al., 1985; Taylor et al., 1985). Cutting at Chi occurs on the upper strand, resulting in the formation of ssDNA fragments having the indicated sizes after complete unwinding of the linear dsDNA. For RecBCD enzyme molecules entering from the left, no Chi-specific nicking occurs (Taylor et al., 1985). The Chi-specific nicking is always superimposed upon the products of the nonspecific nuclease activity of RecBCD enzyme (Ponticelli et al., 1985; Roman et al., 1991).

The Formation of Chi-Dependent Joint Molecules

The Chi-containing linear dsDNA substrates shown in Figure 1 were reacted with purified RecA, RecBCD, and SSB proteins along with homologous supercoiled wild-type pBR322 that does not contain a Chi site (χ°) (Smith et al., 1981a), and the reaction products were analyzed by agarose gel electrophoresis (Figure 2). Subsaturating concentrations of RecBCD enzyme (0.025 functional RecBCD enzyme molecules per linear dsDNA end) were used to ensure that each linear dsDNA molecule is unwound by only one RecBCD enzyme so that, on average, 50% of the linear dsDNA that is unwound is unwound by a RecBCD enzyme molecule approaching Chi in the proper orientation. Since RecBCD enzyme can unwind 30 kb of DNA on average before dissociating (L. J. Roman, A. E. Eggleston, and S. C. Kowalczykowski, submitted), each dsDNA molecule is completely unwound by a single RecBCD enzyme.

Both the ethidium bromide–stained gel and the autoradiograph of these show (Figure 2) that for each specific Chi-containing dsDNA substrate used in a RecABCD reaction, two discrete joint molecules are formed, as well as the heterogeneous joint molecule population previously noted (Roman et al., 1991). By virtue of their stability to gel electrophoresis, these joint molecules are likely to be plectonemically wound. The prominent joint molecule (labeled χ-dep. JM) present in each reaction has a relative mobility that is dependent on the location of the Chi site in the linear dsDNA and has an apparent size that is consistent with the expected length of the "downstream" DNA fragment created by RecBCD enzyme cutting at a correctly oriented Chi site (i.e., the fragment to the left or the 5′ side of the Chi site as depicted in Figure 1). This result suggests that the invasive strand is located to the left of Chi and might be either the "upper" strand (i.e., the strand containing 5′-GCTGGTGG-3′ oriented as written here) or "lower" non-Chi-containing strand. Conspicuous in their absence are discrete species of joint molecules originating from DNA fragments derived from the right side of the Chi site; those fragments would have generated the inverse series of joint molecules whose relative mobility was dependent on the location of the Chi site relative to the right end of the dsDNA. The second discrete joint molecule species (labeled χ-indep. JM) is present at a lower amount,

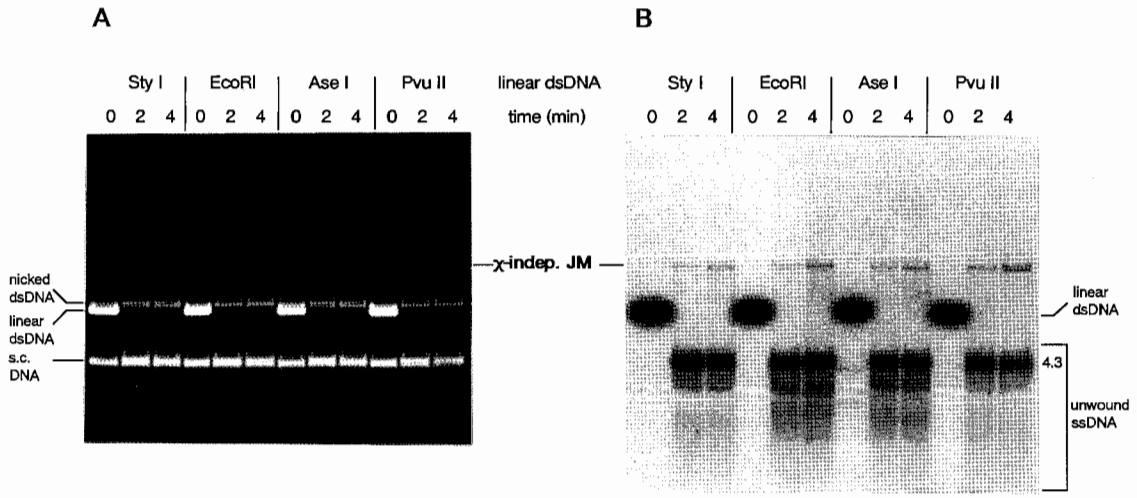

Figure 3. Joint Molecule Formation Using χ° Linear dsDNA

Linear dsDNA was created by cleavage of supercoiled pBR322 (χ°) with the indicated restriction endonuclease, end labeled at the 5′ termini with ^{32}P, and was used in a standard RecABCD reaction as described in Figure 2. The ethidium bromide–stained agarose gel (A) and its respective autoradiogram (B) are shown. All zero time points contained one-half the volume of the remaining time points. The discrete-sized Chi-independent joint molecules (χ-indep. JM), are indicated; this homogeneous species is superimposed upon the heterogeneous joint molecule population.

has a relative mobility that is independent of the location of the Chi site in each reaction, and has the greatest apparent size.

In addition to the joint molecules, the products of RecBCD enzyme DNA unwinding and degradation are visible; these include ssDNA fragments ranging from full-length (4.3 kb) to approximately 400 nucleotides long, reaching the lower limits of detection under these electrophoresis conditions. Also present are ssDNA fragments (labeled χ-ssDNA) generated by cutting at the Chi site by RecBCD enzyme (see Figure 1 for fragment lengths; Ponticelli et al., 1985). The results shown in Figure 2 are unchanged when Chi-containing supercoiled DNA (pBR322 χ^+F) is used as the recipient (data not shown).

Control RecABCD reactions using χ° linear pBR322 dsDNA fail to produce Chi-dependent joint molecules seen under otherwise identical conditions (Figure 3); regardless of the linear dsDNA substrate used, only one distinct joint molecule (labeled χ-indep. JM) is detected. The mobility of this joint molecule species is constant for each reaction and is identical to that of the Chi-independent joint molecules seen in Figure 2. Autoradiography of this control RecABCD reaction using linear dsDNA that is labeled at the 5′ end reveals only a discrete Chi-independent joint molecule species, consistent with the ethidium bromide–stained gel. These results are unchanged when the linear dsDNA is labeled at the 3′ end (data not shown). Identical results are obtained when Chi-containing supercoiled DNA (pBR322 χ^+F) is substituted for χ° supercoiled pBR322 (data not shown), demonstrating that the effect of Chi is manifest only when present in the donor DNA.

To confirm the identity of the invasive strand contained in Chi-dependent joint molecules, Chi-dependent joint molecules from the experiment shown in Figure 2 were isolated from the agarose gel and reelectrophoresed under alkaline conditions to denature the heteroduplex DNA. Figure 4 shows that the joint molecules obtained from reactions using the Chi-containing linear dsDNA substrates SalI-χ^+F, EcoRI-χ^+F, AseI-χ^+F, and PvuII-χ^+F contain ssDNA fragments with lengths of approximately 900, 1600, 2400, and 3800 nucleotides, respectively (Figure 2, lanes 5, 6, 7, and 8). These values are within the experimental error of the predicted nucleotide sizes of Chi-dependent DNA fragments indicated in Figure 1. The ssDNA fragments arise from Chi-dependent DNA cleavage by RecBCD enzyme, since the starting linear dsDNA substrates are free of nicks (Figure 4, lanes 1, 2, 3, and 4). The isolated Chi-independent joint molecules contain ssDNA with a measured size of 4400 nucleotides (data not shown), demonstrating that this species is formed by invasion of a full-length ssDNA molecule.

The quantities of Chi-dependent and Chi-independent joint molecules formed in Figure 2 are approximately 6 ± 0.8% and 1 ± 0.3% of the input linear dsDNA substrate, respectively, as determined by densitometric analysis. The greater amount of Chi-dependent joint molecules seen in Figure 2 is not due to the lack of generated full-length ssDNA, since approximately 10%–20% of the input dsDNA is converted to full-length ssDNA but is not used to form stable joint molecules under these conditions (Figure 2B). This preferential use of ssDNA generated by the interaction between RecBCD enzyme and Chi to create recombinant products suggests that the unwinding of the dsDNA alone is not sufficient to promote efficient recombination.

Chi-Dependent Joint Molecules Contain ssDNA Fragments Derived from the Downstream Side of the Chi-Containing Strand

To determine which strand—either the "upper" Chi-containing strand or "lower" non-Chi-containing strand—

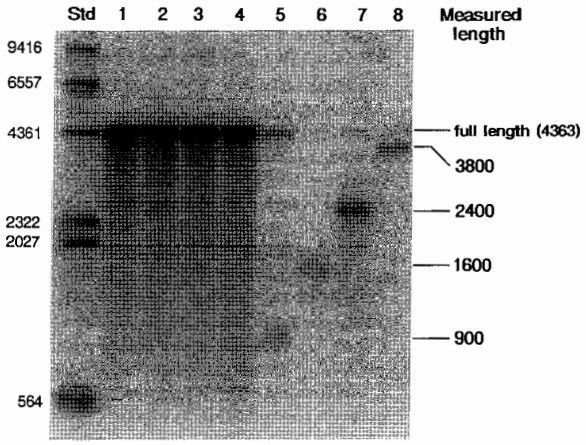

Figure 4. Composition of Chi-Dependent Joint Molecules

Chi-dependent joint molecules formed in RecABCD reactions such as that shown in Figure 2 were isolated from agarose gels and reelectrophoresed under alkaline conditions (see Experimental Procedures). Lanes 1–4 are substrate linear dsDNA isolated from the zero time point of reactions using the following substrates: SalI-χ^+F, EcoRI-χ^+F, AseI-χ^+F, and PvuII-χ^+F, respectively. Lanes 5–8 are Chi-dependent joint molecules isolated from reactions using linear dsDNA substrates SalI-χ^+F, EcoRI-χ^+F, AseI-χ^+F, and PvuII-χ^+F, respectively. The band corresponding to full-length ssDNA seen in lane 5 is spillover from lane 4. The length, in nucleotides, of ssDNA obtained from Chi-dependent joint molecules was determined using the HindIII-digested lambda DNA size markers (Std) to construct a standard curve.

RecA protein uses to promote Chi-dependent joint molecule formation, the linear dsDNA was labeled at either the 5′ or 3′ end. When 5′ end-labeled χ^+ linear dsDNA is used (Figure 2B), the resulting pattern of Chi-dependent joint molecules (and Chi-independent joint molecules) formed on the autoradiograph corresponds to the joint molecules detected when the gel is stained with ethidium bromide (Figure 2A). However, when 3′ end-labeled χ^+ linear dsDNA is used (Figure 5B), autoradiography detects only the Chi-independent joint molecule, even though staining of this gel with ethidium bromide demonstrates the formation of Chi-dependent joint molecules (Figure 5A). These results illustrate that the invasive strand is the fragment derived from the downstream (5′) side of the Chi sequence on the "upper" Chi-containing strand. Consistent with this conclusion, Chi-dependent ssDNA cleavage fragments (labeled χ-ssDNA) are seen in reactions using 5′ end-labeled linear Chi-containing dsDNA (Figure 2B) but are not visible in reactions using 3′ end-labeled DNA (Figure 5B).

The 3′ End of the Chi-Generated ssDNA Fragment Is the Invasive End in Chi-Dependent Joint Molecule Formation

Chi-specific cutting of linear dsDNA by RecBCD enzyme produces a 3′ OH group and a 5′ PO_4 group (Taylor et al., 1985), and DNA strand invasion of supercoiled DNA promoted by RecA protein requires ssDNA that is homologous at its 3′ end (Konforti and Davis, 1987, 1990). These biochemical observations, in concert with considerable genetic evidence, support the hypothesis that RecBCD enzyme initiates recombinational events by creating a substrate suitable for RecA protein (Smith et al., 1981b; Smith, 1990). To determine whether homology at the 3′ end of the invading ssDNA fragment is required in the RecABCD-dependent in vitro reaction, linear dsDNA containing heterology immediately downstream of the Chi sequence (9 bp from the 5′ end of the Chi sequence) was used as a substrate (Figure 6A). These linear dsDNA substrates contain a 961 bp region of M13 DNA inserted into χ^+ and χ° pBR322 DNA.

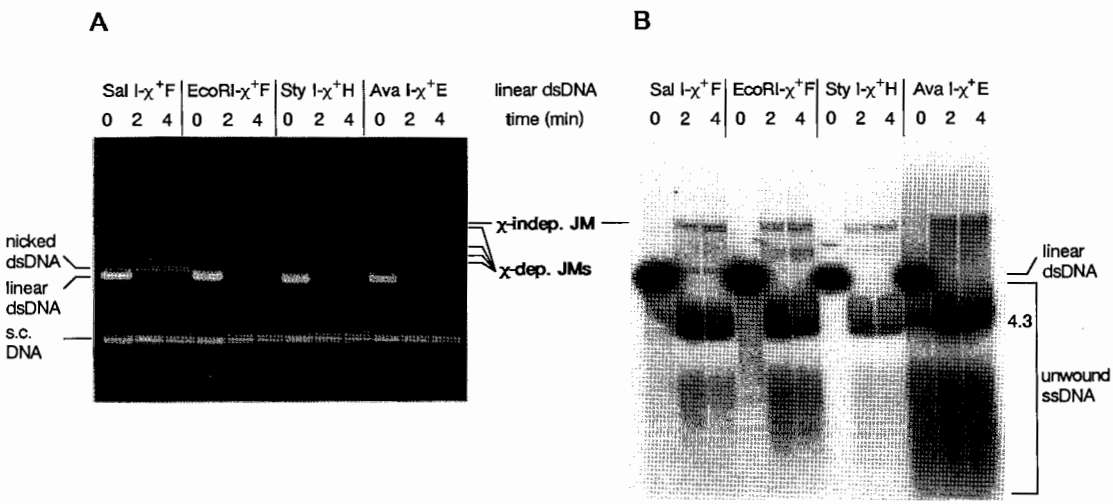

Figure 5. Chi-Dependent Joint Molecule Formation Using 3′ End–Labeled Chi-Containing Linear dsDNA

The linear dsDNA substrates shown in Figure 1 were labeled at the 3′ termini with ^{32}P. Standard RecABCD reactions were performed and analyzed using ethidium bromide–stained agarose gel (A) or its respective autoradiogram (B) as in Figure 2. All zero time points contained one-half the volume of the remaining time points.

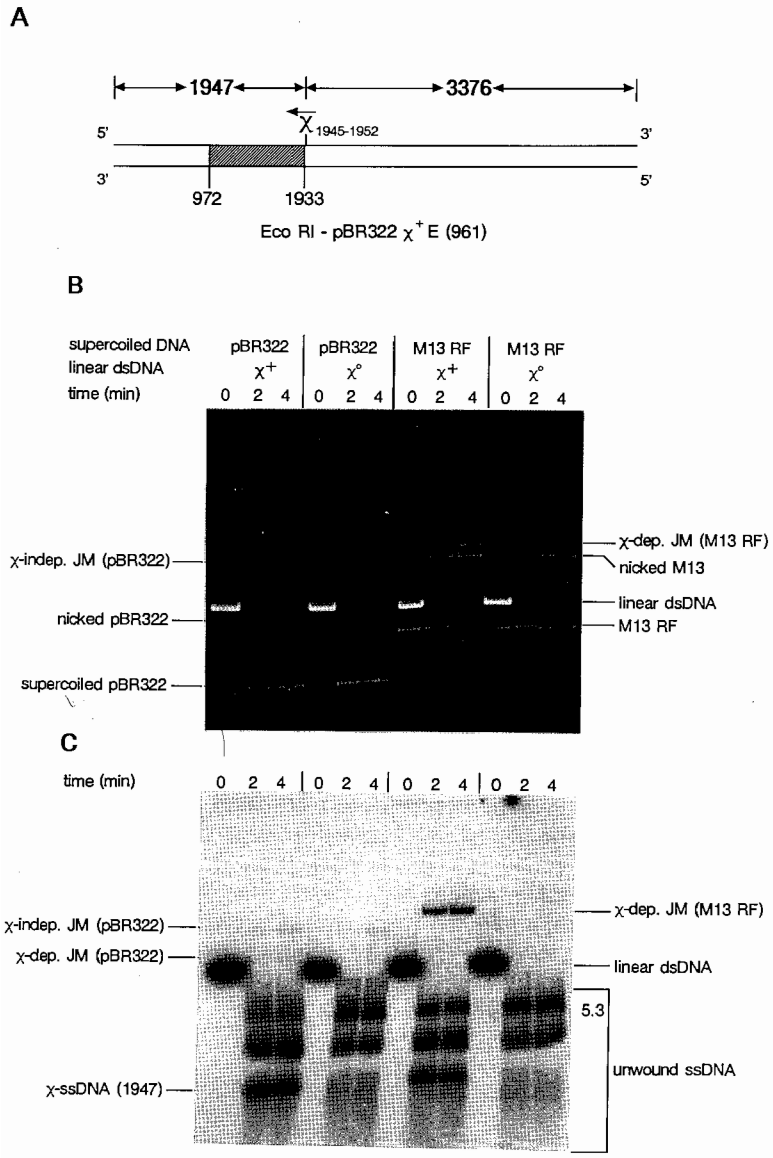

Figure 6. Homology at the 3′ End of the Chi-Dependent ssDNA Fragment Is Required for Chi-Dependent Joint Molecule Formation

(A) DNA substrate EcoRI-pBR322 χ^+E(961) was created by cleavage of the circular form of plasmid pBR322 χ^+E(961) with restriction endonuclease EcoRI. The hatched region represents the 961 bp SspI fragment of M13 DNA located between nucleotide positions 972 and 1933 in the circular plasmid. An identical linear dsDNA substrate that does not contain a Chi site (EcoRI-pBR322 χ°(961); not shown) was used as the χ° control. Chi site location and DNA lengths in nucleotides are described in Figure 1. EcoRI-pBR322 χ^+E(961) and EcoRI-pBR322 χ°(961) linear dsDNA substrates were 5′ end labeled with ^{32}P and used in RecABCD reactions as indicated in Figure 2. Reactions contained either χ^+ or χ° linear dsDNA and either supercoiled pBR322 or M13 replicative form (RF) DNA.

(B) The reaction products analyzed on a 0.75% agarose gel stained with ethidium bromide.

(C) Autoradiogram of the gel in (B). All zero time points contained one-half the volume of the remaining time points. The Chi-dependent ssDNA fragment (χ-ssDNA) with a predicted size of 1947 nucleotides (see Figure 6A) and the full-length (5.3 kb) unwound ssDNA are indicated.

Using the linear dsDNA substrates depicted in Figure 6A, RecABCD reactions were carried out using either supercoiled pBR322 (χ°) DNA or supercoiled M13 replicative form DNA. Figure 6B shows that when supercoiled pBR322 DNA is used in reactions, only a trace amount of Chi-independent joint molecules (labeled χ-indep. JM (pBR322)) containing a full-length (5.3 kb) strand of ssDNA are formed; this is true whether the linear dsDNA is Chi-containing or non-Chi-containing. When 5′ end-labeled substrates are examined (Figure 6C), identical results are obtained, with the exception that a trace (0.6%) of Chi-dependent joint molecule (labeled χ-dep. JM (pBR322)) containing the Chi-dependent cleavage fragment (1.9 kb) is now visible. The identity of the ssDNA contained within this joint molecule, as well as those described below, was confirmed by alkaline gel electrophoresis of the isolated joint molecules (data not shown). It is not known whether this Chi-dependent joint molecule results from a 5′-end or 3′-end invasion of the Chi-dependent cleavage fragment, since there are approximately 21 to 23 nucleotides of pBR322 DNA homology present at the 3′ end depending on the location of the Chi-specific nick (Taylor et al., 1985). Regardless of origin, the amount of this product is very low.

When M13 replicative form DNA is used as the supercoiled DNA substrate, a Chi-dependent joint molecule (labeled χ-dep. JM (M13 RF)) is the most abundant product (Figures 6B and 6C). A Chi-dependent joint molecule results from exposure of M13 DNA at the newly formed 3′ end owing to nicking at the Chi site. No Chi-independent joint molecules are visible using M13 replicative form DNA in either the ethidium bromide–stained gel or its respective autoradiograph, consistent with the expectation that such joint molecules would be paranemic and hence unstable under these assay conditions (Bianchi et al., 1983). Con-

sistent with the results shown in Figure 5, when the linear dsDNA in Figure 6A is labeled at the 3′ end, only Chi-independent joint molecules are present in an autoradiogram when pBR322 is used as the supercoiled recipient, whereas no discrete joint molecule species are detected when M13 replicative form is the recipient (data not shown).

The amount of Chi-dependent joint molecules obtained using supercoiled M13 DNA as the recipient is approximately 40 times higher than the amount of Chi-dependent joint molecules seen using pBR322 DNA (Figure 6C). These results are in agreement with previous findings that show that 3′ ends are 50 to 60 times more reactive than 5′ ends in RecA and SSB protein–catalyzed joint molecule formation between linear ssDNA and supercoiled DNA (Konforti and Davis, 1990). These results demonstrate that the ssDNA end participating in Chi-dependent joint molecule formation is the 3′ OH end created by a RecBCD enzyme–dependent site-specific nick at a Chi site.

Discussion

Using an in vitro reconstituted recombination reaction, we have demonstrated the formation of joint molecules that is dependent upon both the recombination hotspot, Chi, and the combined activities of purified RecA, RecBCD, and SSB proteins. The product of the Chi-specific reaction is a joint molecule formed between a recipient supercoiled DNA molecule and a homologous ssDNA fragment created by the unwinding and Chi-dependent nicking of donor linear dsDNA by RecBCD enzyme. The invading ssDNA is the fragment derived from the Chi-containing DNA strand consisting of the entire portion that is downstream of Chi; its size is directly dependent upon the location of the Chi site in the linear dsDNA molecule. These results are compatible both with genetic data pertaining to Chi stimulation of recombination (Lam et al., 1974; Faulds et al., 1979; Stahl et al., 1980) and with biochemical observations implicating the interaction between RecBCD enzyme and Chi as fundamental to Chi-stimulated recombination events (Smith et al., 1981b; Ponticelli et al., 1985; Taylor et al., 1985). In vitro, the invading ssDNA fragment must contain homology to the supercoiled DNA substrate at its 3′ end for effective Chi-dependent joint molecule formation to occur. This observation is consistent with the known biochemical behavior of RecA protein (Konforti and Davis, 1987, 1990) but appears to contrast with in vivo results showing that Chi-stimulation can occur opposite a heterology in λ phage crosses (Stahl and Stahl, 1975). This difference is readily reconciled by suggesting either that RecBCD enzyme unwinds DNA for long distances beyond the Chi site and occasionally nicks the unwound strand or that other endo- or exonucleases act in vivo to degrade the heterology, allowing plectonemic joint molecule formation. The net effect would be to generate heteroduplex DNA to the left of Chi as observed both in vivo (Cheng and Smith, 1989) and, in this study, in vitro.

Coincident with dsDNA unwinding, the nonspecific nuclease activity of RecBCD enzyme produces random ssDNA fragments varying in size from oligomers to thousands of nucleotides (MacKay and Linn, 1976; Telander-Muskavitch and Linn, 1981; Roman et al., 1991). Consequently, an unanticipated result is the presence of Chi-dependent joint molecules that are derived from the ssDNA fragment downstream of Chi (relative to the entry site of RecBCD enzyme) but not from the fragment upstream of Chi (Figures 2, 4, and 5). This observation holds true even when Chi is situated near (440 nucleotides from) the entry site (Figure 5, AvaI-χ^+E lanes). Not only is there an absence of Chi-dependent joint molecules that might be expected to contain this upstream fragment, but the fragment itself is also absent as the expected 3′ end-labeled ssDNA fragment (Figure 5B). (Although we fail to detect a fragment from the upstream-side of Chi, such a fragment was detected previously [Ponticelli et al., 1985]; we attribute this difference to the greater level of RecBCD enzyme nuclease activity under our reaction conditions, resulting in greater degradation of the upstream fragment [unpublished data].) Furthermore, if the nonspecific cleavage activity of RecBCD enzyme were indeed random, then there would be a bias against the formation of intact downstream Chi-specific ssDNA fragments when the Chi site is close to the entry site (i.e., when the length of the Chi-generated fragment is large). This, also, is not observed.

To reconcile these results, we propose that the interaction between RecBCD enzyme and Chi results in an attenuation of RecBCD enzyme nuclease activity. Before encountering Chi, RecBCD enzyme cleaves the ssDNA ending 3′ at the entry site with a frequency that requires at least one cut within a 440 nucleotide interval. After encountering Chi, the cleavage frequency is reduced to the point that little, if any, cleavage occurs in the largest Chi-dependent fragment examined, 3922 nucleotides. Consistent with this finding is the observation that if RecBCD enzyme cuts at a Chi site, it does not detectably cut at a second properly oriented Chi site on the same DNA molecule (A. F. Taylor and G. R. Smith, personal communication). This proposition reconciles the apparently disparate degradative and recombination functions of RecBCD enzyme and offers a molecular explanation for the stimulatory nature of Chi sites. An alternative hypothesis for the selective loss of the ssDNA fragment derived from the right (3′) side of the Chi site is that this strand is degraded by the single-strand nuclease activities of RecBCD enzyme because it does not participate in joint molecule formation and, as a result, is sensitive to degradation. This potential explanation can be excluded, since in unwinding reactions containing only RecBCD enzyme and SSB protein, this ssDNA fragment is also absent when both RecA protein and supercoiled DNA are absent, demonstrating that degradation of the fragment upstream of Chi and the formation of the downstream fragment are unrelated to homologous pairing (unpublished data).

An additional consideration is the origin of the full-length unwound ssDNA. If both strands are degraded equivalently, then no full-length ssDNA should result, since the consideration above requires a frequency of nucleolytic degradation of at least one cut every 50 to 100 nucleotides (this value is derived from the Poisson distribution and an estimate that the detection limit for the upstream ssDNA

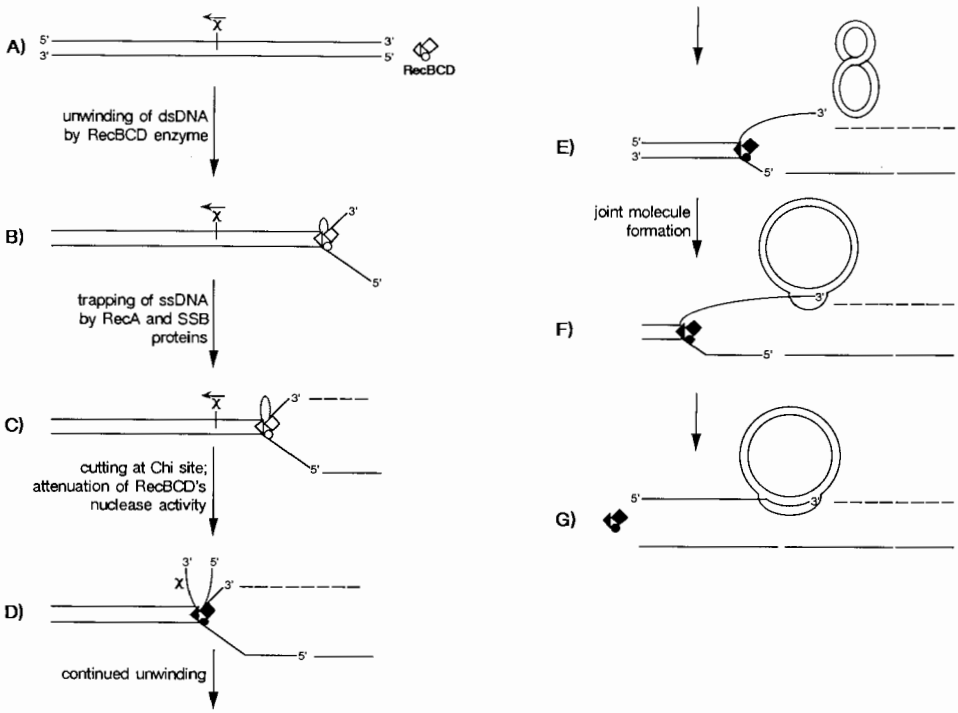

Figure 7. Model for Chi-Dependent Joint Molecule Formation

Details are discussed in the text. The arrow on the Chi site indicates the direction that RecBCD enzyme must approach for recognition of the Chi. RecBCD enzyme is represented by the diamond/triangle/circle. Prior to the interaction with the Chi site, RecBCD enzyme nuclease activity is high, resulting in extensive degradation of the 3′-terminal strand; upon encountering a Chi site, RecBCD enzyme is functionally altered (filled symbol) so that its nuclease activity is attenuated. RecA and SSB proteins are bound to the ssDNA but are not shown.

fragment created by a Chi-dependent nicking event on AvaI-χ^+E dsDNA seen in Figure 5B is 0.5% to 1%). However, full-length ssDNA is clearly produced using either DNA containing a Chi site (Figures 2B and 5B) or not containing a Chi site (Figure 3B); again, using the Poisson distribution and the conversion of 20% of the input dsDNA to full-length ssDNA requires that the 5′-terminal strand is cleaved once every 2700 nucleotides. This observation is explained by proposing that under the conditions used in this study the DNA strands are degraded differentially. Upon binding a dsDNA end, RecBCD enzyme degrades the 3′-terminal strand with a greater frequency than the 5′-terminal strand. Therefore, the 3′-terminal strand is degraded to small ssDNA fragments (less than 50 to 100 nucleotides long), but the 5′-terminal strand is largely intact; this proposal is consistent with the presence of dsDNA molecules containing single-stranded tails as reaction intermediates (MacKay and Linn, 1974). We suggest that upon encountering a correctly oriented Chi site, the RecBCD enzyme 3′-strand nuclease activity is attenuated, whereas the weak 5′-strand nuclease activity remains unaltered. Direct experimental examination of this suggestion is currently in progress.

A specific model incorporating the known biochemical and genetic properties of the RecBCD pathway was proposed by Smith and coworkers (1981b, 1984). Our studies experimentally substantiate the existing premise that RecBCD enzyme can initiate the recombination process at a Chi site by producing a suitable substrate for RecA protein action, and we propose an additional consideration. The activity of the RecBCD enzyme is changed from a recombination-inhibitory nuclease to a recombination-stimulatory helicase with reduced nuclease activity owing to interaction with a Chi site.

A model incorporating the present results and previous findings is shown in Figure 7. RecBCD enzyme initiates the process by binding the end of the linear Chi-containing dsDNA (Figure 7A). RecBCD enzyme begins unwinding the dsDNA, forming a loop on the strand with a 3′ end at the entry point (Braedt and Smith, 1989); immediate binding of the RecA and SSB proteins to the unwound DNA strands (Roman and Kowalczykowski, 1989b; Kowalczykowski and Roman, 1990; Roman et al., 1991) results in the formation of a loop–tail DNA structure (Taylor and Smith, 1980; Telander-Muskavitch and Linn, 1981) (Figure 7B). The nonspecific nuclease activity of RecBCD enzyme degrades the DNA as it unwinds; the 3′-terminal strand is cut often, thereby producing small DNA fragments, while the 5′-terminal strand is cut much less frequently (Figure 7C). Chi recognition occurs only if RecBCD enzyme binds the dsDNA end where, upon translocation through the DNA, it will encounter Chi in the proper orientation (Taylor et al., 1985); recognition results in both the specific nicking of the DNA strand containing the Chi sequence (5′-GCTGGTGG-3′) and an attenuation of RecBCD enzyme 3′ strand–specific nuclease activity (shaded RecBCD en-

zyme) (Figure 7D). This ssDNA produced by cleavage at Chi has a 3′ end homologous to the supercoiled DNA, permitting RecA protein–dependent joint molecule formation to occur (Konforti and Davis, 1987, 1990) (Figure 7E). Continued unwinding of the dsDNA produces an ssDNA fragment whose length is dependent upon the location of the Chi site in the linear dsDNA (Figure 7F). The product Chi-dependent joint molecules consist of a Chi-dependent ssDNA fragment homologously paired to a recipient supercoiled DNA molecule (Figure 7G). Although specific nicking of these D-loop structures by RecBCD enzyme has been previously demonstrated (Wiegand et al., 1977), we fail to detect any nicking of the supercoiled DNA molecule contained within the Chi-dependent joint molecule (unpublished data); presumably this lack of specific nicking is due to the protection of the displaced DNA strand by either RecA or SSB protein (Williams et al., 1981).

These biochemical steps are likely to represent the initial events important to genetic recombination in vivo. The specific biochemical nature of the steps that follow is still unclear, but pairing of the remaining complementary strands (e.g., the displaced strand in the joint molecule and the complementary strand from linear donor DNA) is required to form a Holliday junction. Resolution of this resultant Holliday junction to produce recombinant progeny can occur as described by Connolly and West (1990).

Our model, involving degradation up to a Chi, followed by attenuation of nuclease activity, is consistent with genetic observations. Stahl and coworkers (1990) have recently argued that recombination of bacteriophage λ via the RecBCD pathway involving Chi is nonreciprocal. In genetic crosses involving two parental λ phages, one χ° and the other χ^+, complementary χ^+-containing recombinants are recovered as frequently as the χ° reciprocal recombinants only if the χ^+ parent is in excess of the χ° parent. This genetic observation suggests that the formation of each χ^+ recombinant involves two χ^+ parents. Stahl and coworkers conclude that genetic information to the right of Chi is lost from one of the χ^+ parents, thereby requiring two crossover events to produce viable recombinant progeny. The results presented in this article suggest that RecBCD enzyme-dependent degradation up to Chi is responsible for the loss of genetic information and that both attenuation of nuclease activity and generation of a 3′-invasive end are crucial components of Chi stimulation of recombination.

The molecular basis for the attenuation of nuclease activity upon the interaction with Chi is unknown. However, to explain the genetic properties of the *recBCD*‡ mutations (i.e., a hyperrecombination phenotype in the absence of Chi and a loss of nuclease activity; Chaudhury and Smith, 1984; Amundsen et al., 1986; Lovett et al., 1988), Thaler et al. (1988, 1989) have suggested that the activity of RecBCD enzyme is altered upon interaction with Chi, perhaps by a loss of the RecD subunit, yielding a Chi-activated enzyme. Although conjugational recombination (but not λ recombination; Thaler et al., 1988) in strains containing *recD* mutations has the unique genetic requirement of being dependent on functional *recJ* product (Lovett et al., 1988), our results suggest that the altered nuclease activity of RecBCD‡ mutants may result from a constitutive attenuation of the nuclease activity in the absence of Chi interaction. Direct examination of this hypothesis is underway.

The reaction we describe demonstrates a stimulation of homologous pairing in vitro that is directly dependent upon the presence of the recombinational hotspot, Chi. From a reconstituted recombination reaction utilizing the major components of the RecBCD pathway, we propose a model for the initiation of recombinational events that result in the stimulation of the exchange of genetic material at a Chi site in vivo. The DNA substrates used in this study, linear Chi-containing dsDNA and supercoiled DNA, are representative of the DNA molecules normally involved in the RecBCD pathway of recombination in E. coli (Smith, 1991). Although the experimental data support a model in which the interaction between RecBCD enzyme and Chi results in the production of ssDNA at a Chi-dependent DNA nick and a reduction in nuclease activity, the underlying mechanism of how Chi alters RecBCD enzyme activity remains to be determined.

Experimental Procedures

Enzymes

RecBCD enzyme was purified as described previously (Roman and Kowalczykowski, 1989a). Protein concentration was determined using an extinction coefficient of $4.0 \times 10^5\ M^{-1}\ cm^{-1}$ at 280 nm (Roman and Kowalczykowski, 1989a); the specific activity of the enzyme preparation was 5.4×10^4 nuclease U/mg protein or 1.1×10^4 helicase U/mg protein. The helicase activity of this enzyme preparation saturates at an apparent stoichiometry of 5.4 RecBCD enzyme molecules per dsDNA end (Roman and Kowalczykowski, 1989a). Nuclease units and helicase units were measured as described by Eichler and Lehman (1977) and Roman and Kowalczykowski (1989a), respectively.

RecA protein was purified using a procedure based on spermidine precipitation (Griffith and Shores, 1985; S. C. Kowalczykowski, unpublished data). Protein concentration was determined using an extinction coefficient of $2.7 \times 10^4\ M^{-1}\ cm^{-1}$ at 280 nm.

SSB protein was isolated from strain RLM727 using a protocol provided by Dr. Roger McMacken of Johns Hopkins University (as described in LeBowitz, 1985). Protein concentration was determined using an extinction coefficient of $3.0 \times 10^4\ M^{-1}\ cm^{-1}$ at 280 nm (Ruyechan and Wetmur, 1975).

All restriction endonucleases and DNA-modifying enzymes were purchased from New England BioLabs, Pharmacia LKB, or Bethesda Research Laboratories. The enzymes were used according to Sambrook et al. (1989) or as indicated by the specific vendor.

DNA Substrates

The plasmid pBR322 χ^+H was constructed by inserting a dsDNA oligomer containing the Chi sequence into the wild-type plasmid pBR322. This was accomplished by digesting pBR322 with AseI and PstI; the resulting fragment was removed from the vector by S1000 gel filtration according to a procedure provided by the vendor (Pharmacia LKB). The Chi-containing insert was created by annealing the following two ssDNA oligomers: 5′-TATCTAGACCACCAGCCTCGAGTGCA-3′ and 5′-CTCGAGGCTGGTGGTCTAGA-3′ (Genosys Biotechnologies, Inc.). When annealed, the dsDNA oligomer contains the restriction sites XbaI and XhoI and a Chi site (χ^+H), along with AseI and PstI restriction site overhangs. A ligation reaction mixture was made (Berger and Kimmel, 1987) by adding approximately 0.5 μg (7.4 nM molecules) of AseI–PstI linearized vector pBR322 and a 100-fold molar excess (0.23 μg of duplex oligomer [740 nM molecules]) of the Chi site insert. The mixture was heated for 5 min at 45°C and then allowed to reach room temperature, at which it was made 30 mM Tris–HCl (pH 7.5), 30 mM NaCl, 8 mM $MgCl_2$, 2 mM dithiothreitol, 0.2 mM EDTA, 0.25 mM ATP, 7 mM spermidine, and 100 μg/ml bovine serum albumin. T4 DNA ligase

(2.5 U) was added to a final reaction volume of 25 μl; this was allowed to incubate for 16 hr at 10°C. E. coli strain JM83 (*ara*, Δ*lac-pro*, *strA*, *thi*, *Φ80d lacZ ΔM15*) was transformed with 5 μl of the reaction mixture via $CaCl_2$–mediated transformation (Berger and Kimmel, 1987). Transformants were selected for tetracycline resistance (15 μg/ml) and screened for ampicillin sensitivity (50 μg/ml). Plasmid DNA minipreparations were performed (Sambrook et al., 1989) to verify the presence of the two inserted unique restriction sites (XbaI and XhoI) in the plasmid; this strain was called SKDD003.

The two plasmids pBR322 χ°(961) and pBR322 χ⁺E(961) were constructed such that a region of nonhomologous DNA was placed into pBR322 χ° (wild type) and pBR322 χ⁺E (Smith et al., 1981a), respectively. M13mp7 replicative form DNA was digested with SspI and was then electrophoresed on a 0.8% agarose gel. The region of the gel containing the 961 bp fragment was excised; the DNA was extracted (Sambrook et al., 1989), ethanol precipitated, and dissolved in TE (10 mM Tris–HCl [pH 7.5], 1 mM EDTA). The 961 bp M13 fragment was inserted into the NruI site of pBR322 χ° and pBR322 χ⁺E by blunt-end ligation (Berger and Kimmel, 1987). The site of cleavage by the endonuclease NruI is 9 bp from the 5′ end of the Chi sequence in pBR322 χ⁺E (Sutcliffe, 1978). These two plasmid DNAs were introduced separately into strain JM83 by $CaCl_2$–mediated transformation (Berger and Kimmel, 1987). Recombinant clones were selected for ampicillin resistance (50 μg/ml) and screened for tetracycline sensitivity (15 μg/ml); the proper restriction endonuclease digestion patterns were confirmed. The orientation of the M13 insert has the unique BglII restriction endonuclease site downstream of the Chi site as depicted in Figure 6A. Strains that contain plasmids pBR322 χ°(961) and pBR322 χ⁺E(961) are called SKDD001 and SKDD002, respectively.

The plasmids pBR322 χ°, pBR322 χ⁺E224, and pBR322 χ⁺F225 (Smith et al., 1981a) were prepared from strains S819, AFT450, and S818, respectively, provided by G. R. Smith and A. F. Taylor. All plasmid and replicative form M13mp7 DNAs were purified by CsCl density gradient centrifugation (Messing, 1983; Sambrook et al., 1989). Bacteriophage λ DNA was purchased from New England BioLabs. The molar concentration of dsDNA in nucleotides was determined by using an extinction coefficient of 6290 M^{-1} cm^{-1} at 260 nm. Specific plasmid DNA and DNA molecular weight standards were linearized with appropriate restriction endonucleases and radioactively end labeled either at the 3′ end using the Klenow fragment of DNA polymerase I and appropriate [α-^{32}P]dNTPs (ICN) or at the 5′ end by sequential reactions with calf intestinal phosphatase and then T4 polynucleotide kinase and [γ-^{32}P]ATP (ICN), using methods given by the vendor or Sambrook et al. (1989).

Reaction Conditions

The standard RecABCD reaction mixture consisted of 25 mM Tris acetate (pH 7.5), 8 mM magnesium acetate, 5 mM ATP, 1 mM dithiothreitol, 1 mM phosphoenolpyruvate, approximately 4 U/ml pyruvate kinase, 40 μM nucleotides linear dsDNA, 20 μM nucleotides supercoiled DNA, 20 μM RecA protein, 4 μM SSB protein, 1.25 nM RecBCD enzyme (4.6 helicase U/ml; 0.025 functional RecBCD enzyme molecules per linear dsDNA end based upon a functional enzyme stoichiometry of 5.4 enzyme molecules per dsDNA end). Assays were performed at 37°C and were begun with the addition of RecBCD enzyme after preincubation of all other components for 1 min.

Analysis of Reaction Products

Aliquots of the reaction mixture (40 μl) were taken at the indicated time points and were added to 10 μl of stop buffer (0.1 M EDTA, 2.5% SDS, 40% glycerol, 0.125% bromophenol blue, and 0.125% xylene cyanol) to halt the reaction and to deproteinize the sample. For clarity, all zero time points are one-half the volume of the remaining time points. Reaction time points were electrophoresed on 0.75% agarose gels in the absence of ethidium bromide for 10 hr at 2.1 V/cm in TAE (40 mM Tris acetate [pH 8.0], 2 mM EDTA) and then stained with ethidium bromide (1 μg/ml). The gels were dried and autoradiographed at −20°C with Kodak XAR-5 film and an intensifying screen. Analysis of Chi-dependent and Chi-independent joint molecules was performed by cutting out appropriate DNA bands from a reaction described above. The DNA was extracted from the gel by physical extrusion (Berger and Kimmel, 1987), ethanol precipitated, and redissolved in water. Alkaline agarose gel electrophoresis of the samples was done according to Sambrook et al. (1989) in a 0.8% agarose gel at 2.1 V/cm for 10 hr. Quantitative analysis of joint molecule production was accomplished by scanning the photographic negative or autoradiogram using a Zeineh soft laser densitometer interfaced to a Hewlett-Packard 3390A integrator.

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