Chi Enhances Heteroduplex DNA Levels During Recombination

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ABSTRACT

The major pathway of homologous recombination in *Escherichia coli*, the RecBCD pathway, is stimulated by Chi sites. To determine whether Chi enhances an early or late step in recombination, we measured formation of heteroduplex DNA (hDNA) in extracts of lambda-infected *E. coli*. Chi elevated hDNA levels in these extracts, supporting a role for Chi early (before hDNA formation) in recombination. RecA protein and RecBCD enzyme were both necessary for detection of hDNA, indicating that they, too, act early. Analysis of a panel of *recBCD* mutants indicated that Chi-nicking activity was needed for Chi's stimulation of hDNA formation. These results support a previously proposed model of recombination. Further results suggested that RecBCD enzyme has an additional role late in recombination.

In *Escherichia coli*, the RecBCD pathway is the major pathway of homologous recombination in Hfr crosses, P1 transductions and crosses between *red*- *gam* lambda phage. This pathway mediates events between a linear and a supercoiled DNA molecule and therefore does not effect recombination between plasmids (Smith 1988). Recombination mediated by the RecBCD pathway is enhanced severalfold by Chi sites (5' G-C-T-G-G-T-G-G 3') (Lam et al. 1974; Smith et al. 1981a). This DNA sequence stimulates recombination locally to its left (as written here), with stimulation decreasing about 2-fold for every 2.2 kb from the Chi site (Stahl et al. 1980; Ennis, Amundsen and Smith 1987; Cheng and Smith 1989). Purified RecBCD enzyme nicks DNA at Chi sites (Ponticelli et al. 1985; Taylor et al. 1985), demonstrating that RecBCD enzyme and Chi directly interact. In addition to Chi-nicking, RecBCD enzyme unwinds linear double-stranded DNA (dsDNA) and has double-stranded exonuclease, single-stranded exonuclease, single-stranded endonuclease, and ATPase activities (Taylor 1988).

Smith et al. (1981b) proposed a model for recombination by the RecBCD pathway (Figure 1). In this model RecBCD enzyme binds to a dsDNA end (step A) and travels along the DNA, unwinding and rewinding the DNA, producing single-stranded DNA (ssDNA) loops (steps B and C). Upon encountering a properly oriented Chi site RecBCD enzyme nicks one strand and continues unwinding (step D), freeing a 3' ended ssDNA tail (steps E and F). Aided by RecA and SSB proteins, this tail invades a homologous duplex to form a D-loop (step G). Nicking of the D-loop (by an unknown activity, perhaps RecBCD enzyme) allows a Holliday junction to be formed (step H). Resolution of the Holliday junction yields reciprocal recombinant products (step I). Heteroduplex DNA (hDNA, that composed of one strand from each parent) is present in both intermediates (steps G and H) and recombinant products (step I).

We have addressed predictions of this model by measuring hDNA in phage lambda undergoing recombination by the RecBCD pathway in *E. coli*. If Chi stimulates an early step in recombination (e.g., producing a ssDNA substrate for RecA protein, as suggested above), there would be more hDNA in lambda crosses when Chi is present than when Chi is absent. If Chi stimulates a late step [e.g., resolving Holliday junctions, as suggested by Rosenberg (1987) and Thaler et al. (1988)], the level of hDNA would be the same whether Chi is present or not. In this case

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Chi would change the distribution of hDNA from intermediates to recombinant products, but the overall level of hDNA would be the same with or without Chi. In the experiments reported here we found more hDNA in the presence of Chi (Chi⁺ crosses) than in the absence of Chi (Chi⁻ crosses), supporting a role for Chi early in recombination (i.e., before formation of hDNA). Using strand-specific probes, we addressed whether the Chi-cut tail is used for strand invasion for Chi early in recombination (i.e., yields both complementary recombinant products). Finally, we analyzed hDNA formation in *E. coli* mutants and determined that Chi-nicking activity is required for Chi’s enhancement of hDNA levels.

### MATERIALS AND METHODS

**Bacterial strains, phage and plasmids:** *E. coli* K-12 strains used in this work are listed in Table 1, and the phages and plasmids in Table 2. The *recA56* allele was introduced via P1 transduction by its linkage to *srl-300::TnlO* *Tn10*. Lambda phage were constructed by appropriate vegetative crosses. The presence of relevant alleles was tested by crossing to phage of known genotype. The presence of the *clAH3* allele (deletion of a 564-bp *HindIII* fragment) was verified by restriction enzyme digestion of DNA extracted from phage particles. Note that this deletion is slightly smaller than the 690-bp deletion designated *clAH3* by Lichtten and Fox (1983). Fragments of phage lambda DNA were cloned into the pT3T7lac vector (Boehringer Mannheim) for use in preparing riboprobes. *χ*⁺*C151* is a mutation creating a Chi site (Stahl, Crasemann and Stahl 1975); *χ*⁻*C209* is a mutation destroying this Chi site (Schultz, Swindle and Smith 1981).

**Lambda infections and extraction of DNA:** TB-maltose broth (0.5% NaCl, 1.0% tryptone, 0.1% maltose) was inoculated with 0.02 volume of an overnight culture of *E. coli* and grown at 37° with shaking to an OD₅₅₀ of 0.6 (1.5 × 10⁶ cells/ml). Cells were infected with lambda phage at a multiplicity of infection of 5 or 10 of each phage. Phage were allowed to adsorb 10 min at 37°, then an equal volume of TB-maltose broth was added, and the cultures were shaken at 37°. At various times after addition of broth, 10-ml aliquots were harvested into chilled Corex glass tubes.

Samples were extracted 1-3 times with phenol/chloroform. The aqueous phase was removed to a siliconized tube, and DNA precipitated by the addition of 0.10 volume of 2.5 M saturated ammonium acetate and 2.5 volumes of ethanol. After over-night incubation at −20° DNA pellets were collected by centrifugation at 27,000 x g for 15 min, washed once with 70% ethanol and dried. The pellet was dissolved in 2.5 ml of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Ribonucleases A (boiled to inactivate DNase) was added to 0.5 mg/ml, and the samples incubated 30 min at 37°.

**Southern blots:** Native blots (blotted without prior de-
naturation of the DNA) to detect heteroduplex DNA were performed as previously described (Lichten and Fox 1983). BglII digested DNA (DNA extracted from 1.5 x 10^9 cells per lane) was electrophoresed on 1% agarose gels in TBE buffer (0.1 M Tris-HCl, pH 8.0, 0.083 M boric acid, 1 mM EDTA) containing 5 μg/ml ethidium bromide at 1 V/cm for 20 hr. Denatured blots were performed using standard procedures (Maniatis, Fritsch and Sambrook 1982). Both native and denatured blots were transferred to either nitrocellulose (Schleicher and Schuell) or nylon membranes (Genescreen or Genescreen Plus, New England Nuclear). DNA was fixed to the membrane by baking for 2 hr at 80°C for nitrocellulose and Genescreen or by UV cross-linking using a Stratalinker (Stratagene) for Genescreen Plus. Native blots were probed with the 564-nucleotide (nt) HindIII fragment containing part of lambda’s cl and recA genes (probe A, Figure 2), either the entire dsDNA fragment (32P-labeled using random primers, Boehringer Mannheim) or strand-specific riboprobes (prepared by transcription of pSH3 or pSH4 with T3 RNA polymerase in the presence of [α-32P]CTP, New England Nuclear). Native blots were hybridized 18 hr at 42°C in Stark’s buffer (50% formamide, 5 x SSC, 25 mM Na-PO4, pH 6.5, 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 250 μg/ml salmon testes DNA) containing about 10^7 cpm/ml of probe. Denatured blots were hybridized in Stark’s buffer with the 1184 nt BglII-HindIII fragment riboprobe (probe B, Figure 2, prepared by transcription of pSA95 in the presence of [α-32P]CTP) at 10^6 cpm/ml; probe A (see Figure 2) was used for the denatured blot in Figure 3B. All blots were washed 2 times at 25°C in 2 x SSC, 0.1% SDS, then 3 times at 55°C in 0.1 x SSC, 0.1% SDS. If high background was observed on the autoradiogram, blots hybridized with riboprobes were further treated with ribonuclease A (10 μg/ml in 0.1 X SSC for 30 min at 25°C). For quantitation three gels were loaded from each restriction digest. Two gels were blotted without denaturation and probed with l and r strand specific riboprobes (probe A, Figure 2) to detect hDNA. The third gel was blotted with denaturation and probed with probe B (Figure 2) to measure total lambda DNA (to correct for variations in loading of different digests); probe A was used for the denatured blot in Figure 3B. Hybridization intensity was quantitated by densitometry (whole band analysis) of autoradiograms of native blots or by direct phosphorimage analysis of denatured blots (Molecular Dynamics 400A Phosphorimag er). Relative amounts of hDNA and total lambda DNA were calculated by comparison to dilutions of an artificial hDNA standard. The hDNA standard was prepared by mixing equal amounts of purified DNA from clΔH3 and cl' lambda phage. The DNA was denatured with alkali, neutralized, annealed in the presence of formamide and digested with BglIII. Most autoradiograms included 3-fold dilutions of the standard, spanning the range of signal observed in the samples; in all other cases an appropriate dilution was included. An appropriate larger amount of this hDNA was included as standard for total DNA. The amount of hDNA or total lambda DNA in a sample was estimated by comparison to the signal obtained from the standard dilutions. Because hDNA levels varied between experiments and between time points within an experiment (Figure 4 and Holbeck 1991), all comparisons (chi² to chi», rec» to mutant, l to r) were made between extracts taken at the same time point in the same experiment. When possible, comparisons were made from data obtained from a single blot.

### Table 2

<table>
<thead>
<tr>
<th>Phage or plasmid</th>
<th>Genotype&lt;sup&gt;a&lt;/sup&gt; or description</th>
<th>Source or reference&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1568</td>
<td>b1453 X'C151 susS7</td>
<td>1</td>
</tr>
<tr>
<td>A1569</td>
<td>b1453 X'C151 susS7</td>
<td>1</td>
</tr>
<tr>
<td>A1668</td>
<td>b2 red3 gam210 clΔH3 cl6 susS7</td>
<td>1</td>
</tr>
<tr>
<td>A1725</td>
<td>b143 clΔH3 sus029 susS7</td>
<td>This work</td>
</tr>
<tr>
<td>A1726</td>
<td>b143 clΔH3 sus029 susS7</td>
<td>This work</td>
</tr>
<tr>
<td>A1727</td>
<td>b1453 X'C151 sus029 susS7</td>
<td>This work</td>
</tr>
<tr>
<td>A1730</td>
<td>b1453 clΔH3 X'C151 sus029 susS7</td>
<td>This work</td>
</tr>
<tr>
<td>A1733</td>
<td>X'76 b1453 clΔH3 sus029 susS7</td>
<td>This work</td>
</tr>
<tr>
<td>A1740</td>
<td>X'76 b1453 sus029 susS7</td>
<td>This work</td>
</tr>
<tr>
<td>pSH3</td>
<td>pT3T7lac (Boehringer Mannheim) plus 564 nt HindIII fragment of λ cl-recA genes (nt 36895–37459).</td>
<td>This work</td>
</tr>
<tr>
<td>pSH4</td>
<td>pT3T7lac plus 564 nt HindIII fragment of λ cl-recA genes. Probe generated by T3 RNA polymerase</td>
<td>This work</td>
</tr>
<tr>
<td>probe A</td>
<td>1184 nt BglII-HindIII fragment (probe A, Figure 2), either the entire dsDNA fragment (probe A, Figure 2) to detect hDNA. The third gel was blotted with denaturation and probed with probe B (Figure 2) to measure total lambda DNA (to correct for variations in loading of different digests); probe A was used for the denatured blot in Figure 3B. Hybridization intensity was quantitated by densitometry (whole band analysis) of autoradiograms of native blots or by direct phosphorimage analysis of denatured blots (Molecular Dynamics 400A Phosphorimag er). Relative amounts of hDNA and total lambda DNA were calculated by comparison to dilutions of an artificial hDNA standard. The hDNA standard was prepared by mixing equal amounts of purified DNA from clΔH3 and cl' lambda phage. The DNA was denatured with alkali, neutralized, annealed in the presence of formamide and digested with BglIII. Most autoradiograms included 3-fold dilutions of the standard, spanning the range of signal observed in the samples; in all other cases an appropriate dilution was included. An appropriate larger amount of this hDNA was included as standard for total DNA. The amount of hDNA or total lambda DNA in a sample was estimated by comparison to the signal obtained from the standard dilutions. Because hDNA levels varied between experiments and between time points within an experiment (Figure 4 and Holbeck 1991), all comparisons (chi² to chi», rec» to mutant, l to r) were made between extracts taken at the same time point in the same experiment. When possible, comparisons were made from data obtained from a single blot.</td>
<td></td>
</tr>
</tbody>
</table>

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<sup>a</sup> Phage alleles are described in the following references: b1453, Henderson and Weil (1975); X'C151, Stahl, Crasemann and Stahl (1975); susS7, Goldberg and Howe (1969); X'C209, Schultz, Swindle and Smith (1981); red3, Signer and Weil (1968); gam210, Zissler, Signer and Schaeffer (1971); clΔH3, constructed in our lab (Kathy Heide, personal communication) by deletion of 564 bp of DNA between the HindIII sites in the cl-rex region (nt 36895–37249); susO29, Campbell (1961); b2, Horsy and Landy (1978); X'76, Kobayashi et al. (1982).

<sup>b</sup> 1, Cheng and Smith (1989); 2, Amundsen, personal communication; 3, Ponticelli et al. (1985); 4, Amundsen et al. (1986); 5, Amundsen et al. (1990).
RESULTS

Chi increases hDNA levels: Using the assay shown in Figure 2, we measured hDNA levels during phage lambda recombination proceeding by the E. coli RecBCD pathway. E. coli cells were infected with two lambda parents, one carrying a 564-bp deletion, designated cl\Delta H3, spanning parts of the cl and rexA genes. DNA was isolated from these cells at various times after infection and analyzed on native Southern blots (Lichten and Fox 1983). This assay enriches the hDNA signal due to the single-stranded character of the hDNA formed between wild-type and deletion parents. When blotted to nitrocellulose (without prior denaturation of the DNA), ssDNA binds well to the filter, while dsDNA binds poorly. Furthermore, since the DNA is blotted without denaturation, only pre-existing ssDNA regions should be available to hybridize to the probe (probe A, Figure 2). In practice, manipulations such as ethanol precipitation and fixing of the DNA to the membrane may partially denature the dsDNA and yield a variable hybridization signal from it. Since the native blots do not accurately measure parental dsDNA levels, standard denatured Southern blots were used to quantitate total lambda DNA.

To enable us to study the RecBCD pathway of recombination, we used phage carrying deletions of red, which encodes lambda's own recombination system (Signer and Weil 1968), and gam, which encodes an inhibitor of RecBCD enzyme (Karu et al. 1975). RecBCD enzyme influences the mode of lambda DNA replication by blocking lambda rolling circle replication, presumably due to its ds exonuclease activity (Enquist and Skalka 1973). To avoid this complication, in many crosses lambda DNA replication was blocked by mutation of lambda's O gene. The phage also carried mutations in the S gene, blocking lysis of
Chi Enhances hDNA Levels

**TABLE 3**

Phenotypes of *E. coli* mutants

<table>
<thead>
<tr>
<th><em>E. coli</em> mutant class</th>
<th>Alleles tested for hDNA formation</th>
<th>Relative lambda recombination proficiency</th>
<th>RecBCD in vitro activities</th>
<th>hDNA formation in vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Double-stranded exonuclease</td>
<td>Chi cutting</td>
</tr>
<tr>
<td>recBC*</td>
<td>recB21, recC73</td>
<td>1.0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>recBC null</td>
<td>recA56</td>
<td>0.09</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>recA null</td>
<td>recA56</td>
<td>0.01</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>recD2*</td>
<td>recD1009</td>
<td>3.0-6.0*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>recC*</td>
<td>recC1001</td>
<td>2.0-17.5*</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>recBCD class I</td>
<td>recB2109, recB2152</td>
<td>0.12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>recBCD class II</td>
<td>recC2145, recB2154, recB2155</td>
<td>0.11-0.17</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>recBCD class III</td>
<td>recB2153</td>
<td>0.11</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>recBCD class IV</td>
<td>rec-2123, rec-2140, rec-2148</td>
<td>0.03-0.25</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Class IV, recA56</td>
<td>rec-2123, rec-2148</td>
<td>NT*</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

Data on \( \lambda \) recombination and RecBCD in vitro activities were determined by Chaudhury and Smith (1984) (recD2* mutants), Schultz, Taylor and Smith (1985) (recC* mutants), Holbeck (1991) (recA mutant) and Amundsen *et al.* (1990) (remaining mutants). hDNA was detected using native blots as described in Figure 2 and MATERIALS AND METHODS. Representative blots from which these data were derived are in Holbeck (1991). hDNA was assayed in strains carrying the recBCD alleles on the chromosome as well as in strains with plasmid-borne alleles for all except class I mutants. The data presented for class IV mutants are from plasmid-borne alleles. When attempts were made to move these alleles to the chromosome, they behaved as null alleles (Amundsen *et al.* 1990). Indistinguishable results were obtained with plasmid and chromosomal alleles for all other mutant classes. In all experiments hDNA levels greater than 5% of recBCD* levels would have been detected.

* Measured in \( \chi^- \) crosses; in \( \chi^+ \) crosses, 0.15-0.89 for recD2* and 0.12-0.53 for recC*.

* This hDNA formation is not stimulated by Chi.

* NT, not tested.

the host cells. In most crosses the *E. coli* hosts were deleted for the rac prophage, necessary for the *E. coli* RecF pathway (Gilлен and Clark 1974), and carried the recF143 mutation, inactivating the RecF pathway (Hori and Clark 1973). Thus, the RecBCD pathway was the principal means of recombination active in these experiments.

In the first experiments we analyzed hDNA levels in recA*B*C*D* *E. coli*, which are recombination proficient and which have all in vitro activities of RecA protein and RecBCD enzyme (Table 3). The left panels of Figure 3 show experiments in which the lambda phage were free to replicate. In the native blot (Figure 3A) we found about 10 times more hDNA at 90 min after infection in extracts from a Chi* cross (in which one of the lambda phage had a Chi site) than in extracts from a Chi- cross (in which neither phage had a Chi site). A denatured blot (Figure 3B) showed that Chi stimulated replication severalfold, as reported previously (Henderson and Weil 1975). This stimulation may result from conversion of recombination intermediates into replication forks, as suggested previously (Enquist and Skalka 1973; Smith 1991). After adjusting for the levels of total lambda DNA in the samples, we found that the fraction of total lambda DNA in hDNA, as measured by densitometry, was about 4-fold greater in Chi* crosses than in Chi- crosses.

In the next experiments lambda replication was blocked by mutation of lambda's O gene. The right panels of Figure 3 show native blots from two separate experiments (C and D) and a companion denatured blot for one of them (E). The degree of stimulation of hDNA levels by Chi was determined by quantitation of hybridization intensity on these and similar blots of DNA extracted 90 min after infection. As in the replication-permitted crosses, the ratio of hDNA to total lambda DNA was greater in Chi+ crosses than in Chi- crosses (Figure 3, C-E, and Table 4). As a control, we infected *E. coli* singly with each of the lambda phage. At the time cells were harvested to extract DNA, the singly infected cells were mixed, and their DNA extracted together. In this mixing experiment we found no hDNA (Figure 3C), demonstrating that in the crosses hDNA was formed inside the cells. Thus, in both replication-allowed and replication-blocked crosses Chi increased intracellular hDNA levels.

We report the hDNA levels quantitatively in two ways (Table 4): (i) the fraction of total lambda DNA present as hDNA in each infection and (ii) the ratio of that fraction in a Chi* cross to that in a Chi- cross conducted and analyzed concurrently. By the first measure 0.83% of the total lambda DNA was present as hDNA in the Chi* crosses (cross A, Table 4, mean of six separate experiments) and 0.09% in the Chi- crosses (mean of five separate experiments). While the mean hDNA level was 9.2 times greater in the Chi* crosses than in the Chi- crosses, this measure varied considerably from experiment to experiment (Table 4). The variability between experiments may be due to the transient nature of the hDNA. DNA extracted from a Chi* cross at various times after infection
FIGURE 3.—Chi stimulates hDNA levels. (A and B) *E. coli* strain 594 (rec*) was infected with replication-competent phages 1568 and 1668 (χ+ cross) or 1569 and 1668 (χ− cross). DNA was extracted at the indicated times after infection and analyzed for hDNA (native blot, nitrocellulose, panel A) or total lambda DNA (denatured blot, nitrocellulose, panel B) as described in Figure 2 and MATERIALS AND METHODS. Marker DNA was *BglII*-digested DNA from the non-deletion parent (leftmost lane) and from artificial hDNA (next lane) prepared as described in MATERIALS AND METHODS. (C, D and E) *E. coli* strain V66 (recABCD*) was infected with replication-incompetent phages 1725 and 1726 (χ'C cross), phages 1725 and 1727 (χ'C cross), these phages singly (χ'C mix and χ'C mix), or phages 1725 and 1740 (χ'+76 cross). In the *mix* experiments, singly infected cultures were mixed at the time of DNA extraction. DNA extracted at the indicated times was analyzed for hDNA on native blots (Genescreen, panels C and D) and for total lambda DNA on a denatured blot (Genescreen, panel E). The marker is artificial hDNA prepared as described in MATERIALS AND METHODS.

showed a peak of hDNA, measured as the fraction of total DNA, at 80 min (Figure 4). While hDNA levels rose and fell over time, the reproducibility within an experiment was quite high. Quantitation of the signals from two 80 min samples in this experiment (Figure 4) revealed indistinguishable levels of hDNA (0.40% of total lambda DNA). As expected from these results, the second measure of hDNA levels—the ratio of the hDNA levels in concurrent Chi+ and Chi− crosses—varied less from experiment to experiment; the mean ratio was 6.7 in six separate experiments (Table 4). In all of the 12 such paired experiments analyzed we observed more hDNA in the Chi+ cross than in the Chi− cross (Table 4). The similarity of the two measures indicates that Chi+ crosses produced about 7 times more hDNA than Chi− crosses. For the following experiments blots with like quality were obtained (Holbeck 1991), and only their quantitation is reported.

**Moving Chi to the deletion parent and to the left of the heterology:** The experiments reported thus far were done with Chi in the non-deletion lambda parent (cross A, Table 4). Crosses with Chi in the parent with the *ΔH3* deletion (cross B, Table 4) produced lower levels of hDNA but still higher levels than crosses without Chi. By the first quantitative measure, the fraction of total lambda DNA present as hDNA was 0.23% in cross B (mean of two experiments) or 2.6 times the level in Chi− crosses. By the second measure, the ratio of hDNA in paired Chi+ crosses (cross B) and Chi− crosses was 3.3 (mean of six experiments). Thus, whereas crosses with Chi in the non-deletion parent (cross A) produced 6.7–9.2 times more hDNA than Chi− crosses, crosses with Chi in the deletion parent (cross B) produced 2.6–3.3 times more. These results indicate that Chi in the deletion parent was less efficient in promoting hDNA formation, possibly due to
Chi enhances hDNA levels

**TABLE 4**

Quantitation of DNA levels

<table>
<thead>
<tr>
<th>E. coli allele</th>
<th>Cross</th>
<th>% of λ DNA in hDNA</th>
<th>x⁺hDNA/x⁻hDNA</th>
<th>(hDNA/r hDNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean  Range (n)</td>
<td>Mean  Range (n)</td>
<td>Mean  Range (n)</td>
</tr>
<tr>
<td>recBCD +</td>
<td>A</td>
<td>0.83 0.09-2.06 (6)</td>
<td>6.7 3.6-11.7 (6)</td>
<td>3.2 2.3-4.3 (6)</td>
</tr>
<tr>
<td>recBCD +</td>
<td>B</td>
<td>0.23 0.22-0.25 (2)</td>
<td>3.3 1.4-7.6 (6)</td>
<td>2.6 NA (1)</td>
</tr>
<tr>
<td>recBCD +</td>
<td>C</td>
<td>0.03 NA (1)</td>
<td>NA NA</td>
<td>NT NA</td>
</tr>
<tr>
<td>recBCD +</td>
<td>D</td>
<td>0.05 0.05-0.07 (2)</td>
<td>NT NA</td>
<td>NT NA</td>
</tr>
<tr>
<td>recBCD +</td>
<td>No X</td>
<td>0.09 0.007-0.3 (5)</td>
<td>NT NA</td>
<td>2.8 NA (1)</td>
</tr>
<tr>
<td>recD‡</td>
<td>A</td>
<td>1.04 0.16-2.7 (4)</td>
<td>0.7 0.3-1.4 (5)</td>
<td>NT NA</td>
</tr>
<tr>
<td>recC*</td>
<td>A</td>
<td>0.17 0.15-0.20 (2)</td>
<td>1.2 0.8-1.7 (4)</td>
<td>0.56 NA (1)</td>
</tr>
<tr>
<td>rec-2148 (IV)</td>
<td>A</td>
<td>0.15 0.15-0.18 (2)</td>
<td>0.8 0.5-1.1 (3)</td>
<td>NT NA</td>
</tr>
</tbody>
</table>

Lambda crosses

A: + + x⁺C  +  C: x⁺76 + +  dΔH3 + +  3' l  5' r

B: + + dΔH3  x⁺C  D: x⁺76 dΔH3 + +  r strand hDNA

Variations on the λO⁻ crosses shown in Figure 2 were performed in strain V66 or its derivatives. The relative positions of the relevant alleles are shown (crosses A–D). hDNA levels were quantitated as described in MATERIALS AND METHODS with DNA extracted 90 min after infection in (n) separate experiments. *Ratio of the fraction of total λ DNA in hDNA in a x⁺ cross to that in a x⁻ cross. These comparisons were made between extracts of crosses performed at the same time and run on the same gels.

* Ratio of l strand hDNA to r strand hDNA, as defined in the diagram at the bottom, right. These comparisons were made by quantitating identical blots drooped with either l or r strand specific probes.

NA, not applicable.

NT, not tested.

Chi Enhances hDNA Levels

Chi nicking activity is necessary for Chi stimulation of hDNA: Purified RecBCD enzyme has several activities, including DNA unwinding, Chi nicking, double-stranded exo-, single-stranded exo- and single stranded endonuclease activities (TAYLOR 1988). AMUNDSEN et al. (1990) isolated recBCD mutants with decreased recombination proficiency but retention of one or more of the RecBCD enzymatic activities. To identify which of these activities were needed for hDNA formation, we measured hDNA in replication-blocked crosses performed in these and other E. coli mutants. The results of these crosses, as well as the enzymatic activities and recombination proficiency of these mutants, are listed in Table 3.

As shown earlier (Figure 5), rec⁺ E. coli did form
hDNA in vivo, while we detected no hDNA in recombination deficient recA56 mutants. recBCD null mutants such as recC73, lack all in vitro activities of RecBCD enzyme and failed to make detectable hDNA, indicating that one or more of the RecBCD enzymatic activities was needed for hDNA formation.

The model in Figure 1 predicts that the nicking of DNA at Chi sites is necessary for hDNA formation. Recombination in the absence of Chi has been proposed to proceed by an analogous mechanism, but with nicking at Chi-like sites (Cheng and Smith 1987). This prediction was met with class I and class II recBCD mutants, which lack Chi-cutting activity and failed to make detectable hDNA (Table 3). The fact that these mutants made no hDNA, rather than Chi- levels, supports the view that recombination in the absence of Chi proceeds by the same basic mechanism, although at a lower frequency, as recombination in the presence of Chi. These results also indicate that the DNA unwinding activity (present in class I and class II mutants) was not sufficient for hDNA formation.

The class III mutant recB2153 has all tested activities of RecBCD enzyme, including Chi-cutting, yet this mutant is recombination deficient (Amundsen et al. 1990). We did not detect any hDNA in this mutant (Table 3 and Holbeck 1991). This result indicates that Chi-nicking was necessary but not sufficient for Chi-stimulation of hDNA formation.

Class IV mutants (rec-2123, rec-2140 and rec-2148 from Amundsen et al. 1990) lack both double-stranded exonuclease and Chi-cutting activities. Despite the lack of Chi-nicking, these mutants formed hDNA (Table 3). Formation of hDNA required RecA, suggesting that this hDNA was a recombination intermediate. Similar levels of hDNA were seen in Chi+ and Chi- crosses performed in rec-2148 (Table 4), suggesting that class IV mutants formed hDNA by an altered, Chi-independent pathway. Class IV mutants have varying degrees of lambda recombination proficiency (from 3 to 25% of recBCD+ levels; Table 3), yet all showed similar levels of hDNA. The observation that class IV mutants made hDNA, yet are recombination deficient, suggests that they are blocked at a late step in recombination.

Two other classes of recBCD mutants, recD++ and recC*, exhibit nearly wild-type levels of recombination. As expected, these recombination proficient mutants formed hDNA (Table 3). Extracts of recD++ null mutants have no demonstrable activities of RecBCD enzyme (Chaudhury and Smith 1984; Amundsen et al. 1986) and may recombine by a mechanism different from that in wild-type cells (Lovett, Luisi-Delucca and Kolodner 1988). Extracts of recC* mutants have no detectable Chi-nicking activity but have all other tested activities of RecBCD enzyme (Ponticelli et al. 1985). It has been proposed (Schultz, Taylor and Smith 1983) that they have altered hotspot recognition, in neither recD++ nor recC* mutants does Chi stimulate formation of complete recombinants (Schultz, Taylor and Smith 1983; Chaudhury and Smith 1984). These mutants showed similar amounts of hDNA in Chi+ and Chi- crosses (Table 4). Thus, all recBCD mutants lacking Chi-nicking activity also lack Chi stimulation of hDNA levels and of complete recombinants.
Strand-specific probes reveal a bias in hDNA accumulation: Previous experiments (ROSENBERG 1987, 1988; HAGEMANN and ROSENBERG 1991; SIDDIQI, STAHL and STAHL 1991) have detected a strand bias in hDNA present in recombinant lambda phage. In extracts with detectable hDNA, we looked for a strand bias in hDNA using strand specific RNA probes that hybridize to either lambda’s l strand (which has its 3’ end at the right end of the standard lambda map) or r strand (which has its 5’ end at the right end). The l strand of lambda is nicked at Chi sites (PONTICELLI et al. 1985) (see Figure 1, step D). We define l strand hDNA as that which has the l strand of the non-deletion parent and the r strand of the deletion parent; r strand hDNA has the reciprocal strand arrangement (see Table 4, bottom, for diagrams of l and r strand hDNA). In our standard cross (cross A, Table 4) with Chi in the non-deletion parent, we found 3.2 times more l strand hDNA than r strand hDNA. This is the expected result if the Chi-nicked strand preferentially invades a homologous duplex for initiation of strand pairing (Figure 1, step G). When Chi was present in the deletion parent (cross B), we found 2.6 times more l strand hDNA than r strand hDNA. When neither parent contained a Chi site, a bias (2.8-fold) toward l strand hDNA was also seen. Thus, the strand bias in our experiments is not imposed by Chi. Possible bases for the bias are discussed later.

DISCUSSION

Chi enhances hDNA levels: We initiated these experiments to determine whether Chi stimulates an early step in recombination (before formation of hDNA) or a late step (after formation of hDNA) in E. coli’s RecBCD pathway. By measuring hDNA in lambda phage undergoing RecBCD pathway-mediated recombination, we found that Chi enhanced the level of intracellular hDNA about 5-fold (Table 4). This result indicates that Chi acts early in recombination, as predicted by the model in Figure 1, but does not preclude Chi from having an additional role at a later step.

To conclude that Chi stimulates an early step, we have assumed that we detected hDNA in both intermediates and recombinant products. If we lost hDNA in recombinant products, our interpretation that Chi acts early is unaffected. In fact, it is likely that we did lose products in our extracts. PEARSON and FOX (1988) demonstrated that lambda chromosomes containing a hDNA loop similar to that studied here are destroyed when packaging is attempted. This strengthens our argument that Chi acts early, since if it acted only at a late step it would stimulate the conversion of hDNA into products, which would then be destroyed by lambda packaging. In this case less hDNA might have been observed with Chi than without Chi. Detection of hDNA in intermediates (e.g., D-loops and Holliday junctions), as well as in products, in such extracts would strengthen our interpretation that Chi stimulates an early step in recombination. Two dimensional gels (BELL and BYERS 1982) to look for such intermediates may be fruitful.

Early roles for RecA protein and RecBCD enzyme: RecA protein has been studied extensively (RADDING 1988) and can promote strand transfer, an early step in recombination. We found no detectable hDNA in recA56 (recBCD+) mutant hosts, either in replication-allowed or in replication-blocked lambda crosses. LICHTEN and FOX (1984) also reported a requirement for RecA protein in hDNA formation in lambda red+ crosses. Thus, RecA protein is needed at an early recombination step in vivo, as expected from the in vitro data.

Because purified RecBCD enzyme nicks DNA at Chi sites, RecBCD enzyme and Chi must directly interact (PONTICELLI et al. 1985; TAYLOR et al. 1985). It is therefore expected that they should act at the same stage in the recombination pathway. In crosses in which lambda was free to replicate we unexpectedly found high levels of RecA-independent hDNA in recBC null mutants. Several lines of evidence suggest that the hDNA formed in recBC null mutants was non-recombinogenic material dependent upon lambda rolling circle replication (HOLBECK 1991). When the lambda crosses were repeated using phage unable to replicate due to a mutation in lambda’s O gene, we found no hDNA in extracts of recBC null mutants. This result indicates that RecBCD enzyme, like Chi, acts at an early step in recombination.

Analysis of hDNA formation in class I and class II recBCD mutants indicates that RecBCD enzyme’s early step in recombination requires its ability to nick DNA at Chi sites. Class I and class II mutants lack Chi-nicking activity (AMUNDSEN et al. 1990) and made no detectable hDNA (Table 3; HOLBECK 1991). The absence of hDNA, rather than the low levels seen in a Chi^- recBCD* cross, supports the view that recombination in the absence of Chi proceeds by the same basic mechanism, involving DNA nicking, as recombination in the presence of Chi.

Chi-independent strand bias in hDNA: The model in Figure 1 predicts that the Chi-cut strand (the l strand of phage lambda) invades a homologous duplex. Using strand-specific probes to assay hDNA, we found 3.2 times more l strand hDNA than r strand hDNA (as defined in Table 4) in our standard cross (cross A, Table 4). If nicking at Chi dictated this strand bias, the polarity of the bias should switch when Chi was in the other parent (cross B, Table 4). When this was tested, the l strand bias persisted. An excess of l strand hDNA was also seen in crosses lacking a Chi site. These results indicate that, although Chi initiates
recombination, some other factor influences the strand bias detected here.

There are several ways this bias may have arisen. Mismatch correction might preferentially repair one polarity of hDNA (r strand hDNA to obtain the bias detected here). In cells transfected with heteroduplex phage DNA, large heterologies in lambda’s cl gene are corrected less efficiently than a single bp mismatch at the same locus (DOHET et al. 1987; RAPOSA and FOX 1987). Among phage in which the large heterology had been repaired, there is no bias in repair of l or r strand hDNA. Thus, mismatch correction is unlikely to account for the strand bias we observed; we suggest that the bias arises during the formation of hDNA.

Reciprocal recombination with no gain or loss of nucleotides would yield equal amounts of l and r strand hDNA. Since we observed an excess of l strand hDNA, recombination must not be purely reciprocal. If RecBCD pathway-mediated recombination proceeds through a reciprocal intermediate (e.g., a Holliday junction), then there must be degradation and synthesis, such as that proposed by MESELSON and RADDING (1975), to see the l strand bias detected here.

Figure 6 shows possible mechanisms to obtain the observed strand bias. For simplicity, these have been drawn as proceeding through a Holliday junction; similar mechanisms can be envisioned for nonreciprocal junctions. For both cross A and cross B the initiating event may be the same—invasion by the l strand of the Chi+ parent—with subsequent events dictating the strand bias.

The strand bias may be explained by the action of RecA protein, which promotes strand transfer past a large heterology efficiently when the ssDNA donor contains an insertion (Figure 6A), but poorly when it contains a deletion (Figure 6B) (BIANCHI and RADDING 1983). Analysis of hDNA levels in crosses A and B provides further evidence that the Chi-cut tail of the deletion parent is inefficiently used by RecA. More hDNA was detected with Chi in the non-deletion parent (cross A, Table 4) than with Chi in the deletion parent (cross B, Table 4). Cross A also gave a greater stimulation of hDNA levels by Chi than did cross B. A bias in RecA action may therefore account for both strand bias and the difference seen when Chi was in the deletion vs. the non-deletion parent.

Previous reports have observed a strand bias in hDNA in packaged lambda phage. When hDNA at a
Chi Enhances hDNA Levels

<table>
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<th>Chi independent pathway</th>
<th>Chi stimulated RecBCD pathway</th>
<th>Proposed step of recBCD mutant alteration</th>
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<td></td>
<td>recD: hDNA+, rec+</td>
<td>Chi cut - Class I, Class II: no hDNA, rec-</td>
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<tr>
<td></td>
<td>recC*: hDNA+, rec+</td>
<td>Alter RecBCD activity - recB2153: no hDNA, rec-</td>
</tr>
<tr>
<td>Class IV: hDNA+, rec-</td>
<td>Cut D-loop</td>
<td>Class IV: hDNA+, rec-</td>
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**FIGURE 7.**—Scheme of recombination with proposed alterations in recBCD mutants. Class I and class II recBCD mutants fail to make hDNA because they are unable to nick at Chi. The class III mutant recB2153, which cuts at Chi but forms no hDNA, is proposed to be unable to alter its enzymatic activity after encountering Chi. recD*, recC* and class IV recBCD mutants, which lack Chi-cutting ability but form Chi-independent hDNA, are proposed to make hDNA by a Chi-independent pathway. recD* and recC* mutants are recombination proficient. Class IV mutants form recA-dependent hDNA but are recombination deficient and are thus proposed to be blocked at a late step in the pathway.

Point mutation was measured genetically (ROSENBERG 1987, 1988) or physically (HAGEMANN and ROSENBERG 1991), an excess of hDNA containing the r strand of the Chi* parent was seen. These investigators measured patch recombinants, in which flanking markers are parental (Figure 1, step I, left). SIDDIQI, STAHL and STAHL (1991) measured hDNA in splice recombinants, in which flanking markers are non-parental (Figure 1, step I, right), and found a bias toward hDNA with the l strand of the Chi* parent. Our experiments were designed to detect total hDNA, i.e., hDNA in both patches and splices as well as in intermediates. In all cases, including ours, the bias may be dictated by the heterology used. In the absence of a heterology there may be no bias.

**Non-null recBCD mutants—multiple roles for RecBCD enzyme:** To determine which of RecBCD enzyme's activities are needed for hDNA formation, we analyzed hDNA levels in a panel of recBCD mutants that have decreased recombination proficiency but that retain one or more RecBCD enzymatic activities. The results give insights into RecBCD enzyme's interaction with DNA. In Figure 7 we present a general scheme of recombination with the proposed sites of blockage in the recBCD mutants. Class I and class II mutants lack Chi-cutting activity (AMUNDSEN et al. 1990) and failed to produce detectable hDNA (Table 3). This result supports the view that nicking DNA at a Chi site by wild-type RecBCD enzyme produces ssDNA which can be used by RecA and SSB proteins for strand invasion. It also indicates that unwinding is not sufficient for hDNA formation.

The class III mutant recB2153 has all of the measured activities of RecBCD enzyme yet is recombination deficient (AMUNDSEN et al. 1990) and failed to make detectable hDNA (Table 3). Recent data demonstrate that the activity of RecBCD enzyme is altered after its interaction with a Chi site. After cutting at one Chi site the enzyme does not detectably cut at a second Chi site on the same molecule and has decreased ability to cut at a Chi site on a second molecule (TAYLOR and SMITH 1992). *In vivo* and *in vitro* data suggest that RecBCD enzyme may, under other conditions, degrade DNA up to a Chi site, then cease this
nucleolytic activity (Stahl et al. 1990; Dixon and Kowalczykowski 1991). We propose that the enzyme encoded by recB2153 is not altered by its interaction with a Chi site. If it continued in a nucleolytic mode, it would make no hDNA despite having all assayed in vitro activities.

The class IV mutants made recA-dependent, Chi-independent hDNA yet are recombination deficient (Tables 3 and 4). This result suggests that this class of mutants has two alterations. First, they make hDNA by a pathway different from that of wild-type cells, since these mutants do not cut at Chi (Amundsen et al. 1990) and their hDNA formation was not stimulated by Chi (Table 4). Class IV mutants appear to block lambda rolling-circle replication, while class I mutants, which retain the same enzymatic activities, do not (Amundsen et al. 1990). These results suggest that class IV mutants may have an altered DNA unwinding activity, which might allow hDNA formation by a Chi-independent mechanism. Second, the fact that class IV mutants made recA- dependent hDNA yet are recombination deficient, suggests that RecBCD enzyme has a role late in recombination, in addition to its early role, and that class IV mutants are deficient in this step.

A direct role for RecBCD enzyme in resolution of intermediates is made unlikely by recent observations. RecBCD enzyme is unable to resolve model cruciform structures in a biologically meaningful way (Taylor and Smith 1990). A Holliday junction cleaving activity has been isolated from E. coli and is encoded by ruvC (Connolly et al. 1991). ruvC mutants are only slightly decreased in recombination proficiency, but cells which also carry a mutation in recG are greatly decreased in recombination ability by the RecBCD pathway (Lloyd 1991), implying that either ruvC or recG product is needed to cleave intermediates.

If Holliday junction cleavage is performed by RuvC and RecG products, what might be a late role for RecBCD enzyme? One possibility is cleavage of the D-loop (Figure 1G) to allow formation of a Holliday junction. RecBCD enzyme can cleave D-loops (Wiegand et al. 1977). Alternatively, the single-stranded exonuclease activity of RecBCD enzyme may act after Holliday junction resolution. Some Holliday junction resolving enzymes cleave the junction asymmetrically and leave short ssDNA tails protruding from the duplex, preventing ligation of the strands (West 1992). RecBCD enzyme’s single-stranded exonuclease activity could remove these short tails to allow ligation and thus detection of recombinant products. Class IV recBCD mutants have decreased single-stranded exonuclease activity (Amundsen et al. 1990), consistent with a role for this activity late in recombination.

RecBCD enzyme and Chi have been proposed to act either early (Smith et al. 1981b) or late (Thaler et al. 1988) in recombination. The experiments reported here support a role for RecBCD enzyme and Chi early in recombination and indicate that Chi-nicking activity is necessary for this step. The finding that a class of recBCD mutants form hDNA, but not recombinant products, suggests that RecBCD enzyme may function late in recombination as well.

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