8216–8228 *Nucleic Acids Research, 2016, Vol. 44, No. 17* *Published online 21 June 2016*
doi: 10.1093/nar/gkw541

Unexpected DNA context-dependence identifies a new determinant of Chi recombination hotspots

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Received April 06, 2016; Revised May 10, 2016; Accepted June 03, 2016

ABSTRACT

Homologous recombination occurs especially frequently near special chromosomal sites called hotspots. In *Escherichia coli*, Chi hotspots control RecBCD enzyme, a protein machine essential for the major pathway of DNA break-repair and recombination. RecBCD generates recombinogenic single-stranded DNA ends by unwinding DNA and cutting it a few nucleotides to the 3′ side of 5′ GCTGGTGG 3′, the sequence historically equated with Chi. To test if sequence context affects Chi activity, we deep-sequenced the products of a DNA library containing 10 random base-pairs on each side of the Chi sequence and cut by purified RecBCD. We found strongly enhanced cutting at Chi with certain preferred sequences, such as A or G at nucleotides 4–7, on the 3′ flank of the Chi octamer. These sequences also strongly increased Chi hotspot activity in *E. coli* cells. Our combined enzymatic and genetic results redefine the Chi hotspot sequence, implicate the nuclease domain in Chi recognition, indicate that nicking of one strand at Chi is RecBCD's biologically important reaction in living cells, and enable more precise analysis of Chi's role in recombination and genome evolution.

INTRODUCTION

Special sites that control chromosomal metabolism, such as replication, transcription, and recombination, have been exceptionally useful in elucidating the molecular basis of these events. Determination of the factors that interact with special chromosomal sites reveals not only essential components in these processes but also the activities of components necessary for their role(s) in chromosomal function. A particularly informative example is a class of hotspots of homologous recombination and DNA double-strand break (DSB) repair in the bacterium *Escherichia coli*. These DNA sites, called Chi for crossover hotspot instigator (1), are recognized by the RecBCD helicase-nuclease and activate recombination in their vicinity (2–4). Study of the interaction of RecBCD with Chi, both in cells and with purified components, has been essential in elucidating how the multiple activities of this complex three-subunit enzyme are coordinated to maintain chromosomal integrity and to propel evolution through the reassortment of alleles. Here, we report that Chi sites have a previously unrecognized DNA determinant that interacts with a part of RecBCD not previously considered to be involved in Chi recognition.

Chi was discovered as a set of mutations that enhance recombination of coliphage λ lacking its own recombination-promoting proteins (Red) and an inhibitor (Gam) of the host RecBCD enzyme (1,5,6). Wild-type λ lacks Chi, but spontaneous mutations create Chi at one of several identified sites across the genome (5,7). Comparison of the nucleotide sequences around six Chi sites in λ revealed that all contain 5′ GCTGGTGG 3′ as does the *E. coli* Chi site in *lacZ*, one of ∼1000 Chi sites in the *E. coli* genome (8,9). No base-pair (bp) at a given position flanking the Chi octamer is common to all of these sites, and all observed mutations creating or inactivating Chi occur only within this octamer (8). Thus, it was concluded that Chi is 5′ GCTGGTGG 3′ (10). As expected, insertion of synthetic DNA with this sequence generates a site with Chi activity (11,12). The sequence of the DNA strand with 5′ GCTGGTGG 3′, but not that of its complement, is critical for Chi action (13).

Chi enhances recombination exclusively via *E. coli*'s major pathway for DNA break repair and homologous recombination, the RecBCD pathway (14,15). As diagrammed in Figure 1A, RecBCD initiates DNA unwinding at a free double-stranded (ds) DNA end, rapidly unwinds the DNA with the production of single-stranded (ss) DNA loops (16), nicks the strand with 5′ GCTGGTGG 3′ a few nucleotides to its 3′ side, and continues unwinding (2,3). RecBCD loads the RecA strand-exchange protein onto the newly generated 3′ ss end (17), which invades an intact homologous duplex to generate a D-loop. The D-loop may be converted into a Holliday junction, which is resolved into reciprocal recombinants; alternatively, the D-loop may prime DNA replication and generate non-reciprocal recombinants (18,19).

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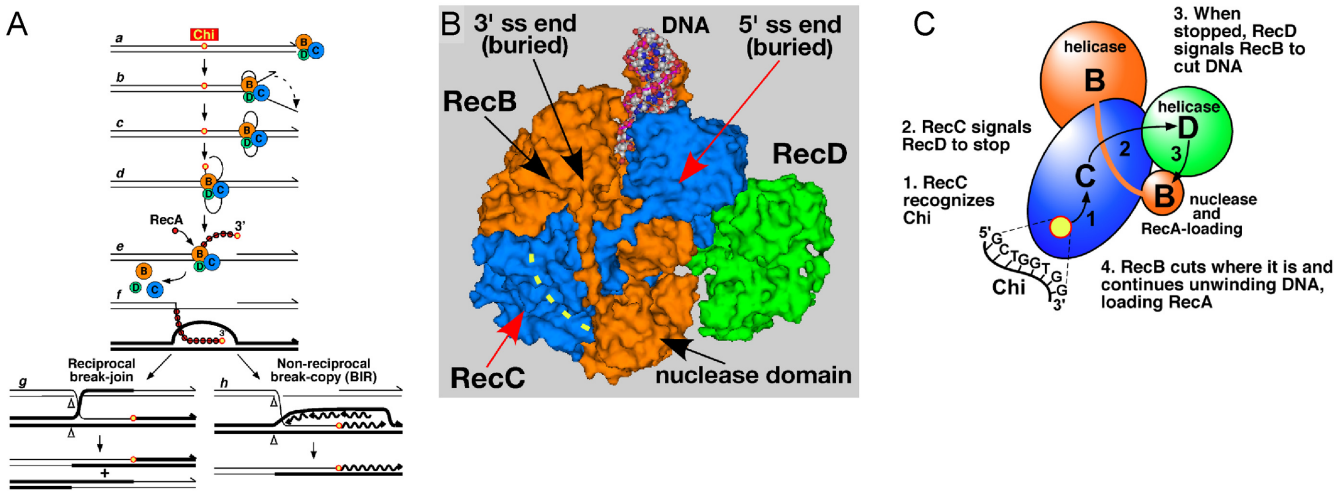

Figure 1. Models for RecBCD's promotion of recombination, and RecBCD crystal structure. See Introduction for description of these models. Adapted from (25). (**A**) Model for recombination promoted by RecBCD and Chi. (**B**) Crystal structure of RecBCD bound to ds DNA [PDB 1W36; (20)]. Chi is inferred to be recognized in a RecC tunnel (yellow dashed line). When Chi is encountered, the RecB nuclease domain is inferred to swing to the position shown from a position to the left of RecC (27). (**C**) Signal-transduction model for Chi's regulation of RecBCD enzyme.

The crystal structure of RecBCD bound to the end of ds DNA (20) is exceptionally informative in giving molecular detail to the enzymatic activities of RecBCD and its interaction with Chi (reviewed in (19,21)). In this structure (Figure 1B) the 5′-ended strand enters a tunnel in RecC and heads toward the helicase domain in RecD, whereas the 3′-ended strand enters the helicase domain of RecB. The RecB helicase tunnel leads to a second tunnel in RecC, where the Chi octamer is likely recognized (4,22).

Chi coordinates the multiple activities of RecBCD enzyme. As noted above, the enzyme unwinds DNA up to Chi, nicks it at Chi, and begins loading RecA. After acting at Chi, RecBCD loses the ability to act at a subsequent Chi site and later (probably at the end of the DNA substrate) disassembles into its three separate, inactive subunits (23,24). The genetic and enzymatic behavior of mutants altered in the RecB helicase domain led to the 'signal transduction' model for Chi's regulation of RecBCD (Figure 1C) (25). In this model, when Chi is in a tunnel in RecC, RecC signals the faster helicase RecD to stop; when RecD stops, it signals the nuclease domain of RecB to nick the DNA at Chi and to begin loading RecA. Unwinding continues by the slower helicase RecB (26). Conformational changes in RecBCD during its action on Chi-containing DNA have supported aspects of this model (27), which is consistent with current genetic and enzymatic data.

Although Chi sites (5′ GCTGGTGG 3′) at different positions in λ clearly stimulate RecBCD-dependent recombination, it is not simple to compare quantitatively the activity of Chi at different sites. This is because the frequency of recombination, and thus the degree of Chi-dependent stimulation (i.e. Chi hotspot activity), depends on the distance between the markers used to assay recombination and the position of Chi within the interval assayed (28). To determine if all Chi sites have the same activity, independent of their DNA sequence-context, we devised a method to determine the DNA sequences at which RecBCD preferentially cuts DNA at Chi. We found a remarkably strong context-dependence, which we then showed has strong effects on recombination-stimulation by Chi in living cells. Our results force a reanalysis of the sequence of Chi and how this sequence interacts with RecBCD enzyme to control its multiple activities. They also provide further evidence that RecBCD's simple nicking of DNA at Chi noted above, rather than degradation up to Chi, is the biologically important reaction. We discuss the implications of our results for DSB repair and the evolution of chromosomes. We also discuss other cases of chromosomal sites with a seemingly unique sequence being more complex than usually considered.

MATERIALS AND METHODS

Enzymes and DNA substrates

RecBCD enzyme was purified as described (26). Other enzymes were from New England Biolabs (Ipswich, MA). To construct the hairpin DNA substrates, a 200-nucleotide Ultramer (Supplementary Figure S1) was obtained from IDT (Integrated DNA Technologies, Coralville, IA) either unpurified or gel purified. Its sequence is 5′ CGGCCATGGAGACGTTTTGC AGCAGCAGTCGCTTCACGTTCGCTCGCGTA TCGGTGATTCATTCTGCTAACCAGNNNNNN NNNNCCACCAGCNNNNNNNNNNCCTCAAC GACAGGAGCACGATCATGCGCACCCGTGGCCA GGACCCAACGCTGCCCGAGATGCGCCGCGTGC GGCTGCTGCGCTCAGGCTATGCCTGAG 3′. Unpurified samples were purified by electrophoresis in an 8% polyacrylamide gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH ∼8.3) in a Bio-Rad Mini Prep Cell. The complementary strand was synthesized from the left-end hairpin using the Klenow fragment (3′→5′ exonuclease-deficient) of DNA polymerase I in CutSmart buffer (New England Biolabs). The polymerase was inactivated by boiling, the ds DNA treated with NcoI,

the DNA boiled again, and the hairpin-terminated duplex substrate purified and recovered as above.

RecBCD reaction conditions

Unless otherwise noted, reactions were in 20 mM MOPS–KOH, pH 7.0, with 3.5 mM $Mg(OAc)_2$. In experiment 1 (Supplementary Figure S2), 25 nM DNA was reacted with 4 nM RecBCD for 4 min. In experiment 2 (Figures 3–5, Supplementary Figures S2 and S3), 25 nM DNA was reacted with the indicated concentrations of RecBCD for 1 min. In experiment 3 (Figure 6, Supplementary Figures S2 and S4), 10 nM DNA was reacted with the indicated concentrations of RecBCD for 1 min. Reactions without SSB contained 2.5 nM RecBCD in 3.5 mM $Mg(OAc)_2$ and MOPS-KOH buffers at pH 7.0 or pH 7.5 as indicated. Reactions with SSB contained 20 mM MOPS–KOH pH 7.5, 1 μM SSB (Promega) and 2 mM $Mg(OAc)_2$ with 5 nM RecBCD, 3.5 mM $Mg(OAc)_2$ with 2.5 nM RecBCD, and 5, 6, 7 or 8 mM $Mg(OAc)_2$ with 1.25 nM RecBCD. Reactions containing SSB were stopped with 20 mM EDTA and 0.05% SDS.

RecBCD enzyme reactions, product purification, and DNA sequencing

RecBCD enzyme was allowed to bind to freshly boiled and cooled substrate at room temperature; enzyme and substrate concentrations were as noted in the figure legends and above. The reaction was started by addition of ATP to 5 mM and was terminated (after 1 min unless otherwise noted) by addition of EDTA to 20 mM, followed by boiling and precipitation with ethanol, aided by PelletPaint (EMD Millipore, Darmstadt, Germany).

For experiment 1, Chi-cut products ('Chi band') and residual full-length DNA ('residual band') were separated by electrophoresis as above but in a 12% polyacrylamide gel. Fractions containing the DNA bands were identified by secondary gel electrophoretic analysis, and the DNA precipitated with ethanol in the presence of PelletPaint. In subsequent experiments Chi band and residual band were separated on a 16 cm wide × 10 cm high × 3 mm thick 12% polyacrylamide gel in TBE buffer. Products were identified by staining small slices of the gel with SYBR Gold, and the unstained gel bands electroeluted in 3350 MWCO dialysis tubing and precipitated with ethanol in the presence of PelletPaint.

Purified Chi band and residual band were reacted with Klenow fragment (3′→5′ exonuclease-deficient) and dNTPs in CutSmart Buffer, boiled, cut with MnlI, and precipitated with ethanol. The desired fragments were isolated on an 8% polyacrylamide gel (two slots wide and 1 mm thick) in TBE buffer in a Novex minigel cassette. DNA was detected by SYBR Gold staining, recovered as above, precipitated with ethanol, dissolved in water, and sequenced on a HiSeq 2500 apparatus (Illumina, San Diego, CA) to give 50 nucleotide paired-end reads, as described in Supplementary Material.

Analytical gel analysis of reaction products

Reaction products shown in Figures 3C and 6B were separated on a 12% polyacrylamide gel in TBE buffer in Novex cassette minigels (1 mm thick) for 75 min at 150 V. Gels were stained with SYBR Gold, imaged on a Typhoon Trion variable-mode imager, and quantified using ImageQuant TL v2005 software.

Bacterial strains, phage, and plasmids

Genotypes and sources of *E. coli* strains and phage are in Supplementary Table S1. Chi (χL) with various surrounding sequences in the λ *gam* gene were constructed by recombineering (29) with oligonucleotides from IDT (Supplementary Table S2) as described in Supplementary Material.

Growth media

Tryptone broth (TB) and agar, LB broth (LB) and agar (LBA) and suspension medium (SM) have been described (4,28). TB top agar contained 0.75% Bacto-Agar (Becton-Dickinson) and TB bottom agar contained 1.0% Bacto-Agar. Tryptone agar plates (BBL YE) for the detection of clear and turbid plaques contained 0.2% yeast extract (Becton-Dickinson).

Chi hotspot crosses in *E. coli*

Crosses between *susJ6 cI857* and cI^+ *susR5* phages (moi of 5 each) homozygous for the indicated χL allele were conducted in *E. coli* strains V66 and V67 as described by Cheng and Smith (28) except that bacteria were grown to a density of 2×10^8 colony-forming units per ml and the diluted phage-bacteria mixture was incubated at 37° for 90 min. The frequency of J^+ R^+ recombinant phage was determined by plating on strain C600 (*supE44*) for total phage and on strain 594 (*sup*$^+$) for recombinants (for active χ^+L alleles) or on strain V3477 (*supE44 recD2741*) for total phage and on strain V222 (*sup*$^+$ *recD1013*) for recombinants (for inactive χ^-L alleles). At least 50, and usually more than 100, plaques on the *sup*$^+$ strain were counted to determine the clear/turbid ratio (Chi activity).

RESULTS

Chi hotspot activity depends on the nucleotide sequences flanking Chi

While studying a class of *recBCD* mutants, we noted that Chi sites at some loci in λ *red gam* mutants enhanced plaque-size, a measure of Chi hotspot activity (5), more than did Chi sites at other loci even though all have 5′ GCTGGTGG 3′. These observations suggested that the DNA sequence flanking Chi affects Chi's activity, but a direct quantitative comparison of these Chi sites was not possible for the reasons noted in the Introduction. We therefore introduced Chi flanked by various random sequences at a new locus (χL) in *gam*. Chi activity was measured as the frequency of recombination in an interval with χL (interval I) relative to the frequency in a control interval without a Chi site to its right (interval II) (Figure 2A). Since Chi acts to its left (as written here) (1,6,28), recombination in interval I is stimulated but that in interval II is not, and the ratio of the recombinant frequency in interval I to that in interval II is a

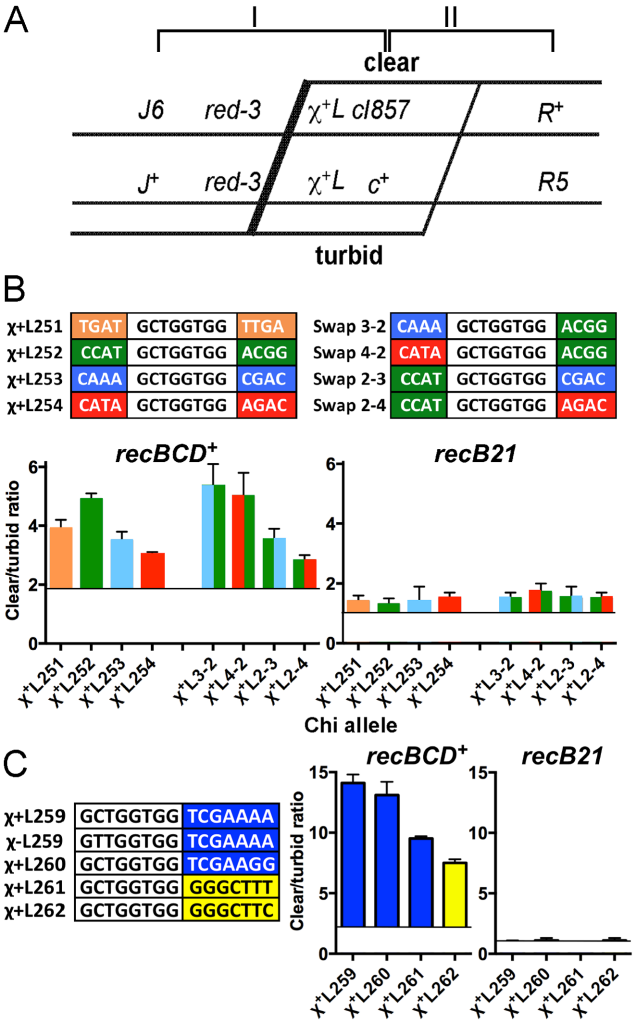

Figure 2. DNA sequences flanking Chi strongly affect Chi hotspot activity during recombination in *E. coli* cells**.** (**A**) Chi hotspot activity was assayed as the ratio of clear (*cI857*) to turbid (cI^+) plaques among selected J^+ R^+ recombinants in the cross shown. Chi with appropriate flanking sequences increases recombination in interval I (*J* – *cI*) relative to that in interval II (*cI* – *R*) and thus the clear/turbid ratio. (**B**) Four χ^+L alleles contain 5′ GCTGGTGG 3′ but have different flanking sequences and different hotspot activities. Swapping the left and right flanking four nucleotides among three of them (*252, 253* and *254*) changes the Chi hotspot activity. Data (clear/turbid ratio among J^+ R^+ recombinants) are the mean ± S.E.M. from >4 crosses with the indicated homozygous χ^+L allele. The black horizontal line is the clear/turbid ratio with the inactive Chi octamer χ^-L252 (1.9 ± 0.1, left panel; 1.1 ± 0.03, right panel). (**C**) Chi hotspot activity in cells correlates with the preference for sequences to the 3′ side of Chi by RecBCD's nicking of DNA at Chi with low Mg^{2+} concentration (Figure 6; Supplementary Table S4). The black horizontal line is the clear/turbid ratio with the inactive Chi octamer χ^-L259 (2.1 ± 0.1, left panel; 1.0 ± 0.1, right panel). *P* values for data in panels B and C are in Supplementary Table S3B.

valid measure of Chi activity. We conducted crosses in wild-type (*recBCD*$^+$) cells, in which Chi is active, and, as a further control, in *recB* null (recombination-deficient) and *recD* null (recombination-proficient) mutant cells, in which Chi is not active (14,30). As expected, Chi strongly stimulated recombination in *recBCD*$^+$ cells but not in the mutant cells. Using this assay, we found that the sequences flanking Chi, particularly those to the right of Chi, indeed have dramatic effects on Chi activity.

In wild-type cells, Chi hotspot activity differed significantly among the four χ^+L sequences tested and ranged from 3.0 ± 0.1 to 4.9 ± 0.2 (Figure 2B; Supplementary Table S3); by unpaired *t*-test, $P < 0.03$ for all but two of the six comparisons. We could not distinguish any notable pattern among these sequences that might account for the differences in Chi hotspot activity. To test whether sequences immediately to the right or to the left of Chi were important, we swapped the four flanking nucleotides to the right (or to the left) among the most active Chi (χ^+L252), the least active Chi (χ^+L254), and a Chi with intermediate activity (χ^+L253). The results showed that sequences to the right (3′) side of Chi had strong effects on Chi hotspot activity, whereas those to the left (5′) side had no significant effect. For example, the right flank of χ^+L252 *increased* the activity of χ^+L254 to that of χ^+L252 (from 3.0 to 5.0), and the right flank of χ^+L254 *decreased* the activity of χ^+L252 to that of χ^+L254 (from 4.9 to 2.8). Swapping the left flanks, however, had no significant effect.

These results showed that flanking sequences have strong effects on Chi activity, but determining the exact nature of the sequence requirements was not feasible by this approach. We therefore sought a method to test vast numbers of random flanking sequences for those that enhance or lower Chi activity.

A method to determine flanking sequences that influence Chi activity

Purified RecBCD enzyme cuts DNA four to six nucleotides to the 3′ side of 5′ GCTGGTGG 3′, but cutting occurs only ∼10–40% of the time that the enzyme traverses Chi (3,24,31). To determine which flanking DNA sequences influence the frequency of this cutting, we used a 185 bp ds DNA substrate with 5′ GCTGGTGG 3′ flanked by ten random bp on each side near the center (Figure 3A, B, and Supplementary Figure S1A). To allow RecBCD to unwind this DNA only from right to left, the direction productive for cutting (3), this substrate had a free (open) ds end on the right and a capped (hairpin) end on the left (24). After limited reaction with RecBCD enzyme, the cut product (Chi band) and the residual, uncut substrate (residual band) were purified by gel electrophoresis (Figure 3C). Consistent with the structure shown for the RecBCD reaction product in Figure 3B, the purified cut product was double-stranded to the left of Chi (sensitive to MnlI) and single-stranded to the right of Chi (resistant to TfiI; Supplementary Figure S1B). The Chi band was filled out using a DNA polymerase to regenerate fully ds DNA (Figure 3B). This DNA and the residual band were subjected to deep-sequence analysis, as was the starting substrate. We expected that sequences *preferentially cut* would be *enriched* in the Chi band and, conversely, *depleted* in the residual band, relative to the starting substrate. Similarly, sequences *infrequently cut* would be *depleted* in the Chi band and *enriched* in the residual band, relative to the starting substrate. These expectations were realized for each of these four comparisons and showed that sequences on the 3′ flank of 5′ GCTGGTGG 3′ do indeed have important roles in determining the activity of Chi.

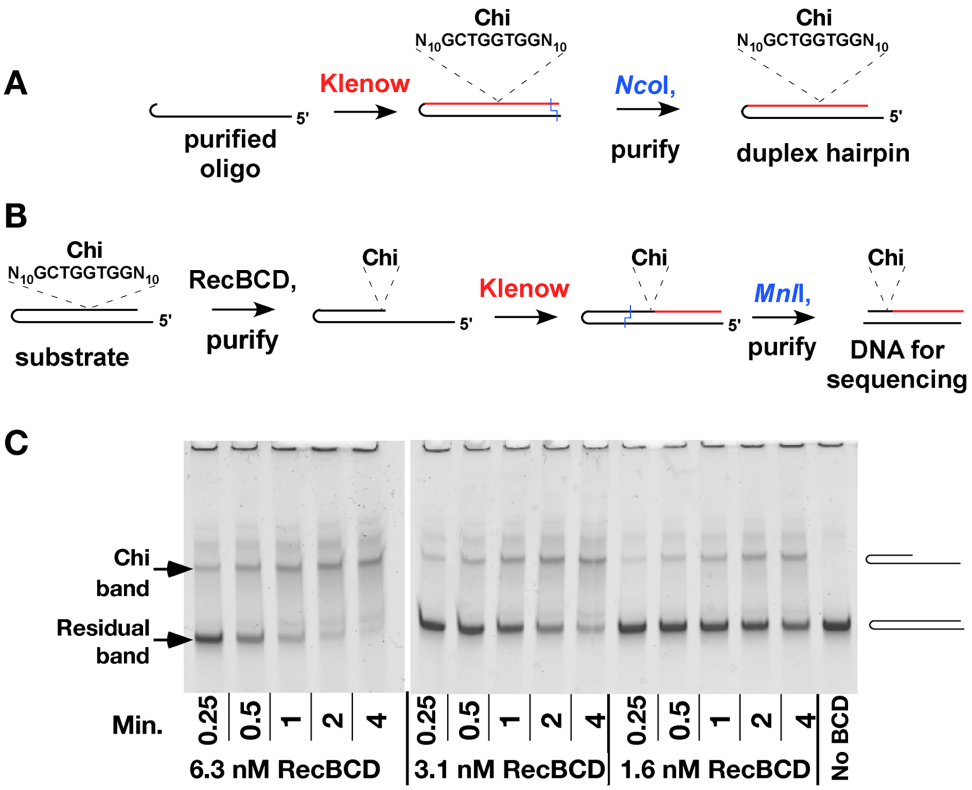

Figure 3. Scheme to generate RecBCD substrates with Chi flanked by random base-pairs, and the products of its limited reaction with RecBCD. (**A**) A 200 nucleotide-long oligonucleotide with a 7 + 7 nucleotide palindrome near its 3′ end (Supplementary Figure S1) was converted to duplex DNA (red) and cut with NcoI. The product had a hairpin left end resistant to RecBCD, a 'sticky' right end to aid RecBCD binding and reaction (65), and, near the center, the Chi octamer (5′ GCTGGTGG 3′) flanked by 10 random nucleotides. (**B**) Cutting at Chi by RecBCD produces a partially duplex DNA, which is converted to completely duplex DNA, cut with MnlI, and deep-sequenced. (**C**) Gel electrophoresis of DNA, without precipitation, after reaction with the indicated concentration of RecBCD for the indicated time with 25 nM DNA. The gel was stained with SYBR Gold. Starting substrate, right-most lane. Arrows indicate the Chi (cut) band and residual (uncut) band as diagrammed on the right.

DNA sequences on the 3′ flank of Chi strongly influence RecBCD's cutting at Chi

Preferred sequences are enriched in the Chi band and depleted in the residual band. An analysis of the products of RecBCD acting on the Chi substrate described above (Figure 3) is shown in Figure 4 and Supplementary Figure S2. The results of these experiments, with similar reaction conditions but with different substrate preparations on different days and sequenced separately, were highly reproducible (Supplementary Figure S2) as were duplicate reactions in the same experiment (Figure 4A). This outcome and the concordance of the four methods of analysis noted above underscore the reliability of the results reported here. In the Chi band, the frequency of certain bases was enriched, relative to their frequency in the starting substrate, at some nucleotide (nuc) positions (Figure 4A and Supplementary Figure S2). Most dramatic was the enrichment for A at positions 4, 5, 6 and 7 to the 3′ (right) side of Chi. Conversely, A was depleted at these positions in the residual band (Figure 4B). At other nuc positions, such as those to the 5′ (left) side of Chi, more limited enrichment or depletion was observed (see below for further analysis). These position-dependent enrichments and depletions, and the high reproducibility of deep-sequencing of a given substrate DNA preparation (Supplementary Figure S2 and Table S4; see also Figure 6 below), demonstrate the significance of the data.

We sought patterns in the preferred sequences by analyzing groups of four or more adjacent bases to the right and left of Chi. In the Chi band, the enrichment, relative to the substrate, of the most frequent tetramer, pentamer, and hexamer beginning at nuc 1, 2, 3, … to the right of Chi was plotted as a function of the distance of its center from Chi (Figure 4C, upper part; Supplementary Table S4). The pentamer (5′ AAGGA 3′) and hexamer (5′ AAGGAG 3′) beginning at nuc 4 were the most highly enriched in each of the duplicate experiments. DNAs with these sequences were respectively enriched 1.72- and 1.93-fold relative to their frequencies in the substrate (Supplementary Table S4). The tetramers 5′ AAAA 3′ and 5′ AAGG 3′ beginning at nuc 4 were the first and second most enriched tetramers in the two experiments and were enriched 1.57- to 1.61-fold. In both experiments 5′ AAGG 3′ was the most enriched tetramer beginning at nuc 5. In one of the experiments the most enriched heptamer (5′ AAGGAGA 3′) was enriched 2.12-fold (Supplementary Table S4). These highly reproducible results show that certain sequences flanking the Chi octamer play an important role in Chi's activation of RecBCD.

Analysis of the residual band confirmed these results. For example, to the right of Chi the base most depleted was A

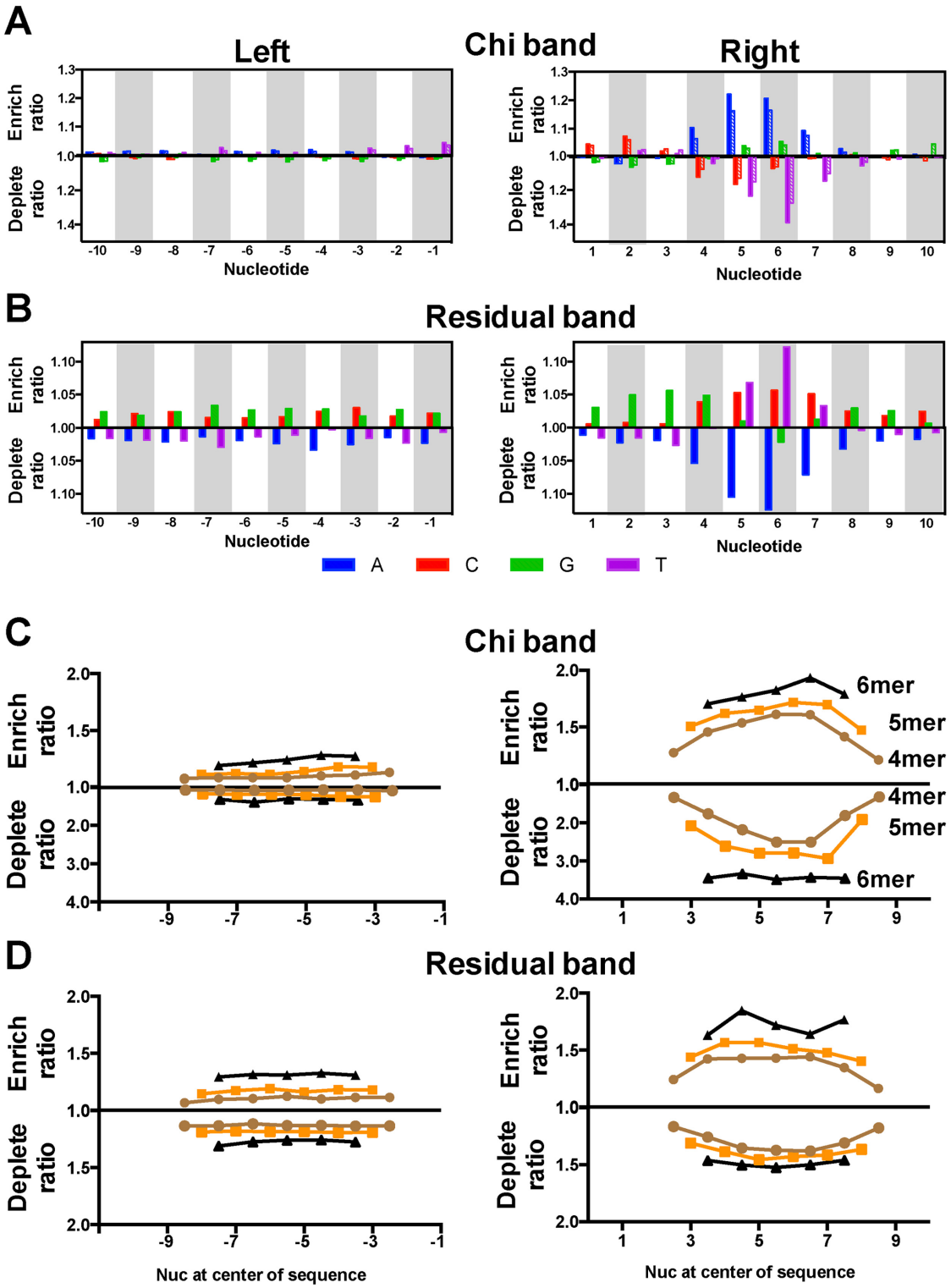

Figure 4. RecBCD nicks DNA with preferred sequences flanking Chi—strong preference to the 3′ side and weak preference to the 5′ side. Data for enriched sequences are the frequency of the indicated base or sequence in the Chi band (or residual band) (Figure 3C) divided by its frequency in the starting substrate; data for the depleted sequences are the reciprocal of this. (**A**) Enrichment or depletion of single bases at the indicated positions to the right (nuc 1 to 10) or left (nuc −1 to −10) of Chi in the Chi band after limited reaction (using 1.6 nM RecBCD and 25 nM DNA). Data from two replicate experiments done on different days are indicated by solid and hatched bars (see also Supplementary Figure S2). (**B**) Analysis of the residual band after extensive reaction (using 6.25 nM RecBCD and 25 nM DNA; companion to solid-bar experiment in A). (**C**) Extent of enrichment (top part) or depletion (bottom part) of the most enriched tetramer, pentamer, and hexamer, with centers at the nuc position indicated, in the Chi band after limited reaction (using 1.6 nM RecBCD). Data are from the experiment in panels A (solid bars) and B. (**D**) Analysis as in panel C of the residual band after extensive reaction (using 6.25 nM RecBCD). Data are from the experiment in panels A (solid bars) and B.

at nuc 4–8 (Figure 4B), and the tetramer most depleted was 5′ AAAA 3′ beginning at nuc 4 (Figure 4D, lower part); it was depleted by a factor of 1.37 relative to the substrate (Supplementary Table S4). The most depleted pentamer (5′ TTAAA 3′) and hexamer (5′ TTAAAA 3′) each began at nuc 3 and were depleted by factors of 1.46 and 1.53, respectively. These results are consistent with those of the Chi band and reinforce the conclusion that RecBCD preferentially cuts at Chi flanked by certain nucleotide sequences.

Lesser, but still noticeable, enrichments in the Chi band and depletions in the residual band were seen for certain sequences immediately to the left of Chi. Among single bases, T was among the most enriched in the Chi band at nuc −1, −2 and −3 (Figure 4A and Supplementary Figure S2) and, conversely, was among the most depleted in the residual band (Figure 4B and Supplementary Figure S2). The most enriched tetramer (5′ CATT 3′) in the cut product was immediately adjacent to the Chi octamer (i.e. with its 3′ end at nuc −1) and was enriched by a factor of 1.13 (Figure 4C, upper part; Supplementary Table S4) ($P << 0.0001$ by contingency chi-square test). The most enriched pentamer (5′ ACATT 3′) and hexamer (5′ CACATT 3′) also began at nuc -1 and were enriched by factors of 1.18 and 1.27, respectively ($P << 0.0001$ by contingency chi-square test for both values). The most enriched tetramers, pentamers, and hexamers beginning at nuc −2 had the same sequences and fold-enrichments as those beginning at nuc −1. Conversely, these or closely related sequences were correspondingly depleted in the residual band (Figure 4D, lower part). Since these enrichment and depletion factors were seen in repeat experiments (Supplementary Figure S2), we conclude they are significant, though considerably less than the corresponding data to the right of Chi. Thus, there is a slight preference for RecBCD to cut at Chi with certain sequences to its left.

Disfavored sequences are enriched in the residual band and depleted in the Chi band. To find sequences at which RecBCD prefers *not* to cut, we analyzed sequences most enriched in the residual band and most depleted in the Chi band. The results were fully consistent with those above. The residual band was enriched for T at nuc 5, 6 and 7 and for C at nuc 4 to 7 to the right of Chi; G was enriched at nuc 1 to 4 to the right of Chi and to a lesser extent to the left of Chi (Figure 4B). The tetramer 5′ GTCG 3′ beginning at nuc 5 to the right of Chi was the most enriched in the residual substrate, by a factor of 1.44 relative to the starting substrate (Figure 4D, upper part; Supplementary Table S4). Conversely, the tetramer most depleted in the Chi band 5′ TTTT 3′ began at nuc 5 and was depleted by a factor of 2.50 (Figure 4C, lower part; Supplementary Table S4). Pentamers and hexamers were also correspondingly enriched in the residual band and depleted in the Chi band, by factors of up to 3.50. Lesser, but significant, enrichments and depletions for tetramers, pentamers, and hexamers were also seen to the left of Chi (Supplementary Table S4).

Collectively, these results show that DNA with Chi flanked by certain tetramers, such as 5′ AAGG 3′ to the right of Chi, were cut by RecBCD up to 4.0 times more frequently than those with other sequences, such as 5′ TTTT 3′. DNA with certain pentamers and hexamers to the right of Chi were cut by RecBCD up to 7.4 times more frequently than others. These factors are about the same as the activity of Chi in a standard hotspot cross: the geometric-mean stimulation of recombination by the χ^+D and χ^+76 sites in λ is ∼6.0 (14,32). Our results thus have uncovered a new DNA sequence feature of Chi as strong as that of Chi itself (see Discussion).

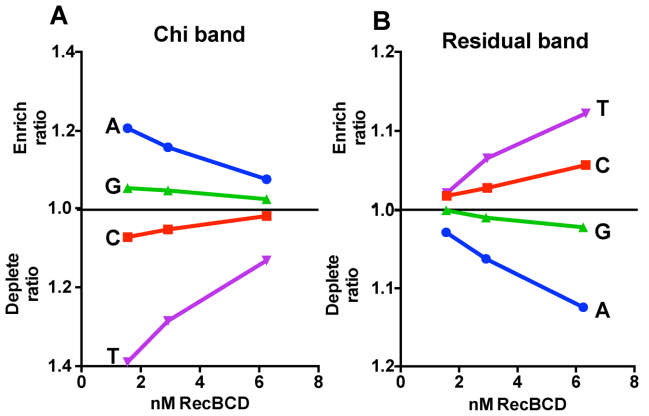

Figure 5. The extent of RecBCD reaction determines the degree of enrichment and depletion of preferred and disfavored sequences flanking Chi. (**A**) Analysis as in Figure 4A of nuc 6 of the Chi band with the indicated concentrations of RecBCD and 25 nM DNA. Analyses of tetramers, pentamers, and hexamers gave similar results (Supplementary Figure S3). (**B**) Analysis as in Figure 4B of nuc 6 of the residual band with the indicated concentrations of RecBCD and 25 nM DNA.

Preferred sequences in the Chi band are most prominent after limited reaction, and disfavored sequences in the residual band are most prominent after extensive reaction

Since RecBCD cuts DNA with less than unit frequency when it passes Chi during unwinding (3), we predicted that preferred sequences would be most enriched in the Chi band with very limited enzyme reaction (i.e. when only the most preferred sequences had been cut). The degree of enrichment would decrease with increasing extent of reaction (until all the DNA had been cut). Similarly, disfavored sequences would be increasingly enriched in the residual band with more extensive reaction (when the most disfavored sequences had at last been cut). We found these predictions to be true. For this analysis, we used variable enzyme concentration while keeping the substrate concentration and reaction time constant (see Materials and Methods). We analyzed nuc 6 to the right of Chi, at which large differences were seen in Figure 4A and B.

The enrichment of A in the Chi band decreased monotonically from 1.21-fold with 1.6 nM RecBCD to 1.07-fold with 6.3 nM RecBCD (Figure 5). Conversely, the depletion of A in the residual band increased from 1.03- to 1.12-fold. Similarly, the enrichment of T in the residual band increased from 1.02- to 1.12-fold, while the depletion of T in the Chi band decreased from 1.38- to 1.13-fold. As expected, tetramers, pentamers, and hexamers showed similar changes, but of greater magnitude, in enrichment and depletion (Supplementary Figure S3). These results reinforce the conclusion that RecBCD prefers to cut at Chi with certain flanking sequences and disfavors cutting at Chi with others.

Chi context-dependence is observed at low, but not at high, magnesium ion concentration

The action of RecBCD on DNA with Chi depends on the reaction conditions, most notably the ratio of Mg^{2+} to ATP (Figure 6A). With excess ATP, RecBCD simply nicks the 'top' strand, as drawn in Figure 3, a few nucleotides to the 3′ side of 5′ GCTGGTGG 3′ (3). With excess Mg^{2+}, RecBCD degrades the top strand up to Chi, cuts the bottom strand at Chi, and degrades the bottom strand during continued unwinding (31,33,34). Which of these reactions is more relevant to the action of RecBCD and Chi in living cells has been controversial (19) (see Discussion). We therefore investigated the effect of changing the Mg^{2+} concentration on the sequence preference at low and high Mg^{2+} concentrations.

We carried out reactions in the presence of *E. coli* single-stranded DNA binding protein (SSB), necessary for the recovery of products at higher Mg^{2+} concentrations, and at pH 7.5 typically used for such reactions (31,34). The change of pH and addition of SSB had little qualitative effect on the reaction products at low Mg^{2+} (Supplementary Figure S4). We detected the Chi and residual bands up to at least 7 mM Mg^{2+} (Figure 6B and C). SSB interfered with purification of the partially single-stranded Chi band, so we analyzed the residual band for depletion of preferred sequences or enrichment of disfavored sequences to the right of Chi, as in Figure 4B. We conducted side-by-side experiments with 2, 3.5, 6, 7 and 8 mM Mg^{2+}, each with 5 mM ATP. At the lowest Mg^{2+} concentration (2 mM), A was depleted at nuc 3–7, and T was enriched at nuc 5 and 6 (Figure 6D), as shown above (Figure 4B and Supplementary Figure S2). As the Mg^{2+} concentration was raised to 3.5 mM, these biases decreased. Most importantly, no significant preference for any base at any position was seen at the higher Mg^{2+} concentrations (6, 7, and 8 mM), even though both Chi and residual bands were observed up to at least 7 mM Mg^{2+} (Figure 6B and C). These data show that the DNA sequence context is important for RecBCD's cutting of DNA at Chi only at low Mg^{2+} concentration (see Discussion).

Influence of 3′ sequences flanking Chi in *E. coli* cells parallels that with purified RecBCD enzyme at low magnesium concentration

To test in living *E. coli* cells the sequences preferred or disfavored by purified RecBCD acting at Chi, we measured Chi hotspot activity as the clear/turbid ratio among selected J^+ R^+ recombinants (Figure 2A). We conducted crosses with the Chi octamer at the χL locus flanked on its right side by two of the most preferred and two of the most disfavored 3′ flanking sequences. As predicted, the preferred sequences, in χ^+L259 (with flanking 5′ TCGAAAA 3′) and χ^+L260 (5′ TCGAAGG 3′), gave remarkably high Chi hotspot activities—14.1 and 13.2, respectively (Figure 2C; Supplementary Table S3). The disfavored sequences, in χ^+L261 (with flanking 5′ GGGCTTT 3′) and χ^+L262 (5′ GGGCTTC 3′), gave significantly lower Chi hotspot activities—9.5 and 7.5, respectively. As expected, the very high Chi hotspot activity of χ^+L259 requires the Chi octamer 5′ GCTGGTGG 3′: changing it to 5′ GTTGGTGG 3′ reduced the Chi hotspot activity from 14.1 to 2.1. In *recB21* and *recD1013* null mutants no hotspot activity was observed (Supplementary Table S3). We note that the Chi hotspot activities of the χ^+L alleles described earlier were even lower, ranging from 2.8 to 5.3 (Figure 2B), but three of these Chi sites had different flanking sequences both to the right and to the left of the Chi octamer (Supplementary Table S5). We suppose that complex interactions, by rules yet to be worked out, between certain flanking sequences left and right of the Chi octamer produce the Chi hotspot activities as measured in these crosses. Nevertheless, these results show that sequences flanking the Chi octamer strongly affect Chi's activity in cells. The dramatic effects on hotspot activity parallel those reported above with purified enzyme acting with low Mg^{2+} (Figure 6). The collective results indicate that nicking of DNA is the relevant reaction of RecBCD at Chi in cells (see Discussion).

DISCUSSION

DNA context-dependence – a new feature of Chi recombination hotspots

We report here a newly recognized aspect of Chi recombination hotspots—the activity of Chi depends on its local DNA sequence context. A role for nucleotides flanking the Chi octamer (5′ GCTGGTGG 3′) with wild-type RecBCD enzyme has not, to our knowledge, been previously tested by comparing the activity of two Chi sites differing by a limited number of flanking bp (see below for a related example). We tested the effects of the flanking bp and found dramatic, parallel effects on Chi activity both with purified RecBCD enzyme and on RecBCD-dependent phage λ recombination in *E. coli* cells (Figures 2–6). The concordance of the results from these two different methods of analysis provides further evidence that in cells RecBCD simply nicks DNA at Chi, as discussed below. The 3′ flanking sequences had much more effect than those on the 5′ side (Figures 2 and 4; Supplementary Tables S1 and S2). The Chi octamer has been proposed to interact with RecC, including certain amino acids lining one of its tunnels (4,20,22), and we propose that the 3′ flanking sequence interacts with the nuclease domain of RecB, as discussed below. DNA with certain 3′ flanking sequences was up to seven times more active than DNA with other sequences (Figure 4; Supplementary Table S4), showing that the flanking sequence (with Chi) can stimulate RecBCD by a factor as great as that of the Chi octamer itself. Our results force a reanalysis of the activity of Chi sites in promoting recombination and genome evolution.

Molecular basis of Chi recognition and context-dependence

The available evidence indicates that the Chi octamer (5′ GCTGGTGG 3′) is recognized as ss DNA on the strand with 5′ GCTGGTGG 3′ as it passes through a tunnel in RecC (Figure 1 B) (19,21). Presumably, certain amino acids lining that tunnel contact specific bases of the DNA (20,22) and upon the correct fit send a signal to other parts of RecBCD to alter its activity (Figure 1C). Our data indicate that optimal signaling requires more than 5′ GCTGGTGG 3′, in particular nucleotides 4–7 to the 3′ side of this octamer. These nucleotides are about the correct distance from the Chi octamer to extend into the RecB nucle-

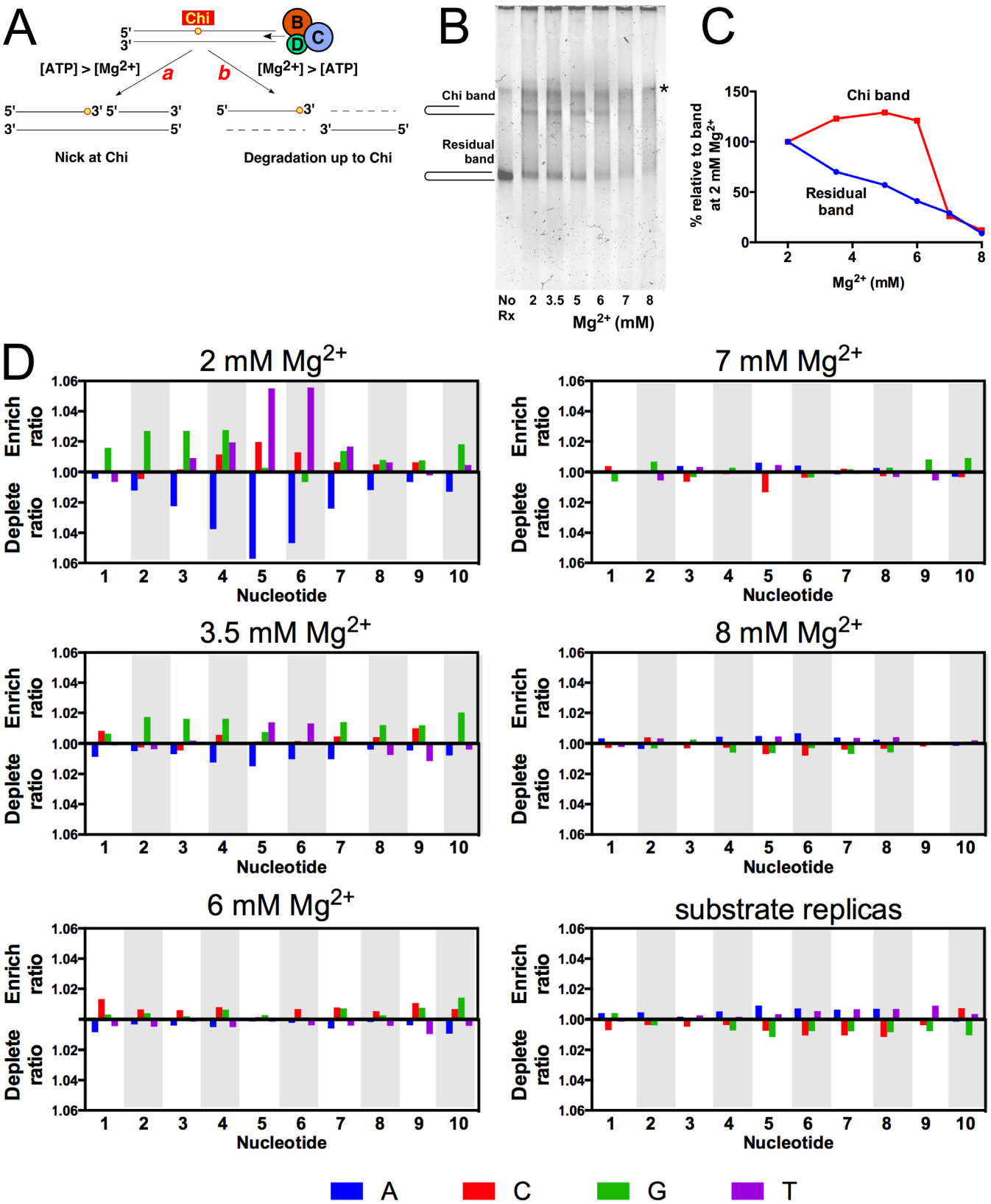

Figure 6. Flanking sequences affect RecBCD's cutting at Chi at low but not at high magnesium ion concentration. (**A**) Diagram of the reaction of RecBCD with Chi-containing DNA with excess ATP (*a*) or excess Mg^{2+} (*b*). See Results for description. From (19). (**B**) Analysis as in Figure 3C of DNA (10 nM) after reaction with RecBCD (1.25–5 nM; see Materials and Methods) at the indicated concentration of $Mg(OAc)_2$ with 5 mM ATP and 1 μM SSB. Note that the band (*) above the Chi band is present in all lanes and may be residual oligonucleotide substrate in this preparation. (**C**) Quantification of the bands in panel B, relative to the yields with 2 mM $Mg(OAc)_2$. (**D**) Data from the residual bands in the reactions in panel B are the enrichment or depletion of the indicated bases to the right of Chi, as in Figure 4B.

ase domain, which in the crystal structure is near the exit of the RecC tunnel (Figure 1B) (20). The preference for certain sequences, such as 5′ AAGG 3′ or 5′ AAAA 3′ (Figure 4; Supplementary Table S4), a few nucleotides to the right (3′ side) of Chi may reflect the ease with which the RecB nuclease can cut this flanking sequence. Indeed, at the two Chi sites analyzed nicking occurs most frequently to the 3′ side of nucleotides 4, 5 and 6 (or 5 and 4) to the right of Chi (3). Since nicking of DNA is essential for completion of the signal transduction, measured as RecA loading (35), we conclude that the nucleotides to the right of Chi are important not just for DNA cutting but also for Chi's control of RecBCD via signal transduction.

Our data showed a significant, but weaker, preference for nucleotides to the left (5′ side) of Chi. The preference was less focused at particular nucleotides than that to the right of Chi (Figure 4). We propose that certain nucleotides, or sequences of nucleotides, slow the progression of RecBCD along the DNA and provide more time for Chi to be recognized and to initiate and complete the signal transduction. The available evidence indicates that the signal transduction is a series of conformational changes in the subunits of RecBCD (27). In particular, upon RecBCD's encountering Chi its nuclease domain appears to swing from the left side of RecC, where it has no access to DNA, to the other side, adjacent to the RecC tunnel's exit for DNA to the right of Chi (Figure 1B). This step may take appreciable time and be reversible but, once taken and the DNA nicked, the remaining changes, including RecA loading, may ensue without reversal. Thus, we envisage that nucleotides to the left of Chi slow RecBCD to enable Chi recognition and nucleotides to the right of Chi allow DNA nicking to complete the transfiguration of RecBCD from a helicase into a RecA-loading machine (as well as helicase).

A precedent for the 3′ flank influencing a Chi-like site is that of the mutant RecBC^{1004}D enzyme, which does not respond to Chi but does respond to a closely related sequence, called χ^* (5′ GCTGGTGCTCG 3′) (4,36). Activation of RecBC^{1004}D requires each of the 3′ nucleotides CTCG (36). χ^* weakly stimulates cutting of DNA and loading of RecA protein by the purified RecBC^{1004}D mutant and wild-type RecBCD enzymes. It increases the plaque and burst sizes of λ *red gam* phage in *recC1004* mutants and in *recBCD*$^+$ cells but, curiously, does not confer detectable recombination hotspot activity in either strain (36,37). Why χ^* lacks hotspot activity is unclear, but the requirement for the 3′ nucleotides may reflect their alteration of the nuclease domain, and thus plaque and burst size, without stimulation of RecA-loading activity required for hotspot activity.

In vitro Mg^{2+}-dependence implies intracellular nicking of DNA by RecBCD enzyme

Two contrasting reactions of purified RecBCD on DNA with Chi have been reported, depending on the reaction conditions (Figure 6A). Most critical is the ratio of Mg^{2+} to ATP, which chelates Mg^{2+} and leaves little free Mg^{2+} to hyper-activate the nuclease unless the concentration of Mg^{2+} exceeds that of ATP. At low Mg^{2+} concentration (i.e. less than the ATP concentration), the enzyme unwinds DNA up to Chi and simply nicks the top strand a few nucleotides to the 3′ side of Chi (3). At high Mg^{2+} concentration (i.e. greater than the ATP concentration), the enzyme intermittently nicks the top strand during unwinding up to Chi, at which it makes its final cut on the top strand; it then cuts the bottom strand at Chi and continues to cut this strand during continued unwinding (31,33,34). Which of these two reactions is more relevant to the action of RecBCD in cells has been controversial, largely because of the uncertainty of the effective intracellular concentrations of Mg^{2+} and ATP (19).

In reactions with purified RecBCD, we found that the preference for sequences flanking the Chi octamer was strong with low Mg^{2+} but was reduced as the Mg^{2+} concentration approached that of ATP (Figure 6D). With Mg^{2+} in excess over ATP no significant preference was detectable. In *E. coli* cells preferred sequences strongly enhanced Chi hotspot activity during recombination (Figure 2). Thus, certain flanking sequences enhance Chi's activity both in cells and with purified RecBCD but only with low Mg^{2+} concentration, a condition in which RecBCD simply nicks at Chi. Therefore, our results strongly indicate that in cells RecBCD nicks DNA at Chi. Numerous comparisons of the action of RecBCD and Chi in cells and with purified components have led to the same conclusion (19).

Implications for Chi's role in genetic recombination and genome evolution

The context-dependence of Chi, shown here, implies that the ∼1000 Chi sites in the *E. coli* genome (9) are not equivalent: some are likely much more active than others. Indeed, we found 31 *E. coli* Chi sites with right flanks (nuc 4–7) of 5′ AAAA, AAAG, AAGG, or AAGA 3′, tetramers that confer the highest Chi activity (Figure 4 and Supplementary Table S4), when only 16 are expected based on random association of the equal-frequency four bases. Thus, *E. coli* may have evolved to have maximal activity of at least some of its Chi sites, but the full spectrum of activities remains to be elucidated. This unknown bears on arguments about the role of Chi in repair of DNA damage, such as that at replication forks (38). For example, it has often been noted that the orientation of Chi on the chromosome is about 3 times more frequent in the direction productive for repair of blocked replication forks than in the opposite direction (9,39,40). The effective factor may be considerably more or less than 3 when the activity of individual Chi sites is taken into account.

After formation of a DSB on one sister chromatid during replication of *E. coli*, RecA protein is bound preferentially at and near Chi sites in the surrounding region of ∼100 kb (41). The amount bound at each Chi decreases with the site's distance from the DSB, showing that in cells RecBCD acts at Chi with less than unit frequency as does purified RecBCD (3,24). We predict that the magnitude of Chi's effect depends on the sequence-context of each Chi site. In addition, the relation between genetic and physical distance is likely to be influenced by the activity of individual Chi sites. Thus, a more complete determination of the rules of Chi's context-dependence, enabled by our results, will deepen our understanding of DNA break repair and recombination in *E. coli*.

Bacterial chromosomes rapidly evolve to acquire immunity by incorporating bits of invading DNA via the CRISPR-Cas machinery (42). It was recently reported that *E. coli* integrates into the CRISPR locus DNA to the 5′ side of Chi less frequently than DNA from the rest of the genome (43). This may be because this disfavored DNA is single-stranded and loaded with RecA protein by RecBCD after acting at Chi. Our data (Figure 6, discussed above) imply that DNA to the 3′ side of Chi (not disfavored) is double-stranded as in Figure 1A (i.e. not degraded), as required for DNA integration by purified Cas1–Cas2 complex (44). We predict that the reduced frequency of pick up to the 5′ side of Chi will be correlated with the 3′ flanking sequence at each Chi site. If so, Chi's flanking sequences may play an important role in immunity acquisition, both in *E. coli* and in other species, as noted next.

Implications of context-dependence of Chi in other bacterial species

DNA sequences with properties related to those of Chi in *E. coli* have been determined for several bacterial species, both Gram-negative and Gram-positive. Chi (5′ GCTGGTGG 3′) confers hotspot activity to λ crosses in Salmonella cells and in *E. coli* cells expressing RecBCD enzyme from numerous enteric species, including Vibrio, Klebsiella, Serratia and Proteus, but not from more distantly related species, such as Pseudomonas (45–47). Thus, Chi (5′ GCTGGTGG 3′) is likely important in DNA break repair and recombination in most or all enteric bacteria, but the importance of the flanking sequences in these species remains to be determined.

In other bacterial species, sequences with some features of Chi have been determined by assaying high molecular weight (HMW) DNA forms of a plasmid that accumulate after the plasmid replication is switched to the rolling circle mode (48–50). HMW DNA formation depends on a short DNA sequence designated 'Chi,' which is different for distantly related species. In the case of *Bacillus subtilis*, the sequence 5′ AGCGG 3′ stimulates both HMW DNA formation and cutting of DNA by purified AddAB (the RecBCD analog) but does not, to our knowledge, stimulate RecA loading by the enzyme (51). We suspect that some or all of these 'Chi' sites would display context-dependence, but this remains to be tested except for a limited analysis suggesting that the *B. subtilis* site is responsive to the 3′ context (52). It should be noted, however, that only the *Lactococcus lactis* sequence (5′ GCGCGTG 3′) has been shown to stimulate recombination (in *E. coli* expressing the *L. lactis* AddAB enzyme) (53), but localized stimulation (i.e. hotspot activity) was not reported. Since the χ^* sequence stimulates formation of HMW DNA in *E. coli* but is not a hotspot of recombination (36,37), as noted above, the sequences called Chi in the non-enteric species may not be crossover hotspot instigators, the definition of Chi (1). Thus, caution is needed in extending the properties of Chi to these sequences.

Context-dependence of chromosomal sites appears widespread

The activity of Chi is determined both by its local nucleotide sequence context, as shown here, and by its longer-range context in the genome—a Chi site in λ is active only if it is properly oriented with respect to the *cos* site from which DNA packaging is initiated (54). This interaction can extend over half the chromosome length, or >25 kb, and stems from RecBCD initiating unwinding only at the right end of the λ chromosome, the Chi sequence being asymmetric, and only one strand being recognized (10,13,16,54). Similar local and long-distance context effects are exhibited by other chromosomal sites noted below.

A dramatic case of local context-dependence of a chromosomal site unrelated to Chi is provided by the sites cleaved by restriction enzymes. For example, the canonical recognition sequence for EcoRI is 5′ GAATTC 3′, but the five sites in λ DNA are cleaved at rates covering a ten-fold range for the wild-type enzyme and up to a 100-fold range for certain EcoRI mutants (55,56). The flanking three bp, and perhaps more, are critical in determining the rate of cleavage of the canonical sequence in different contexts (56). Similar context-dependences are seen for many other restriction enzymes (refs. cited in (56)).

Each of the classes of chromosomal sites noted in the Introduction is subject to context-dependence, either local (i.e. flanking bp) or more distant (e.g. sequences up to 100 kb or more). For example, in *Saccharomyces cerevisiae* origins of replication can change from late-firing to early-firing when placed in a different chromosomal context (57). Promoters of transcription often depend on distant enhancer sequences and change activity when moved (58). In *Schizosaccharomyces pombe* the meiotic recombination hotspot *M26* was initially deduced to be determined by 5′ ATGACGT 3′ (59) and bound by the transcription factor Atf1-Pcr1 (60). Later, it was shown that nucleotides as far as seven bp to the side of this sequence can influence the hotspot activity of *M26* (61,62). Furthermore, the activity is often lost when a several kb-long DNA fragment with *M26* near its middle is moved to another chromosomal locus (63,64). Thus, the *M26* hotspot is subject to the same types of context-dependence as we report here for Chi hotspots. Chromosomes very likely have evolved to optimize the activities of their various parts, which can overlap and result in context-dependence.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We are grateful to Lynn Thomason and Don Court for valuable advice and materials; Knox Young for generating new Chi alleles; Bonny Brewer, Kyle Fowler, Steve Hahn, Randy Hyppa, Lise Raleigh and Sarah Zanders for helpful discussions; and Meriem El Karoui, David Leach, Mridula Nambiar, and Walt Steiner for helpful comments on the manuscript. This research was supported by grant GM031693 from the National Institute of General Medical Sciences.

FUNDING

National Institutes of Health (NIH) of the United States of America [GM031693 to G.R.S.]. Funding for open access charge: NIH [GM031693].
Conflict of interest statement. None declared.

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