Chi-Dependent Intramolecular Recombination in *Escherichia coli*

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ABSTRACT

Homologous recombination in *Escherichia coli* is enhanced by a *cis*-acting octamer sequence named Chi (5′-GCTGGTGG-3′) that interacts with RecBCD. To gain insight into the mechanism of Chi-enhanced recombination, we recruited an experimental system that permits physical monitoring of intramolecular recombination by linear substrates released by *in vivo* restriction from infecting chimera phage. Recombination of the released substrates depended on *recA*, *recBCD* and *cis*-acting Chi octamers. Recombination proficiency was lowered by a *xonA* mutation and by mutations that inactivated the RuvABC and RecG resolution enzymes. Activity of Chi sites was influenced by their locations and by the number of Chi octamers at each site. A single Chi site stimulated recombination, but a combination of Chi sites on the two homologs was synergistic. These data suggest a role for Chi at both ends of the linear substrate. Chi was lost in all recombinational exchanges stimulated by a single Chi site. Exchanges in substrates with Chi sites on both homologs occurred in the interval between the sites as well as in the flanking interval. These observations suggest that the generation of circular products by intramolecular recombination involves Chi-dependent processing of one end by RecBCD and pairing of the processed end with its duplex homolog.

GENETIC recombination in wild-type *Escherichia coli* cells requires RecA, RecBCD, SSB and enzymes that resolve recombination intermediates (Clark and Margulies 1965; Howard-Flanders and Theriot 1966; Emmerson 1968; Glassberg *et al.* 1979; Lloyd 1991). Recombination is stimulated by a *cis*-acting octamer (5′-GCTGGTGG-3′) named Chi, which interacts with RecBCD in an orientation-dependent manner (reviewed in Smith *et al.* 1984; Myers and Stahl 1994). RecA catalyzes pairing of either 3′- or 5′-ending single-stranded DNA (ssDNA) with homologous double-stranded DNA (dsDNA) resulting in reciprocal strand exchange (reviewed in West 1992; Kowalczykowski *et al.* 1994). RecBCD is a multifunctional heterotrimer, encoded by the *recB*, *recC* and *recD* genes. *In vitro*, it loads at DNA double-stranded ends and promotes DNA-dependent ATPase and ATP-dependent helicase activities. Its helicase activity is frequently associated with exonuclease and endonuclease activities (reviewed in West 1992; Kowalczykowski *et al.* 1994).

Current hypotheses assume a role for Chi and RecBCD in the generation of ssDNA substrates for the homologous pairing reaction. This proposition is supported by *in vitro* studies that demonstrate a change in recBCD activity upon interaction with Chi (Taylor *et al.* 1985; Taylor and Smith 1992; Dixon and Kowalczykowski 1993; Anderson and Kowalczykowski 1997). Both Chi-dependent and Chi-independent RecBCD activities are greatly influenced by assay conditions. Depending on the molar ratio of ATP to Mg^{2+}, RecBCD activity at Chi may generate a nick at the 3′ side of the sequence (as written above), attenuate RecBCD exonuclease activity or switch the polarity of its exonuclease activity from degrading the 3′-ending strand to degrading the 5′-ending strand (Dixon and Kowalczykowski 1995; Taylor and Smith 1995; Anderson and Kowalczykowski 1997).

The mode of RecBCD function and the nature of the homologous pairing reaction that prevails *in vivo* are not known, and alternative models postulate different mechanisms for the presynaptic stage of recombination (Thaler *et al.* 1988; Rosenberg and Hastings 1991; Myers and Stahl 1994; Dixon and Kowalczykowski 1995; Taylor and Smith 1995; Anderson and Kowalczykowski 1997). In an attempt to resolve this problem and test predictions of the alternative models we recruited an experimental system that facilitates efficient and synchronous release of linear recombination substrates within *E. coli* cells (Nussbaum *et al.* 1992; Silberstein *et al.* 1993). We employed this system for physical monitoring of the recombining molecules and structural analysis of primary products. In this paper we report on the genetic requirements of intramolecular recombination by the linear substrates and the dependence of this mode of recombination on Chi elements and DNA double-stranded breaks. We also determined the distribution of recombination

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events along the homology and physically monitored the fate of the recombining substrates and the accumulation of the recombination products.

MATERIALS AND METHODS

Bacterial strains and growth conditions: *E. coli* strains (Table 1) were grown on LB medium (Luria and Burrous 1957). Plasmid-carrying strains were grown in media supplemented with the appropriate antibiotics (100 μg of ampicillin per milliliter, 20 μg of kanamycin per milliliter). Unless otherwise indicated, infected cultures harbored pMB4 (Betlach *et al.* 1976). Infection protocol and growth conditions for cultures infected by phage precursors of the linear recombination substrates were as described previously (Silberstein *et al.* 1993).

Chimera phage and plasmids: Chimera phage with intramolecular recombination substrates cloned between *Eco*RI sites (Table 2) were constructed by ligating linearized substrates to λEMBL4 (Frischauf *et al.* 1983) phage DNA arms as described previously (Silberstein *et al.* 1993). The orientation of the cloned substrate in the phage vector was verified by restriction analysis with *Hin*dIII endonuclease. Where indicated, oligomers containing one to three Chi octamers were inserted into the *Xho*I (χ*Xho*) or *Bcl*I (χ*Bcl*) sites on the recombination substrate (see Figure 1) or into both of them. The Chi octamers at χ*Xho* and χ*Bcl* were inserted into the *luxA luxB* fragment of pAP601 (Nussbaum and Cohen 1988) and pMS804 (Silberstein *et al.* 1993), respectively, and the respective fragments were assembled into intramolecular recombination substrates as described previously (Silberstein *et al.* 1993). To insert a single Chi octamer into the *Xho*I or *Bcl*I sites, complementary oligomers (A and B in Table 3) containing a Chi octamer, a *Not*I restriction site and the appropriate ssDNA overhangs were synthesized, annealed and ligated into the respective site. To insert a second Chi octamer, oligomers containing *Kpn*I and *Pst*I restriction sites, a Chi octamer and *Not*I ssDNA overhangs (C in Table 3) were inserted into the *Not*I site of the plasmids containing the first Chi octamer. The third Chi octamer was inserted into the cloned *Kpn*I site, using an oligomer containing a *Bam*HI restriction site, two Chi octamers in opposite orientations and *Kpn*I ssDNA overhangs. Chi octamers in all substrates depicted in Table 2 were in the functional orientation with respect to the adjacent *Eco*RI site on the chimera phage. The sequence and orientation of Chi in substrates containing a single Chi octamer were verified by sequence analysis. Chi orientation in all substrates was verified by PCR analysis (Innis *et al.* 1990).

In experiments designed for the determination of recombinant frequency and isolation of plasmid recombination products, substrates with pACYC184 (Chang and Cohen 1978) origin of replication were used (Ori^+ substrates). For physical monitoring of recombination by Southern blot hybridization, substrates devoid of a replication origin (Ori^- substrates) were used. All phage harboring Ori^+ substrates were isogenic to λMS805 and all phage harboring Ori^- substrates were isogenic to λZS820 (Silberstein *et al.* 1993). Ori^- substrates were derived from the appropriate plasmids and inserted into the phage vector by the methodology described previously (Silberstein *et al.* 1993). Maintenance and propagation of chimera phages harboring intramolecular recombination substrates were described previously (Silberstein *et al.* 1993). The cloned fragment in λRF936 has a single copy of the *luxA luxB* genes. λRF936 was constructed by

TABLE 1

***E. coli* strains used**

Strain	Relevant genotype[a]	Reference or source
AB1157	*rec*$^+$	Bachmann 1987
JC2926	*recA13*	A. J. Clark
JC5519	*recB21 recC22*	Willetts and Clark 1969
JC15502	*recD1009*	A. J. Clark
BT125	*recJ 284::*Tn*10*	Rinken *et al.* 1992
WA818	*xonA2*	Rinken *et al.* 1992
WA820	*xonA2 recJ284::*Tn*10*	Rinken *et al.* 1992
CS85	*ruvC53 eda51::*Tn*10*	Shurvinton *et al.* 1984
N2731	*recG258::kan*	Lloyd 1991
N3398	*recG258::kan* *ruvC53 eda51::*Tn*10*	R. G. Lloyd
AC224	*recB21 recC22 sbcA23* [λ(ind$^-$)]	Silberstein *et al.* 1993
AC227	*rec*$^+$ [λ(ind$^-$)]	λ(ind$^-$) lysogen of AB1157
AC258	*recD1009* [λ(ind$^-$)]	λ(ind$^-$) lysogen of JC15502
AC259	*recJ 284::*Tn*10* [λ(ind$^-$)]	λ(ind$^-$) lysogen of BT122
AC260	*xonA2 recJ284::*Tn*10* [λ(ind$^-$)]	λ(ind$^-$) lysogen of WA820
AC267	*xonA2* [λ(ind$^-$)]	λ(ind$^-$) lysogen of wA818
AC275	*recB21 recC22* [λ(ind$^-$)]	λ(ind$^-$) lysogen of JC5519
AC278	*recA13* [λ(ind$^-$)]	λ(ind$^-$) lysogen of JC2926
AC302	*ruvC53 eda51::*Tn*10* [λ(ind$^-$)]	λ(ind$^-$) lysogen of CS85
AC306	*recG258::kan* *ruvC53 eda51::*Tn*10* [λ(ind$^-$)]	λ(ind$^-$) lysogen of N3398
AC307	*recG258::kan* [λ(ind$^-$)]	λ(ind$^-$) lysogen of N2731

[a] All strains are isogenic derivatives of AB1157. Other markers are *thi-1 his-4* Δ(*gpt-proA*)*62 argE3 thr-1 leuB6 kdgK51 ara14 lacY1 galK2 xyl5 mtl-1 tsx-33 supE44 rpsL31*.

TABLE 2

Chimera phage harboring intramolecular recombination substrates

Phage	$Ori_{pACYC184}$[a]	Chi at χ*Xho*[b]	Chi at χ*Bcl*[b]	Reference or source
λMS805	+	0	0	Silberstein *et al.* 1993
λRF918	+	1	0	This work
λRF919	+	0	1	This work
λRF919[c]	+	0	1	This work
λRF915	+	1	1	This work
λRF944	+	2	0	This work
λRF943	+	0	2	This work
λRF943[c]	+	0	2	This work
λRF942	+	2	2	This work
λRF950	+	3	0	This work
λRF949	+	0	3	This work
λRF951	+	3	3	This work
λMS820	−	0	0	Silberstein *et al.* 1993
λRF920	−	1	1	This work
λRF946	−	2	2	This work
λRF953	−	3	3	This work

All phages harboring Ori^+ substrates are isogenic derivatives of λMS805 and all phages harboring Ori^- substrates are isogenic derivatives of λZS820. The cloned recombination substrate in all phages except for λRF919a and λRF943a were in the same orientation, with the χ*Xho* site at a distance of 837 nucleotides from the 21.5 kb λEMBL4 arm (see Figure 1).

[a] Indicate the presence (+) or absence (−) of pACYC184 replication origin on the cloned substrate.

[b] Indicate the number of Chi octamers at the insertion site (Figure 1).

[c] λRF919a and λRF943a harbor the same substrates as λRF919 and λRF943, respectively, except that the orientation of the cloned substrate in λRF919a and λRF943a was inverted to that in λRF919 and λRF943.

deleting pACYC184 replication origin from pRF934 and ligating the linear *Eco*RI fragment to λEMBL4 arms as described (Silberstein *et al.* 1993). pRF934 is an isogenic derivative of pMS804 (Silberstein *et al.* 1993) except that it contains a single *Eco*RI restriction site in the inactivated *Nde*I site of the *luxA* gene as in pZS824 (Silberstein *et al.* 1995) and Chi octamer triplets at the *Xho*I and *Bcl*I sites. λAN612 has been described previously (Nussbaum *et al.* 1992).

Determination of recombinant frequency and structural analysis of plasmid recombination products: To select for Kan^R recombinants, cells were infected by the appropriate chimera phage, and samples taken at 120 min following infection were plated on kanamycin-supplemented medium as described (Nussbaum *et al.* 1992). To minimize the occurrence of intermolecular recombination events or multiple recombination events within the same infected cell, the multiplicity of infection (MOI) was 0.2. Recombinant frequency was defined as the ratio of Kan^R recombinants per infected cell. Chi activity was defined as the recombinant frequency in cultures infected by phage harboring Chi^+ substrates divided by the frequency in cultures infected by phage harboring Chi^0 substrates. To determine the distribution of recombinational exchange along the homology, isolated colonies were inoculated into kanamycin-supplemented liquid medium. Plasmid DNA preparations of overnight cultures were made by the rapid boiling method (Holmes and Quigley 1981) and subjected to restriction endonuclease analysis using *Bgl*II, *Hin*dIII, and a combination of *Not*I and *Sal*I endonucleases (see Figure 1 for the location of the corresponding restriction sites). Recombinant frequency at each genetic interval was calculated by multiplying total recombinant frequency by the fraction of recombinational exchange occurring within the interval. Recombinant frequency at each interval was divided by the physical length of the interval and is presented as "recombinant frequency/bp."

Physical monitoring of recombination: Cells were infected at an MOI of 2. Total DNA preparations of samples taken at the indicated times following infection were digested by *Sal*I endonuclease and subjected to Southern blot hybridization as described (Silberstein *et al.* 1993).

RESULTS

Chi-dependent recombination: Linear intramolecular recombination substrates were cloned between *Eco*RI sites on phage λ vectors, delivered by infection into *E. coli* cells, lysogenic for λ(ind−) and released from the chimera phage by *in vivo* restriction (Nussbaum *et al.* 1992). The cloned 13.6 kb substrate (Figure 1) had a direct terminal repeat of 3.5 kb, consisting of *Vibrio fischeri luxA luxB* genes, with each copy of the repeated sequence mutated at a different site (Nussbaum and Cohen 1988). An intramolecular recombinational exchange within these substrates would yield circular products with a single copy of the *luxA luxB* genes. To facilitate selection for infected cells harboring circular recombination products, the linear substrates also contained a pACYC184 origin of replication and a *kan* gene (Figure 1). To investigate the dependence of intramolecular recombination on Chi elements, oligomers containing one to three Chi octamers in the functional orientation with respect to the *Eco*RI-induced break (Stahl *et al.* 1980), were inserted into either *Xho*I, *Bcl*II or both sites. These sites are located at a distance of 837 and 1565 nucleotides from the *Eco*RI-induced DNA break,

TABLE 3

Oligomers used for substrate construction

Oligomer	Sequence
A	TCGAGCGGCCGCCCACCAGC
	CGCCGGCGGGTGGTCGAGCT
B	GATCGCTGGTGGGCGGCCGC
	CGACCACCCGCCGCGCTAG
C	GGCCGCGTGCAGGGTACCGCTGGTGGGC
	CGCACGTCCCATGGCGACCACCCGCCGG
D	CGCTGGTGGGGATCCCCACCAGCGGTAC
	CATGCGACCACCCCTAGGGGTGGTCGC
E[a]	*Not*I *Pst*I *Kpn*I *BamH*I GCTGGTGGGCGGCCGCTGCAGGTACCGGCTGGTGGGGATCC CGACCACCCGCCGGCGACGTCCATGGCGACCACCCCTAGG CCACCAGCGGTACCGCTGGTGGGCGGCCGC GGTGGTCGCCATGGCGACCACCCGCCGGCG *Kpn*I *Not*I

Oligomers A and B were used to insert the first Chi octamer into the *Xho*I and *Bcl*I sites, respectively (Figure 1). Insertion of oligomer C into the cloned *Not*I site of oligomers A and B generated substrates with two Chi octamers. The third Chi octamer was inserted with oligomer D into the cloned *Kpn*I site on oligomer C.

[a] The inserted oligomer contains three Chi octamers in the functional orientation and one Chi octamer in the opposite orientation. Restriction sites and Chi octamers are indicated by vertical and horizontal arrows, respectively.

respectively (Figure 1). The effect of these insertions on recombinant frequency was determined by scoring Kan^+ cells in cultures infected by the respective chimera phage. Infection of wild-type *E. coli* cells by chimera phage harboring substrates devoid of Chi octamers (Chi^0 substrates) did not yield a detectable number of Kan^+ recombinants ($<1 \times 10^{-5}$ recombinants/infected cell). This low frequency is compared with recombinant frequencies of about 6% and 15% in infected *recB recC sbcA* and *recD* mutants, respectively (Table 4). Insertion of Chi octamers at either one of the two sites, or at both of them, led to an increase in recombinant frequency (Figure 2). A maximal recombinant frequency of 10% was achieved with substrates having three Chi octamers at each site. Recombination also depended on *Eco*RI-induced DNA breaks. The frequency of Kan^+ recombinants in infected cells that did not express *Eco*RI was 1000-fold lower than that in infected cells that expressed *Eco*RI from a resident pMB4 plasmid (Table 4).

Presumably, a Chi site at one end is sufficient to promote RecBCD-mediated generation of a recombinogenic single-stranded end, and this end may pair with the duplex homolog. However, with the substrate depicted in Figure 1, Chi may have a role at both ends: if pairing is by strand invasion, Chi may stabilize the noninvading end by modulating RecBCD exonuclease activity. Furthermore, with these substrates, a circular product may also be generated by a single-strand annealing (SSA) mechanism that depends on processing of both ends (Silberstein *et al.* 1993). To test whether a Chi site on both ends is required, we determined recombinant frequencies with sets of substrates having Chi octamers at a single site (substrates χ*Xho* and χ*Bcl*) or at both sites (substrates χ*XhoBcl*) (Figure 2). Insertion of Chi octamers at either one of the two sites enhanced recombination, and recombinant frequency increased with the number of Chi octamers at the site. Chi octamers at one site (χ*Xho*) gave a higher recombinant frequency than at the other site (χ*Bcl*). Recombinant frequencies of substrates having Chi octamers at both sites (χ*XhoBcl*) were consistently higher than the sum of the frequencies by substrates having Chi octamers at a single site (χ*Xho* + χ*Bcl*). Thus, a single Chi site was sufficient to stimulate intramolecular recombination, but a combination of two Chi sites was synergistic. These observations, the distribution of recombinational exchanges (see Figure 4), and the dependence on Holliday junction resolution systems (see Figure 6D) argue against an SSA recombination mechanism (see discussion). Assuming that pairing is by strand invasion, these observations suggest that Chi elements play a role both at the invading and at the noninvading ends.

We considered the possibility that the difference in Chi activity between substrates having Chi octamers at χ*Xho* and χ*Bcl* may be related to the orientation of the cloned substrate in the chimeric phage. This may have

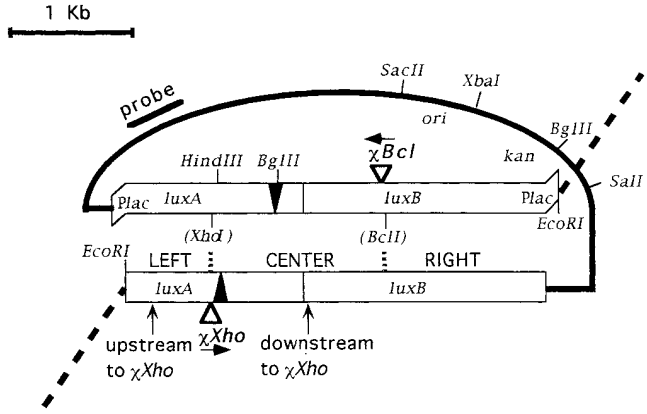

Figure 1.—A substrate prototype for Chi-dependent intramolecular recombination. *In vivo* restriction of the infecting phage DNA by *Eco*RI releases a linear fragment with a direct terminal repeat. For determination of recombinant frequency and the distribution of recombinational exchanges, fragments with pACYC184 origin of replication (*ori*) were used. For physical monitoring of recombination, the *Sac*II-*Xba*I fragment containing the plasmid replication origin was deleted. The location of Chi insertion sites (χ*Xho* and χ*Bcl*), relevant restriction sites and genes are indicated. Homology is designated by parallel boxes, phage λ arms (not to scale) by broken lines and mutations in the *luxA luxB* genes by triangles. The homology to the probe used in the hybridization experiments (probe) and the genetic intervals defined by the Chi sites (left, center, right) are indicated. The orientation of the inserted Chi octamers (5′-GCTGGTGG-3′) is indicated by 3′ to 5′ arrows; sequences located "upstream" and "downstream" of Chi are defined here and elsewhere in the paper as sequences on the 3′ and the 5′ sides of the Chi octamer (5′-GCTGGTGG-3′), respectively.

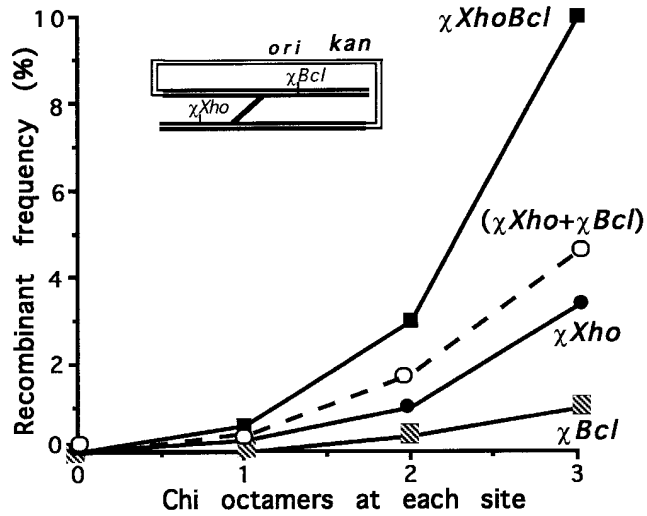

Figure 2.—Dependence of recombinant frequency on the location of Chi sites and the number of Chi octamers at each site. A derivative of AC227 (*rec*$^+$), containing pMB4 (*Eco*RI$^+$) was infected by chimera phage, harboring intramolecular recombination substrates with the indicated number of Chi octamers at χ*Xho* (●), χ*Bcl* (▨), or at both insertion sites (■). The frequency of Kan$^+$ recombinants was determined as described in materials and methods. The sum of the frequencies by substrates having Chi octamers at a single site (○, χ*Xho* + χ*Bcl*) is presented.

occurred if sequences on the λ phage vector, in substrates generated by partial restriction, contributed in any way to recombinant formation. To check for this possibility, we cloned substrates with a single and a double Chi octamer at the χ*Bcl* site in the λ vector in both orientations (Table 2) and compared recombinant frequencies in cultures infected by the two sets of chimeric phages (Table 5). Recombinant frequency was not affected by the orientation of the substrate in the vector. In both orientations, a single Chi octamer at χ*Bcl* had no detectable activity, and the activity of two Chi octamers at χ*Bcl* was similar to that of a single Chi octamer at χ*Xho*. We note that while a single Chi octamer at χ*Bcl* had no activity, it enhanced recombination when combined with a single Chi octamer at χ*Xho*.

A single DNA molecule, released *in vivo* by *Eco*RI restriction, can presumably undergo intramolecular recombination to yield a circular product. To test whether the release of a single molecule is sufficient for recombinant production, we determined the dependence of recombinant production on MOI (Figure 3). It is clear that the observed dependence on MOI is better approximated by the theoretical curve corresponding to a single hit being sufficient for recombination than that requiring at least two hits.

Genetic requirements: The RecBCD pathway, as defined in transduction and conjugational recombination, depends on functional *recA* and *recBC* genes (Clark 1973). To test whether our recombination system has similar requirements, we infected the appropriate mutants by the chimeric phage precursor of the Chi$^+$ substrate and scored Kan$^+$ recombinants. Kan$^+$ recombinants were not detected in infected *recA* or *recBC* mutants ($<10^{-5}$ recombinants/infected cell) (Ta-

TABLE 4

The effect of Chi octamers and host genotype on recombinant frequency

Host genotype	Chi[a]	*n*[b]	Recombinant frequency (%)[c]
Wild type	0	4	<0.001
recD	0	4	15.2 ± 0.35[d]
recD	+	4	17.5 ± 0.57
recB recC sbcA	0	3	5.8 ± 0.6
Wild type	+	6	9.9 ± 0.6
recA	+	2	<0.001
recB recC	+	2	<0.001
Wild type (*Eco*RI$^-$)	+	2	0.01

[a] All Chi$^+$ substrates had three Chi octamers in the functional orientation at each insertion site.

[b] *n* represents number of experiments.

[c] Recombinant frequency is the number of Kan$^+$ recombinants per infected cell.

[d] Plus/minus represents standard deviation.

TABLE 5

The orientation of the cloned substrate in the chimera phage does not affect recombinant frequency

Infecting phage[a]	Chi at *χXho*	Chi at *χBcl*	*n*[b]	Recombinant frequency (%)[c]
λRF918	1	0	5	0.25 ± 0.05[d]
λRF919	0	1	4	<0.001
λRF919a	0	1	4	<0.001
λRF915	1	1	5	0.67 ± 0.1
λRF944	2	0	5	0.83 ± 0.1
λRF943	0	2	4	0.33 ± 0.05
λRF943a	0	2	4	0.28 ± 0.025
λRF942	2	2	4	2.95 ± 0.18

[a] λRF919a and λRF943a harbor the same substances as λRF919 and λRF943, respectively, except that the orientation of the cloned substrate in λRF919a and λRF943a was inverted to that in λRF919 and λRF943.

[b] *n* represents number of experiments.

[c] Recombinant frequency is the number of Kan$^+$ recombinants per infected cell.

[d] Plus/minus represents standard deviation.

ble 4). The 10^4-fold decrease in recombination proficiency caused by the *recBC* mutations was more severe than that in conjugation or transduction in which recombination proficiency of *recBC* mutants is 0.1–1.0% of that in *recBC*$^+$ cells (Clark 1973; Mahajan 1988). As expected, production of Kan$^+$ recombinants in *recD* mutants was independent of the presence of Chi octamers on the substrate (Table 4). The effect of host genotype on recombination kinetics is presented below (see Figure 6).

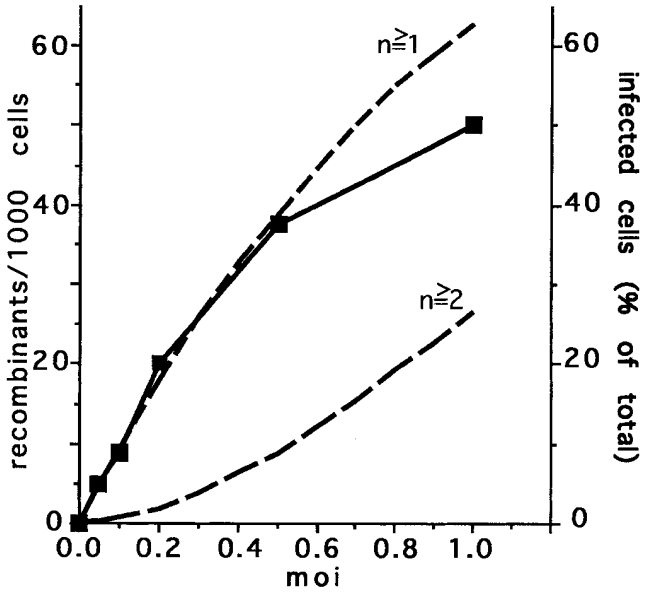

Figure 3.—Dependence of recombinant production on MOI. A derivative of AC227 containing pMB4 was infected at the indicated MOI by chimera phage harboring intramolecular recombination substrates with three Chi octamers at each insertion site (λRF951) and the number of Kan$^+$ recombinants per 1000 cells was determined (■). Theoretical curves (– – –) representing the percentage of cells infected by one or more ($n \geqq 1$) or by two or more ($n \geqq 2$) phage particles are presented as a reference. These theoretical curves were computed under the assumption that the number of phage DNA molecules entering a cell follows a Poisson distribution.

The distribution of Chi-stimulated recombinational exchanges: The distribution of recombinational exchanges in substrates with Chi octamers inserted at either *χXho*, *χBcl* or both sites (see Figure 1) was investigated. Kan$^+$ clones were selected by plating infected cultures on kanamycin-supplemented medium, and *kan* plasmids of the selected clones were subjected to restriction analysis. All plasmid products, generated by intramolecular recombination of linear substrates with Chi octamers at a single site, lost the Chi octamers in recombination (100 independent plasmids products analyzed). This loss of Chi is consistent with models that postulate that Chi stimulates recombination downstream of its 5′ end (Thaler *et al.* 1988). However, it does not contradict the finding that some Chi-mediated exchanges in phage λ crosses are upstream to the 3′ end of Chi (Cheng and Smith 1989), since in the experiments reported here, Chi was inserted within heterologies of 30–76 nucleotides. These short heterologies may have prevented recombinational exchanges at the 3′ end of Chi.

The distribution of genetic exchanges in substrates with Chi octamers at both the *χXho* and *χBcl* sites was investigated. The two Chi sites define three genetic intervals: a central interval between the Chi insertion sites and two flanking intervals between each site and the adjacent end (see Figure 1). Restriction analysis of plasmid recombination products indicated that genetic exchanges in substrates with Chi octamers at both sites occurred in all three intervals (Figure 4). Exchange frequencies in the central interval were higher than those in the flanking intervals. This distribution of recombination events is different from that in *recD* mutants (insert in Figure 4), where similar frequencies were measured in all three intervals.

Physical monitoring of recombination: In an attempt to follow the fate of linear recombination substrates, we cloned substrates devoid of plasmid replication origins in phage λ vectors and delivered them by infection into λ(ind$^-$) lysogens expressing *Eco*RI from a resident plasmid (pMB4). Total cellular DNA preparations of samples taken at time intervals after infection were digested by *Sal*I endonuclease and analyzed by Southern blot hybridization as described previously (Silberstein *et al.* 1993; Figure 5). Three fragments with homology to the probe (see Figure 1) were observed in samples taken immediately following infection. These fragments corresponded in molecular length to the expected *Sal*I products of linear phage DNA that escaped *Eco*RI restriction (lower phage band), circular phage DNA (upper phage band) and *Eco*RI-restricted phage DNA (substrate).

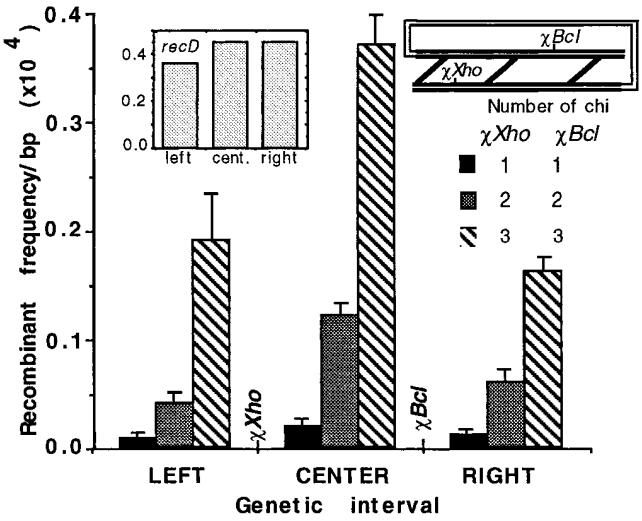

Figure 4.—The distribution of recombinational exchange along the homology. Substrates having one to three Chi octamers at each site were delivered to lysogenic cells as described. Recombinant frequencies were determined by scoring Kan^R cells in the infected cultures, and the distribution of recombinational exchanges along the homology was determined by restriction analysis of plasmid recombination products. Exchange frequencies at each interval were normalized for the length of the interval. Error bars represent standard deviation. The distribution of exchange frequencies along the homology in *recD* mutants (inset) is presented as a reference.

Incomplete restriction by the endogenous *Eco*RI endonuclease would generate two molecular species. Digestion of these products by *Sal*I endonuclease resulted in two molecules that are identical to the *Sal*I products of linear and fully restricted phage DNA, respectively.

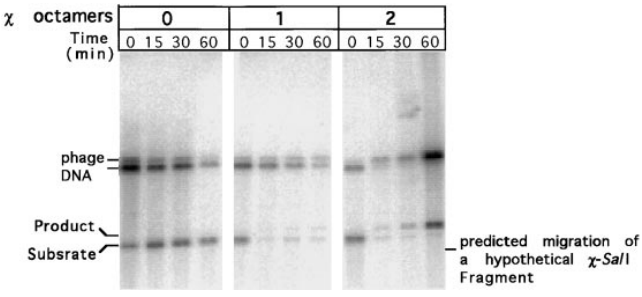

Figure 5.—Physical monitoring of Chi-dependent intramolecular recombination. Substrates were released from infecting chimera phage by *in vivo* restriction, and samples were taken at the indicated times after infection. *Sal*I-digested total cellular DNA preparations were subjected to Southern blot hybridization with the probe depicted in Figure 1. The number of Chi octamers at each site on the recombining substrates (Figure 1) is indicated. The locations of *Sal*I digestion products of circular and linear phage DNA (upper and lower λ DNA bands, respectively), *Eco*RI-restricted phage DNA (substrate) and the circular recombination product (product) are indicated. The predicted migration of a *Sal*I product of a hypothetical intermediate (a *Sal*I-*χXho* fragment) is indicated.

Recombination products were not detected by hybridization in cells infected by phage harboring Chi^0 substrates at any timepoint. Insertion of Chi octamers at the *χXho* and *χBcl* sites led to the generation of circular recombination products in the infected cells. These circular products were first detected 15 min following infection, and their amount increased thereafter (Figure 6A). Increasing the number of Chi octamers at each site from one to two enhanced the rate of circular products formation. However, a third Chi octamer at each site did not affect recombination kinetics (Figure 6A). Consistent with results presented in Table 4, the generation of circular products depended on *in vivo* restriction by *Eco*RI and on functional *recA* and *recB recC* genes. Recombination in *recD* mutants was independent of Chi and occurred at a higher rate than in $recD^+$ cells (Figure 6B).

Another class of enzymes that affect recombinant frequency in some systems are ssDNA-specific exonucleases (Feng and Hays 1995; Miesel and Roth 1996; Razavy *et al.* 1996). *xonA* and *recJ* products degrade ss-DNA from the 3′ and the 5′ ends, respectively. Recombination rate was lowered by a *xonA* mutation but was not affected by a *recJ* mutation. Recombination rate in *xonA recJ* double mutants was consistently lower than that in single *xonA* mutants (four independent experiments). Unlike recombination in $recD^+$ cells, recombination in *recD* mutants depended on *recJ* activity (R. Friedman-Ohana, unpublished results).

The linear substrates used in this study may generate circular products by either one of two recombination mechanisms: One involves strand exchange and the other SSA (reviewed in Kolodner *et al.* 1994; Eggleston and West 1996). Unlike the strand exchange pathway, the SSA pathway does not generate Holliday junction intermediates and therefore should not depend on their enzymatic resolution. To test the dependence on proteins that resolve Holliday junctions, we determined recombination kinetics in *recG* and *ruvC* mutants. Single *recG* or *ruvC* mutations had a small effect on recombination proficiency, but a double *recG ruvC* lowered the recombination rate by about 10-fold (Figure 7C). This result is consistent with a mechanism that involves generation and resolution of Holliday intermediates. The residual activity in *ruvC recG* double mutants may indicate that a small fraction of the recombination events occur via a pathway that does not depend on the formation and resolution of Holliday junctions. Alternatively, Holliday junctions were formed, but resolved by a mechanism that is independent of RecG and RuvABC.

We did not observe molecular intermediates predicted by models that postulate attenuation of RecBCD exonuclease activity at Chi. Possible reasons for our inability to detect these putative intermediates are discussed below.

The longevity of the linear substrates in the infected cells was monitored by hybridization, as seen in Figure

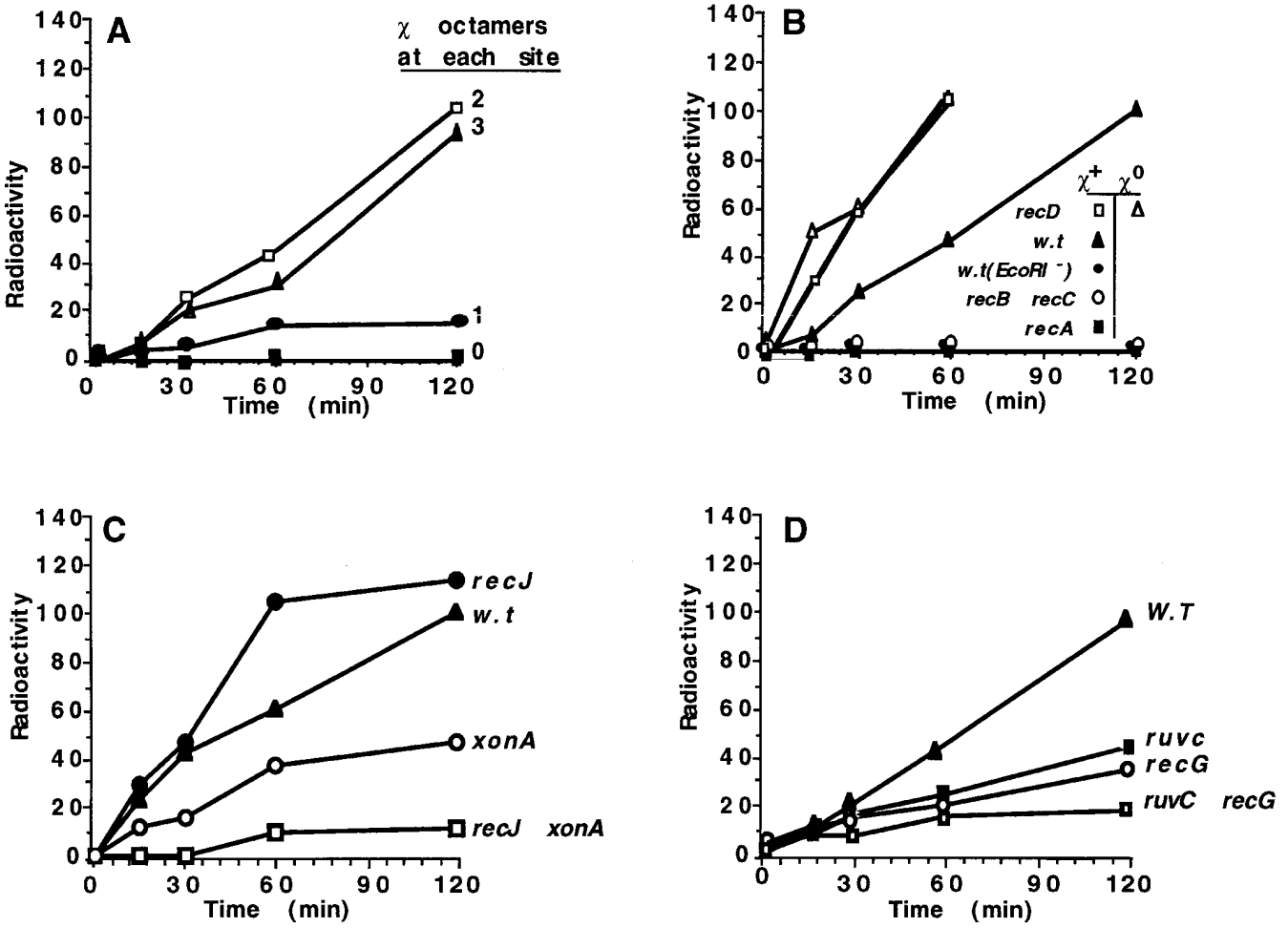

Figure 6.—The effect of the number of Chi octamers (A) and host genotype (B–D) on recombination kinetics. The kinetics of product formation in infected wild-type cells and in the indicated mutants was determined by phosphoimaging analysis of Southern blot hybridization experiments like the ones depicted in Figure 5 and is expressed as arbitrary units of radioactivity. The experiments in (A) were with wild-type cells (AC227), and the number of Chi octamers at each site on the delivered substrate is indicated. Substrates with three Chi octamers at each site (RF953) were used in most experiments depicted in (B) to (D). A phage harboring a Chi^0 (χ^0) substrate (λZS820) was used where indicated. Except where indicated, infected cells harbored pMB4. To monitor recombination in cells that do not express *Eco*RI, the infected cells harbored pBR322 (wild-type *Eco*RI^-). The effect on recombination kinetics of *recA* and *recBCD* mutations and the dependence on *in vivo* restriction is depicted in (B). The effect of mutations inactivating single-strand-specific exonucleases (*xonA* and *recJ*) on recombination kinetics is depicted in (C) and the effect of mutations that inactivate Holliday junction resolution systems (*recG* and *ruvC*) is depicted in (D). Experimental procedure is described in text.

5. The density of the substrate band decreased with time (Figure 7A). However, the persistence of linear fragment did not increase because of the inserted Chi octamers. Furthermore, the persistence of the Chi^0 substrate was consistently higher than that of substrates with Chi octamers at both insertion sites (12 independent experiments).

Linear DNA fragments, generated *in vivo* by λ terminase cleavage of cosmid DNA, were degraded in *E. coli* cells in a RecBCD-dependent fashion and Chi sites increased the survival of these fragments (Kuzminov *et al.* 1994). Since Chi octamers had an opposite effect on the persistence of the *Eco*RI-generated linear recombination substrates (Figure 7A), we considered the possibility that this difference is the result of the engagement of Chi-specific end-processing products in homologous pairing. An alternative explanation is that the observed difference reflects an intrinsic difference between the two methodologies for generation of linear DNA fragments within *E. coli* cells. To test for the latter possibility, we infected cells that express *Eco*RI with chimeric phage harboring DNA fragments that cannot serve as intramolecular recombination substrates. The effect of Chi octamers on the longevity of the *Eco*RI-generated fragments was ascertained by comparing Southern blot hybridization patterns of DNA preparations from cells infected by chimeric phage harboring Chi^+ and Chi^0 DNA fragments. A fragment with triple Chi octamers in the functional orientation at a distance of 382 and 973 nucleotides from the substrate's ends (RF936) was de-

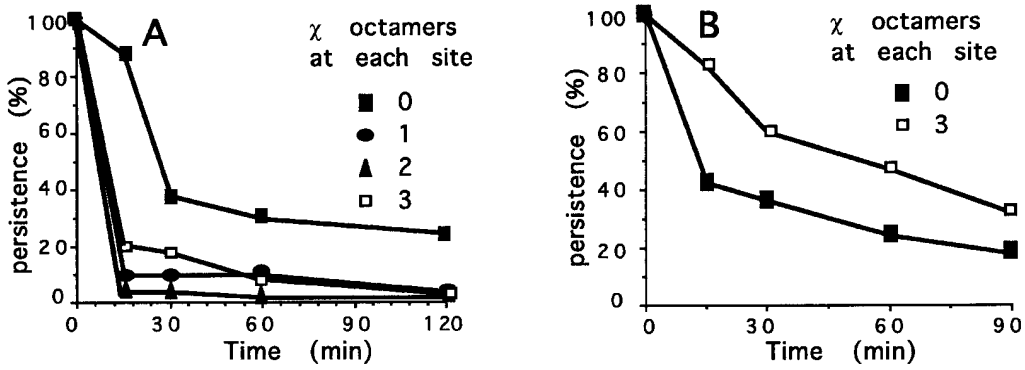

Figure 7.—The effect of Chi octamers on the longevity of linear fragments that could circularize by recombination (A) and on recombinationally inert fragments (B). Cells were infected by chimera phage harboring intramolecular recombination substrates (A) or linear fragments with no terminal or internal repeats (B). The persistence of the *Eco*RI-generated fragments, following infection by phage harboring recombination substrates with the indicated number of Chi octamers at each site, was determined by phosphoimaging analysis of Southern blot hybridization experiments like the ones depicted in Figure 5. The persistence of the linear fragments is expressed as the percent of radioactivity associated with the "substrate" band at time zero.

graded more slowly than a Chi^0 substrate (AN612) (Figure 7B). This observation supports the proposition that the apparent failure of Chi octamers to stabilize *Eco*RI-generated linear intramolecular recombination substrate is related to the efficient engagement of Chi-specific intermediates in homologous pairing.

As indicated above, the substrate hybridization band (see Figure 5) contains *Sal*I digestion products of phage DNA that has been fully or partially restricted *in vivo* by *Eco*RI. To monitor the persistence of each one of these two molecular species, we subjected total nondigested DNA preparations of samples taken at timepoints after λRF953 infection to Southern blot hybridization analysis (Figure 8) and quantitated the corresponding hybridization bands (Figure 9). The density of both hybridization bands decreased with time. However, the density of the band representing partially restricted phage DNA decreased more rapidly than that of the band representing fully restricted phage DNA. This difference suggests that part of the partially restricted phage DNA observed at time zero is further restricted to yield fully restricted phage DNA. This is consistent with the observed increase in density of the band representing fully restricted phage DNA between 0 and 5 min after infection (Figure 9).

DISCUSSION

The effect of Chi octamers on intramolecular recombination of linear substrates with direct terminal repeats was investigated. Linear substrates, released by *in vivo* restriction of infecting chimera phage DNA, recombined in wild-type *E. coli* cells by a mechanism that depended on *recA*, *recBCD* and Chi sites. These requirements indicate that recombination of these substrates proceeded by the RecBCD pathway, perceived first for transductional and conjugational recombination (Clark 1973). Recombination also depended on DNA double-stranded breaks, since *in vivo* restriction increased recombinant frequency by about 1000-fold (Table 4).

The synchrony and high efficiency of the experimental system facilitated physical monitoring of recombination by Southern blot hybridization (Figure 5). The generation of recombination products depended on *in vivo* restriction of the chimeric phage, the presence of Chi octamers on the substrate and functional *recA* and *recBCD* genes. The similarity of these requirements to the requirements for Kan^+ recombinants formation by the Ori^+ substrates (Table 4) suggests a common recombination mechanism. Chi-specific intermediates, predicted by models that postulate attenuation of RecBCD exonuclease at Chi and observed in an *in vitro* system (Dixon and Kowalczykowski 1993) were not detected by Southern blot hybridization. This may be the result of one or more of the following reasons: (1) the putative intermediates were rapidly engaged in homologous pairing and therefore did not accumulate, (2) the intermediates were substrates for other *E. coli* nucleases (Myers *et al.* 1995) and/or (3) the intermediates did accumulate but were not detectable by Southern blot hybridization. The latter may be the case if the putative intermediates were not of a discrete length or if they comigrated with another band in electrophoresis. Comigration of Chi-specific products with the substrate band is predicted by the "nick at Chi" model (Smith *et al.* 1984).

Linear substrates with direct terminal repeats may yield circular products by either one of two distinct intramolecular recombination mechanisms. In recombination by the strand exchange mechanism, one end is processed to a single-stranded overhang that invades the homologous dsDNA sequence. Conversely, in recombination by the SSA mechanism, resectioning of both ends generates complementary single-stranded overhangs and annealing of these strands yields a circular deletion product (reviewed in Kolodner *et al.* 1994; Eggleston and West 1996). The following evidence argues for the strand exchange mechanism. First, Chi is presumably essential for RecBCD-mediated formation of a recombinogenic end. The observation that Chi

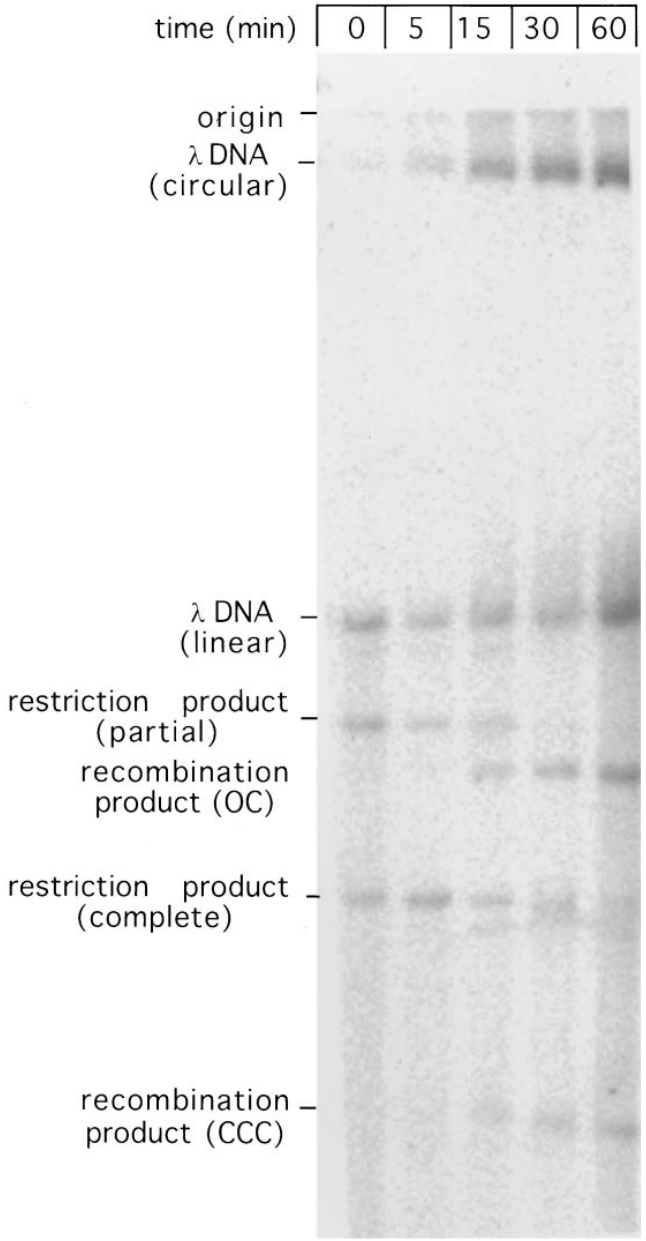

Figure 8.—Southern blot hybridization analysis of nondigested DNA preparations from cells infected by a phage harboring a Chi$^+$ substrate (A). Experimental procedure is as described in the legend to Figure 5, except that the DNA preparations were not digested prior to the Southern blot hybridization analysis. The infecting phage (λRF953) harbored a substrate with three Chi octamers at each site. The locations of circular and linear phage DNA, phage DNA that had been partially or fully restricted, covalently closed (ccc) and nicked (oc) forms of the circular recombination products are indicated.

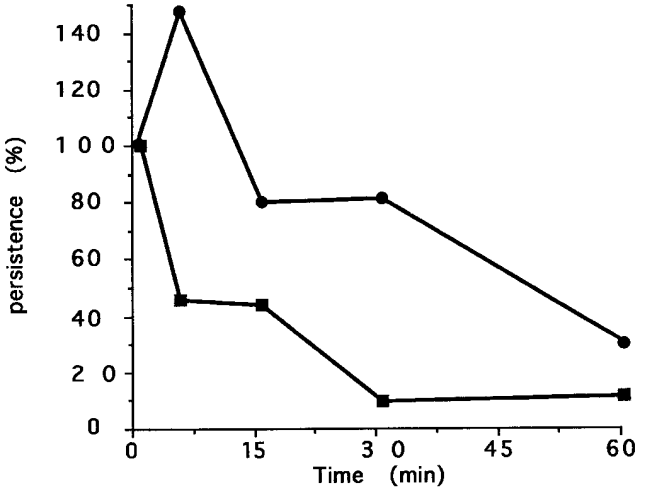

Figure 9.—The persistence of fully and partially *Eco*RI-restricted phage DNA. The appropriate hybridization bands in the experiment depicted in Figure 8 were scanned by phosphoimaging analysis. The persistence of the fully restricted (●) and partially restricted (■) phage DNA products is presented as the percent of radioactivity associated with the corresponding bands (see Figure 8) at time zero.

octamers induced recombination when present at only one site (Figure 2; Table 5) suggests that processing of only one end is sufficient. Second, the observation that in recombination of substrates with two Chi sites about half of the recombinational exchanges occur in the flanking intervals (Figure 4) also argues against a mechanism that requires RecBCD- and Chi-dependent processing of both ends. If the loss of Chi in recombination is associated with end processing or with pairing of a processed end, both Chi sites are expected to be lost in recombination by an SSA mechanism. Third, Holliday junction intermediates are expected in the strand exchange pathway but not in recombination by the SSA mechanism. The dependence of recombination on *ruvC* and *recG* (Figure 6D) is consistent with a mechanism that involves formation and resolution of Holliday junctions.

While the genetic requirements are in general agreement with the requirements for recombination by the RecBCD pathway, this recombination system is more sensitive than others to some genetic deficiencies. For example, *recBC* mutations, which lower conjugation and transduction proficiencies by about 100-fold (Clark 1973; Mahajan 1988), lowered recombinant frequency in the investigated system by more than 10,000-fold, and a *xonA* mutation, which by itself has little effect on transduction or λ recombination (Miesel and Roth 1996; Razavy *et al.* 1996), lowered the recombination rate by about 10-fold. The difference in the requirement for Chi octamers is discussed below. The high sensitivity of this recombination assay may be related to the short length of homology (about 3 kb) of the recombining sequences. It has been suggested that the high sensitivity of "short homology" assays stems from the requirement to complete the pairing event before the recombining sequences are degraded (Miesel and Roth 1996).

The requirement for *xonA* activity in phage λ DNA recombination and in the short homology transduction assay was interpreted as evidence for a role for exonu-

clease I in the generation of homologous pairing substrates with 5′-ended strands (Miesel and Roth 1996; Razavy *et al.* 1996). This does not seem to be the case in our system since exonuclease I deficiency impairs a recombination pathway that involves pairing of a 3′-ending strand (data not shown). It seems more likely that exonuclease I role in recombination is enhancing the rate of strand exchange by degrading the displaced strand (Konforti and Davis 1992; Bedale *et al.* 1993; Miesel and Roth 1996; Razavy *et al.* 1996).

Enhancement of recombination by the inserted Chi octamers was strongly influenced by the location of the insertion site and the number of inserted Chi octamers. The observed difference in Chi activity between Chi octamers inserted at *χXho* and *χBcl* (Figure 2; Table 5) was not related to the orientation of the cloned substrate in the chimeric phage, since inversion of the substrate in the phage vector did not affect Chi activity (Table 5). We also considered the possibility that the difference in Chi activity between the two insertion sites was the result of a difference in the distribution of Chi-like sequences (Schultz *et al.* 1981; Cheng and Smith 1984, 1987) along the homology. Sequence analysis of the linear substrate revealed the presence of a single Chi-like sequence (5′-CCTGGTGG-3′), in the functional orientation with respect to the adjacent end, at a distance of 332 nucleotides upstream to *χXho* (R. Friedman-Ohana, unpublished results). This sequence had no residual Chi activity in phage λ recombination (Schultz *et al.* 1981). The possibility that Chi-like sequences that differ from the Chi octamer by two nucleotides (two sequences on each side) contribute to the difference in Chi activity between *χXho* and *χBcl* cannot be ruled out. *χXho* and *χBcl* are located at a distance of 837 and 1565 nucleotides from the substrate's ends, and the length of homology downstream of these sites is 2700 and 1977 nucleotides, respectively. The possibility that the differences between the distances from the respective ends and/or the lengths of homology downstream of Chi affect Chi activity at the two sites is being investigated.

The observation that multiple Chi octamers are required for maximal Chi activity is consistent with the finding that interaction of RecBCD with a single Chi octamer is only about 50% efficient (Yagil and Stromas 1985; Stahl *et al.* 1990; Taylor and Smith 1992). Recombination efficiency, measured by determining the rate of product accumulation at the first 2 hr after infection, increased by three- to six-fold as the number of Chi octamers at each site increased from one to two, but an additional Chi octamer at each site did not affect recombination kinetics (Figure 6A). Conversely, addition of a third Chi octamer at each insertion site tripled recombinant frequency (Figure 2). The effect of a third Chi octamer on recombinant frequency, but not on recombination kinetics in the first 2 hr after infection, suggests that this octamer may contribute to the persistence of the recombination process. This may occur whether increasing the number of Chi octamers contributes to the stability of the restriction-generated substrate or to its continuous production. Stabilization of linear DNA fragments by multiple Chi octamers has been reported (Dabert *et al.* 1992; Kuzminov *et al.* 1994) and is also apparent from the data depicted in Figure 7B. A mechanism that may provide linear recombination substrates continuously in the experimental system described here is suggested by the following observation: phage DNA that escaped restriction replicates by a mechanism that generates linear multimers, and the newly synthesized multimers are only partially resistant to *in vivo Eco*RI restriction. This mode of replication depends on *recA* and the presence of multiple Chi elements on the chimeric phage. It is independent of phage or plasmid replication origin (I. Karunker, R. Friedman-Ohana and A. Cohen, unpublished results). It is conceivable that linear substrates, generated by partial restriction of the linear multimers, may contribute to recombinant production at a later period following infection.

Chi increases the persistence of linear DNA that cannot undergo intramolecular recombination (Kuzminov *et al.* 1994; Figure 7B), but not of linear fragments that could circularize by recombination (Figure 7A). One interpretation of this structure-dependent difference is that Chi-specific intermediates that comigrate with the linear substrate are converted into circular recombination products. Alternatively, recombination-mediated pairing of the processed ends with circular phage DNA that escaped restriction may prime synthesis of phage DNA linear multimer, and restriction of the multimer would yield "linear substrates" as described above. If linear multimer synthesis is primed by an intermolecular recombination event, the engagement of the processed end in intramolecular recombination may reduce the efficiency of this process. This explanation does not account for the observed difference in longevity between Chi^+ and Chi^0 substrates of intramolecular recombination (Figure 7A).

A single Chi site is sufficient to stimulate intramolecular recombination, but a combination of two is synergistic (Figure 2). Assuming that Chi elements at only one site are involved in each recombinational exchange, this synergistic effect suggests a role for the Chi octamers at the other site. It is likely that a Chi site on the noninvasive end enhances recombination by attenuating RecBCD nuclease activity and thereby increases the survival of the linear restriction product (Dabert *et al.* 1992; Kuzminov *et al.* 1994). However, since multiple Chi sites also enhance phage DNA linear multimer synthesis, we cannot rule out the possibility that recombination enhancement by Chi octamers at the nonpairing end is an outcome of continuous generation of recombination substrates by the mechanism described above.

Whereas in a *recD* mutant, genetic exchanges were evenly distributed along the short homology, in wild-type cells, exchange frequencies were higher in the interval between the Chi sites than in the flanking intervals (Figure 4). Since the central interval is downstream of both Chi sites and each flanking interval is downstream of only one, this observation is consistent with the proposal that, in this system, all recombination events occur downstream of Chi. Exchange frequencies in the central interval approximated the sum of the frequencies in the flanking intervals. This suggests that in most recombination events at least part of the interval distal to the pairing end is available for strand exchange. If RecBCD degrades DNA from the substrate's end up to the Chi site, this distribution would indicate that in most recombining substrates, only one end interacted with RecBCD.

Chi activity is defined by the ratio of recombinant frequency in a given genetic region containing Chi, compared to that in the same region without Chi (Stahl and Stahl 1977). The maximal Chi activity in the experimental system presented here is about 100-fold higher than in gene replacement by a short linear DNA fragment (Dabert and Smith 1997), and several hundredfold higher than in RecBCD-mediated phage λ recombination (Lam *et al.* 1974; Stahl *et al.* 1980). These marked differences in Chi activity probably reflect the relatively low efficiency of Chi-dependent recombination in the gene replacement system and the high background of RecBCD-mediated Chi-independent recombination of λ DNA. The difference in the efficiency of Chi-dependent recombination between the intramolecular recombination and the gene replacement systems may be the result of the difference in the configuration of the respective recombination substrates. While in intramolecular recombination only one recombinogenic end is required for recombinant formation, two ends may be required for gene replacement. It is also conceivable that the search for homology by the short substrates is more efficient in intramolecular recombination than in the replacement of a gene on the bacterial chromosome.

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