Recombination initiation: **Easy as A, B, C, D... χ?**

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The octameric Chi (χ) sequence is a recombination hotspot in *Escherichia coli*. Recent studies suggest a singular mechanism by which χ regulates not only the nuclease activity of RecBCD enzyme, but also the ability of RecBCD to promote loading of the strand exchange protein, RecA, onto χ-containing DNA.

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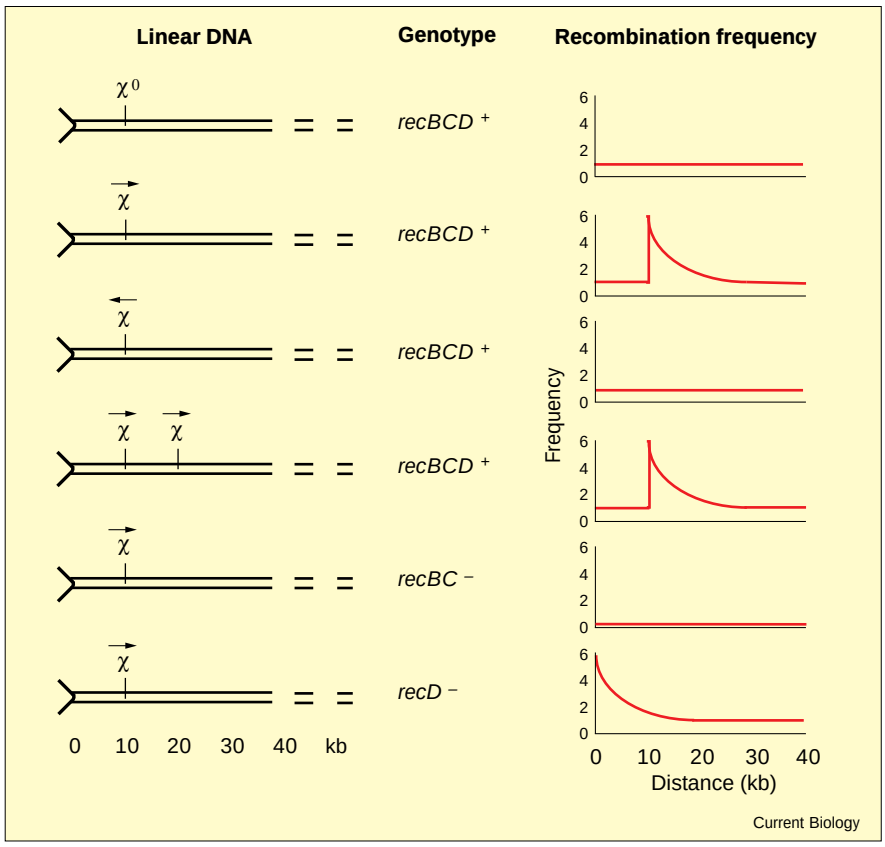
More than 20 years ago, phage λ geneticists identified certain mutations, termed Chi (χ), which increase the frequency of recombination in their vicinity (reviewed in [1]). A large body of experiments in phage λ and *Escherichia coli* established a number of properties of χ-dependent stimulation of recombination (Figure 1). The effect occurs on linear molecules, requiring a specific orientation between the χ site and a double-stranded DNA end; the stimulation is confined to one side of the χ site, and is exerted over many kilobases. Multiple χ sites do not have additive effects, and the stimulation depends on the host recombination genes, *recB* and *recC*. DNA sequencing showed that the octanucleotide 5′-GCTG-GTGG-3′ is χ, although it was only recently shown that this single-strand sequence, and not its complement or duplex form, is sufficient for χ activity [2]. Recent work has shed new light on the mechanism by which χ stimulates recombination, which turns out to involve changes in the activity of the RecBCD enzyme.

Regulation of RecBCD activity by χ

The dependence of χ activity on the host *recBC* gene products indicated that these components of the recombination system have a role in eliciting the stimulatory effect, although it was noteworthy that the *recD* gene product, the third subunit of RecBCD enzyme, was not required for χ activity. In fact, in a *recD* mutant, the RecBCD enzyme

Figure 1

Properties of χ in RecBCD-dependent recombination. A series of linear DNA molecules are shown in the first column. Unwinding initiates from the left end of the molecule (arrowhead). The absence or presence of χ is indicated (χ^0 and χ, respectively), with an arrow showing the direction the enzyme must travel to recognize χ. In the centre column, the relevant host genotype is listed. On the right, an idealized plot of the recombination frequency *versus* distance from the end for each pair of linear DNA and host genotype is shown.



behaved as if it was constitutively χ-activated (Figure 1). Genetic studies had defined an early role for RecBCD in both recombination and the repair of double-strand DNA breaks, but the nature of this role was enigmatic. RecBCD was initially described as a nuclease capable of degrading linear double-stranded and single-stranded DNA molecules to oligonucleotide products. Yet RecBCD is required for recombination, a process in which its ability to degrade double-stranded and single-stranded DNA would seem to be antagonistic, as it would obliterate the substrate required for subsequent DNA strand exchange. Later, the RecBCD enzyme was found also to be a highly processive helicase capable of unwinding duplex DNA from blunt, or nearly blunt, ends (for review, see [3]).

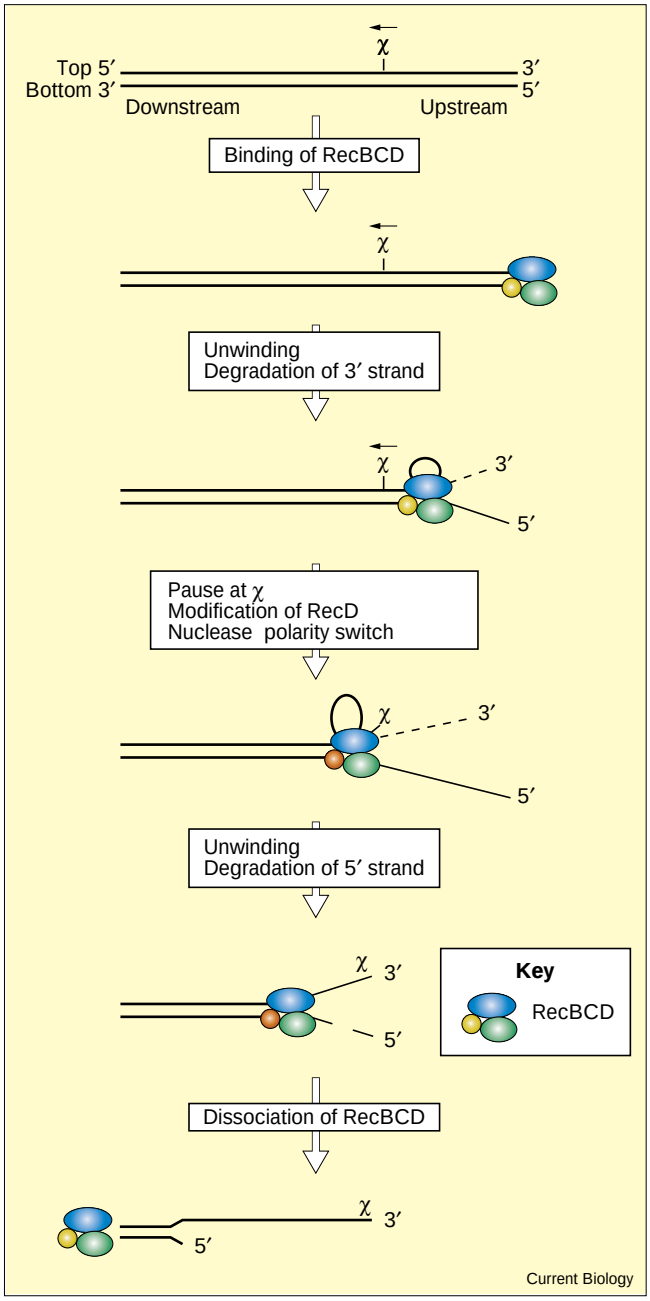
The first indication of a direct relationship between χ and RecBCD came when it was found that incubation of a linear DNA molecule containing a χ site with RecBCD, under conditions that suppressed the enzyme's normally high (and non-specific) nuclease activity, resulted in the introduction of a nick 4–6 base pairs to the 3′ side of χ [4]. Despite this demonstration of nicking localized to the vicinity of the hotspot, however, it was not immediately obvious how this activity resulted in a higher frequency of recombination. Smith and colleagues [4] proposed that the 3′ end liberated by the nick at the χ site served as the invasive tail in the initial step of DNA strand invasion, but this model side-stepped the question of why the 3′ terminus at the DNA end from which unwinding initiated was less recombinogenic.

Increasingly detailed studies of the effects of χ on RecBCD enzymatic activity provided the necessary insight. It was found that, as RecBCD enzyme unwound DNA lacking χ sequences (χ^0 DNA), it nicked the strand that contained the 3′ terminus of the initially bound end more frequently than it did the 5′ terminal strand [5] (Figure 2). When RecBCD encountered a correctly oriented χ site, however, unwinding stopped momentarily; it is thought that this pause accounts for the elevated frequency of nicking on the top strand adjacent to χ. Remarkably, after unwinding resumed, degradation of the 3′ strand was attenuated. In addition, a weaker 5′–3′ nuclease activity was upregulated [6], resulting in a nick on the bottom strand near the χ complementary sequence [7,8] and further degradation downstream of χ. One obvious consequence of this switch in the apparent strand polarity of nuclease activity is that, as unwinding continues past χ, a single-stranded DNA tail having χ at the 3′ end is generated. Moreover, the opposite strand, a potential competitive partner for reannealing behind the enzyme, is eliminated by nucleolytic degradation (Figure 2).

Regulation of RecBCD–RecA interactions by χ

At the time, the nuclease attenuation model appeared to address how recombination was stimulated by χ sites: χ

Figure 2



Regulation of the polarity of RecBCD nuclease activity. The products of RecBCD-mediated unwinding of χ-containing DNA are defined as originating in the top or bottom strand (with the top strand containing the χ octamer, 5′-GCTGGTGG-3′), and from upstream or downstream of χ (upstream being to the 3′ side of χ). If RecBCD begins unwinding from the right end, it degrades the top strand (which has the 3′ terminus of the duplex end) but leaves the bottom (5′ terminus) strand intact. Upon interaction with a properly oriented χ site (indicated by arrow), RecBCD stalls, the RecD subunit is altered (signified by a change in colour), and the polarity of nuclease activity is reversed. Thus, when unwinding resumes, the top strand is released intact and possesses χ at its 3′ end, while the bottom strand is degraded. If unwinding is initiated from the left end, the 3′ strand would be completely degraded, and the 5′ strand would be released as a full-length molecule.

modulated the nuclease activities of RecBCD enzyme to favour the production of a 3′ terminal single-stranded DNA tail. Such an overhang was postulated in recombination models to provide a scaffold upon which RecA protein, which catalyzes the processes of DNA pairing and strand exchange, could initiate binding. Nevertheless, this model did not fully explain some *in vitro* observations from studies in which RecBCD-mediated unwinding was coupled to RecA-dependent joint molecule formation [9]. In particular, it was noted that, although the amount of unwound full-length single-stranded DNA — generated from the 5′ terminal strand when the enzyme does not encounter a correctly oriented χ site (see Figure 2) — was significantly greater than that of the fragment having χ at its 3′ end, the χ-dependent fragment was over-represented in the population of joint molecules (Figure 3a). Why were χ-containing fragments preferentially used in these pairing reactions?

The solution to this puzzling observation was presented in a recent paper by Anderson and Kowalczykowski [10]. By coupling RecA and RecBCD activities in a single *in vitro* reaction, they examined the effects of varying the order of addition of the various proteins — RecA, RecBCD, and a single-strand DNA binding protein (SSB) — on the yield and nature of the joint molecules. They found that preferential incorporation of the fragment having χ at its 3′ end was eliminated when RecA-promoted pairing was uncoupled from RecBCD-dependent unwinding. Under these conditions, the downstream χ fragment (Figure 2) was used with the same, lower efficiency as the full-length fragment, which was incorporated at identical efficiencies in both coupled and uncoupled reactions. To probe the requirements of preferential pairing further, a single-strand specific nuclease, exonuclease I (Exo I), was added to the reaction to determine which fragments were sensitive to degradation. Quite dramatically, only the downstream χ-specific fragment was protected from degradation when RecA and SSB were present in the coupled reaction; if RecA was omitted, or if RecA and SSB were added after the DNA had been unwound by RecBCD, all of the unwound single-stranded DNA was Exo I-sensitive.

These results led Anderson and Kowalczykowski [10] to propose a model in which SSB and RecA proteins compete for binding to single-stranded DNA (Figure 3b). In the coupled reaction, and presumably in the cell, before RecBCD encounters χ, the higher affinity of SSB for

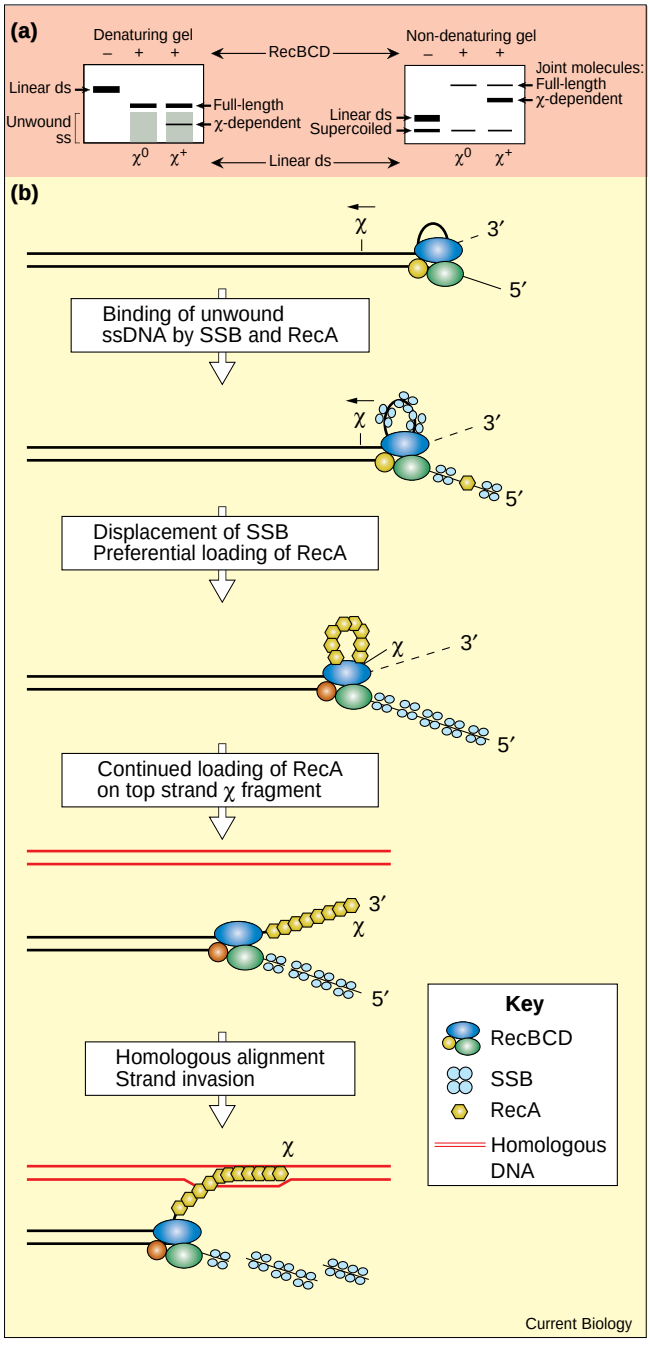


Figure 3

χ stimulates preferential loading of RecA by RecBCD. (a) A paradox was observed in reactions coupling RecBCD unwinding and RecA pairing. When the products of RecBCD-mediated unwinding of a linear, double-stranded (ds)DNA molecule were examined on both native and denaturing gels, the population of full-length single-stranded (ss)DNA molecules was significantly greater than that of the downstream χ-dependent molecules. The same linear DNA was then co-incubated with homologous supercoiled DNA, RecBCD, RecA, and SSB proteins. Analysis of the joint molecules produced in this coupled reaction on a non-denaturing gel, however, revealed that the χ-dependent fragment was preferentially incorporated into joint molecules. (b) To explain these results, a model has been proposed whose central premise concerns competition for ssDNA binding sites between RecA and SSB proteins. The ssDNA produced upstream of χ can be bound by both RecA and SSB. As SSB has a higher affinity for ssDNA, it binds to the unwound DNA and few RecA filaments are assembled on the DNA. The interaction with χ alters RecBCD enzyme so that it promotes the nucleation of RecA protein on the downstream χ-containing strand. Thus, this fragment, rather than the other ssDNA fragments which may be bound by a mixture of SSB and RecA proteins, displays heightened pairing ability.

single-stranded DNA results in the unwound DNA being bound predominantly by SSB, such that active RecA filaments are not readily formed. If RecBCD is activated by χ, however, it undergoes a change that causes the enzyme to promote the binding of RecA, to the exclusion of SSB, on the downstream top-strand χ fragment as RecBCD translocates along the DNA. These data appear to have uncovered a previously unrecognized level in the control of recombination and double-strand break repair — the directed loading of RecA protein by a translocating RecBCD enzyme onto a single-stranded DNA overhang containing χ at its 3′ end.

Further links between χ, RecA and RecBCD

By modulating RecBCD's nuclease activity, χ serves an important role in protecting the genome from extensive degradation when a double-strand break occurs. On the basis solely of the composition of the *E. coli* genome, it was initially estimated that χ would occur on average once every 5–10 kilobases. Given the high processivity of RecBCD helicase activity — it can unwind greater than 30 kilobases *in vitro* — such a distribution of χ sites seemed sufficient to prevent significant loss of genetic information caused by RecBCD-mediated DNA degradation before the enzyme encounters a properly oriented χ site.

This argument now appears to have been naive. With access to the complete sequence of the *E. coli* genome [11], the positions of 1,009 χ sequences have been identified, a number that is several-fold higher than previous predictions. In addition, the orientations and locations of the sites are distinctly non-random. Approximately 75% of the χ sites are oriented towards the replication origin (S.C. Kowalczykowski, personal communication), although it has been suggested to us that this bias may relate more to codon usage and to a preference for transcription to be oriented with respect to replication than to a property of χ itself (G.R. Smith, personal communication). The χ sequences appear to be embedded in statistically-defined GT-rich islands comprising 17% of the genome [12].

In analyzing these regions, a repeating pattern of TGG was found. As the χ sequence can arise from a single nucleotide mutation in this pattern, it is possible that this association is fortuitous. Nevertheless, recent studies indicate that RecA protein pairs GT-rich DNA with greater efficiency than DNA rich in other dinucleotide pairs, and possesses a higher binding affinity for these sequences [13]. The level of enhancement with GT-rich DNA is modest, however (less than five-fold). Genomic χ sites may therefore be located within regions that are optimally suited for recombination by virtue of enhanced stability, although the functional significance of these GT-rich sequences has not been established *in vivo*. Further studies are required to determine whether this sequence context has any influence on χ activity.

Outlook

Given our still-evolving appreciation of the various levels of control by which the activities of RecBCD enzyme and RecA protein are influenced by χ, it is unlikely that we understand all there is to know about χ-dependent recombination. Perhaps the most pressing mystery remains the nature of the molecular change in RecBCD enzyme that alters the polarity of its nuclease activity. For years, the favoured explanation for χ-activation of RecBCD enzyme was dissociation of the RecD subunit. Despite the efforts of several laboratories, however, conclusive evidence of the validity of this model has not been obtained. In fact, both *in vivo* and *in vitro* data now suggest that χ-interaction causes an alteration of the activity of the RecD subunit, rather than its loss [6,14–16]. Although, in Figure 2, the modification in the RecD subunit is shown to be reversed following dissociation of the enzyme from the DNA, the question of whether and when this occurs *in vivo* is also not yet fully understood; *in vitro*, however, conditions can be manipulated to either permit or inhibit reversal of the change in RecBCD induced by χ.

The mechanism by which RecBCD directs loading of RecA onto DNA remains cryptic. One possibility is that the interaction between RecBCD and χ alters the structure of the enzyme such that protein–protein contacts are made between RecBCD and RecA. Alternatively, the loading of RecA may occur indirectly through the generation of an unusual DNA structure formed by χ-activated RecBCD as it translocates.

The interplay between χ, RecBCD and RecA protein has proven to be much more complex than originally anticipated. Although progress to a fuller understanding of the way in which recombination is initiated in *E. coli* is being made, the secrets of RecBCD action have revealed themselves more slowly than hoped. There undoubtedly remain unimagined mysteries in this process, and the keys to unlock them are likely to require the development of more complete *in vitro* recombination systems than are presently available.

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