ABSTRACT

Title of dissertation: LOCATION-SPECIFIC RECOGNITION OF THE RECOMBINATION HOT-SPOT "CHI" (χ) BY THE *ESCHERICHIA COLI* RECBCD ENZYME.

Avanti Kulkarni, Doctor of Philosophy, 2004

Dissertation directed by: Professor Douglas A. Julin

Department of Chemistry and Biochemistry

RecBCD is an ATP-dependent helicase and exonuclease, which generates 3'-single-stranded DNA (ssDNA) ends used by RecA for homologous recombination. Critical to this function is its encounter with a regulatory DNA octamer called the Chi sequence, 5'-GCTGGTGG-3', which is known to stimulate recombination by the RecBCD pathway. RecBCD undergoes an unexplained modification following Chi encounter that results in attenuation of its 3'-5'exonuclease activity. There are several aspects of the Chi-RecBCD interactions that are still not understood, and most studies

addressing these issues have done so by employing Chi- bearing duplexes in various biochemical assays for RecBCD functions. The recognition of Chi on exclusively single-stranded substrates has never been tested before, even though Chi is known to be recognized as the single stranded sequence 5' GCTGGTGG 3'. This study tests the effect of ssDNA oligonucleotides, having a Chi sequence (Chi^+) or a single base mutant of the Chi sequence (Chi^o), on the enzymatic activities of RecBCD. The results obtained show that Chi is specifically recognized by RecBCD even when part of a single-stranded substrate. However its location within the single strand is important; specific recognition and distinction from the mutant sequence occurs only when Chi is in the middle of the construct, flanked by DNA at either end. The activities affected included the helicase, and consequently the exonuclease and Chi-recognition activity itself. The effects observed were also found to be dependent on the length of the oligonucleotides. The longer oligonucleotides affect the enzymatic function of RecBCD better than the shorter ones. Preincubation of DNA substrate with RecBCD abolishes the inhibitory effect of the oligonucleotides on RecBCD function however; preincubation of the oligonucleotides themselves with RecBCD does not enhance inhibition by them. This study also presents some preliminary results from photocrosslinking of oligonucleotides to RecBCD, which suggest that RecC may play an important role in binding and recognizing Chi. The results lead to the proposition of a model that explains the location-specific (flank-dependent) recognition of the Chi sequence by RecBCD and discusses the possibility of the existence of a specific site on RecBCD for Chi.

LOCATION SPECIFIC RECOGNITION OF THE RECOMBINATION HOT-SPOT "CHI" (χ) BY THE *ESCHERICHIA COLI* RECBCD ENZYME

By

Avanti Kulkarni

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2004

Advisory Committee:

Professor Douglas Julin, Chair
Professor Dorothy Beckett
Professor Norman Hansen
Professor Jason Kahn
Professor David Straney

ACKNOWLEDGMENTS

I would like to express my heartfelt gratitude to all those people who have helped make my journey towards the completion of this degree, an enjoyable and memorable learning experience. I have enjoyed being a part of the department and the University itself, and would like to start by thanking the department, for giving me this wonderful opportunity to work towards my degree on its premises.

I would like to thank all my committee members. Dr Norman Hansen, Dr Jason Kahn, and Dr Dorothy Beckett for their advice and guidance during the course of these five years. I am especially grateful to Dr Jason Kahn and Dr Dorothy Beckett for their valuable inputs about future prospects and direction of study, their encouraging and supportive stance during annual committee meetings and for their support in providing me with recommendations, which have played a big role in my being able to continue working in this field. I am very grateful to both of them for extending their cooperation in this regard. I would like to thank Dr David Straney for accommodating my request and making the time to serve as the dean's representative for my committee.

Most of all, I would like to thank my advisor Dr Douglas Julin, to whom I am truly indebted for all his support, advice, encouragement and patience over the years. I thank him, for giving me this chance to work for him, and believing in my commitment and dedication to the completion of this degree. In addition to his valuable advice and guidance, his timely encouragement in times of doubt, and his infinite patience has made working towards this degree a thoroughly memorable and rewarding experience. I would like to thank him for being approachable at all times, for giving a patient ear to my innumerable queries and doubts and for all the help and advice he has given me in resolving research complications whenever they have made their presence felt, which was more often than not. I feel extremely privileged to have been given this chance to work for someone like him, who I can look upto not just as an academic advisor and professor, but also as a wonderful human being. I thank him sincerely for everything that I have learnt from him and for all his kindness and patience without which I would never have been able to achieve this end today.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BPB	Bromophenol blue
CPM	Counts per minute
dsDNA	Double stranded DNA
EDTA	Ethylene diamine tetracetic acid
FPLC	Fast performance liquid chromatography
LB	Luria-Bertani
OriC	Site for origin of replication
RPM	Rotations per minute
SDS-PAGE	Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis
SSB	Single stranded DNA binding protein
ssDNA	Single stranded DNA
TE	Tris-EDTA (50mM, pH 8.0)
TLC	Thin layer chromatography
UV	Ultraviolet
DTT	Dithiothreitol
TCA	trichloroacetic acid

CHAPTER I

INTRODUCTION

The RecBCD enzyme of *Escherichia coli* functions in the first step of homologous recombination called initiation. It is involved in the generation of 3'single- stranded stretches of DNA from regions of the chromosome interrupted by double-stranded DNA damage [1; 2]. The 3' single-stranded ends are the substrate for RecA protein, which then uses these ends to repair the damage by the process of homologous recombination [3; 4]. The first part of this chapter provides a brief introduction to the process of homologous recombination, its mechanism and significance to both repair of damaged DNA and replication, while outlining the different models proposed which incorporate the collaborative role of approximately 25 different gene products involved in the process. The second part of the chapter outlines the RecBCD enzyme itself, its biochemical activities and its mechanism of action. The final part of the introduction discusses the relevance of the octameric regulatory element "Chi" to RecBCD mediated recombination, and discusses the particular aspect of Chi interaction under investigation in this study.

1.1 **Homologous recombination**:

Homologous genetic recombination, an event critical to the maintenance of genetic stability and integrity and the generation and propagation of genetic diversity, is characterized by the exchange of genetic information between homologous or identical sections of the chromosome. This kind of an exchange of genetic material typically occurs during the process of DNA repair and during "crossing over" or chiasma formation, a crucial event occurring during the meiotic phase of replication.

The complex biochemical, genetic and molecular mechanistic details of this process have been extensively studied in *E. coli*, with at least 25 different gene products implicated in various aspects of the process [1;5]. These include products of the Rec family of genes, primarily RecA, RecBCD, RecF, RecO, RecQ, RecR, RecE, RecG, RecJ, members of the Ruv family (Ruv ABC), helicases, gyrase, DNA-binding proteins, topoisomerases, SSB, members of the replicative machinery including DNA Pol III and priA. Until recently, recombination was only thought to be responsible for repairing damaged DNA (double-stranded damage) and for the generation of genetic diversity [6]. However, new work and studies now suggest there is a more intricate connection between recombination and replication as well [7; 8]. The two processes employ the same biochemical machinery and work in conjunction to maintain the genome. New studies now show that recombination is needed to initiate oriC-independent replication, which arises out of collapse of the replication fork due to imperfections in the DNA template (the imperfections themselves may arise due to exogenous DNA damaging agents/radiation, or naturally by the release of free radicals from the metabolic pathways). The process is referred to as

"recombination-dependent –replication" (RDR) and is proposed to occur in almost every replication cycle to repair collapsed or stalled replication forks [1; 9]. The interdependence of the two processes is further exemplified by the "replication-dependent-recombination" process, which is proposed to occur during conjugation or transduction whereby a linear DNA fragment is integrated into the bacterial host genome.

The prototypical genetic model for recombination was proposed by Holliday [10], and it illustrates the repair of a single-stranded break occurring in two homologues. Many variations of the Holliday model have been proposed since, to encompass the repair of a single-stranded break in one of the homologs [11] or repair of double-stranded break in one of the homologs (called DSBR-double-stranded break repair model) [12 13]. The key steps involved in these events, along with the enzymatic machinery regulating these steps is reviewed next.

1.1-1 DNA repair and the genetics of recombination: Recombination is the only means by which cells can repair double-strand DNA breaks where information is lost from both the strands of the duplex. These kind of breaks may occur naturally during DNA replication when the replication fork encounters single-stranded gaps in the template causing the replication fork to collapse [14; 15], or they may be induced by artificial means like ionizing radiation [16; 17; 18]. It is estimated that under normal aerobic growth conditions, *E. coli* suffers an average 3000-5000 such DNA lesions, per cell, per generation [9]. The process of repair involves several events starting with the priming of the damaged strand for recombination (in some cases) to the formation of a four-stranded recombination intermediate that is resolved to give repaired recombinants. The important models

illustrating these events (the Holliday model, the Meselson-Radding model and the DSBR-double-stranded break repair model) are summarized below.

A) The Holliday model: [5; 10] The prototypical genetic model for generalized recombination was first proposed by Holliday in 1964 , much before the actual identification and discovery of the enzymes involved in the process. The model was proposed to explain the observed gene conversions in fungi. The model illustrates the formation of a recombinant DNA molecule starting with the repair of two homologs each with a single-strand nick (figure 1.1). The first step involves alignment of the two homologs such that genetic exchange is facilitated through the nicks. The nicked strands are aligned to face each other such that they can then move, and crossover to pair with their region of complementarity in the opposing chromosome. This movement of the nicked strands towards the homologous region in the facing chromosome, followed by base pairing in the complementary region, leads to the formation of a four-stranded structure with a heteroduplex joint which is referred to as the "Holliday" structure. The structure is capable of extension and movement along the heteroduplex joint, a phenomenon that is called "branch migration" and is facilitated by RuvAB, RecG and PriA [9]. Resolution of this structure by RuvC [5;20] to give two recombinant duplexes is the final step in the process, and it can be achieved in two ways depending on the strands

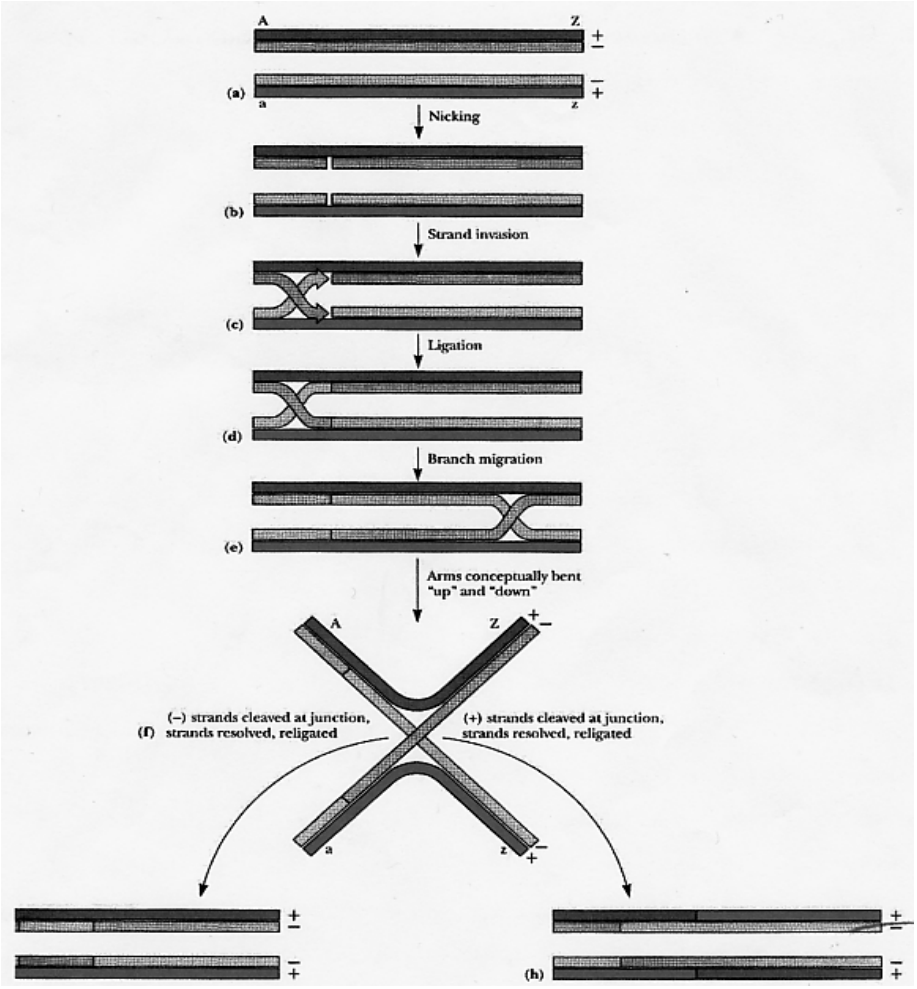


Figure1.1

Holliday Model for Genetic Recombination

a) Alignment of homologs. b) Nicking of facing strands.c) Strand exchange and cross over. d) Ligation of crossover point to form heteroduplex recombination intermediate called “Holliday intermediate”. e) Branch migration. f) Resolution of Holliday intermediate by nicking and resealing of exchanging strands (patched) or non-exchanging strands (spliced). (Sourced from [19])

cleaved at the crossover point. Cleavage of the two single-strands that crossed over results in the original duplexes, which have exchanged flanking markers from the crossover event. These are called "patched recombinants." Cleavage of the non-exchanging strands results in formation of "spliced recombinants" which are traditional recombinant molecules with sections of DNA from both chromosomes.

B) The Meselson-Radding model: [11] The Meselson-Radding model, is a variation of the Holliday model, proposed to explain repair originating from a strand nick in one of the homologs. The basic steps again involve strand-invasion by the nicked strand to form a D-loop (displacement loop), followed by degradation of the D-loop and recipient strand and finally DNA replication of the invading strand to form a heteroduplex capable of branch-migration and resolution to yield patched or spliced recombinants as in the Holliday model (figure 1.2).

C) The double-stranded break repair model (DSBR): [12; 13] The model (shown in figure 1.3) explains the recombinational repair of a double-stranded break in DNA. It involves the conversion of double-stranded ends to 3' single-strands (carried out in *E. coli* by RecBCD and the regulatory Chi sequence), followed by invasion of the homolog by the 3' single-strand and RecA, leading to D-loop formation. The model involves two rounds of single-strand synthesis, followed by homologous pairing, DNA strand exchange and formation of a heteroduplex capable of extension and migration (figure 1.3). The final step involves resolution of the joints to give either the patched or spliced recombinants.

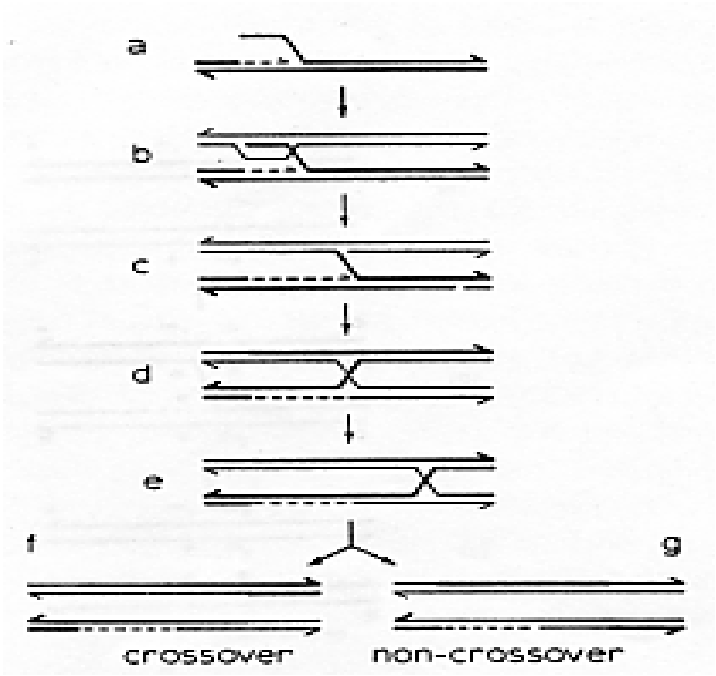


Figure 1.2[13]:

The Meselson Radding model for homologous recombination:

A) A single-strand nick in a duplex acts as the starting point. **B**) The 3' end of the nick invades the homolog, displacing the "D-loop". **C**) The D-loop and recipient strand is degraded, and invading strand is resynthesized. **D & E**) Ligation seals the gaps to form the heteroduplex joint molecule, which is capable of branch migration along the joint. **F & G**) Resolution of joint yields recombinants.

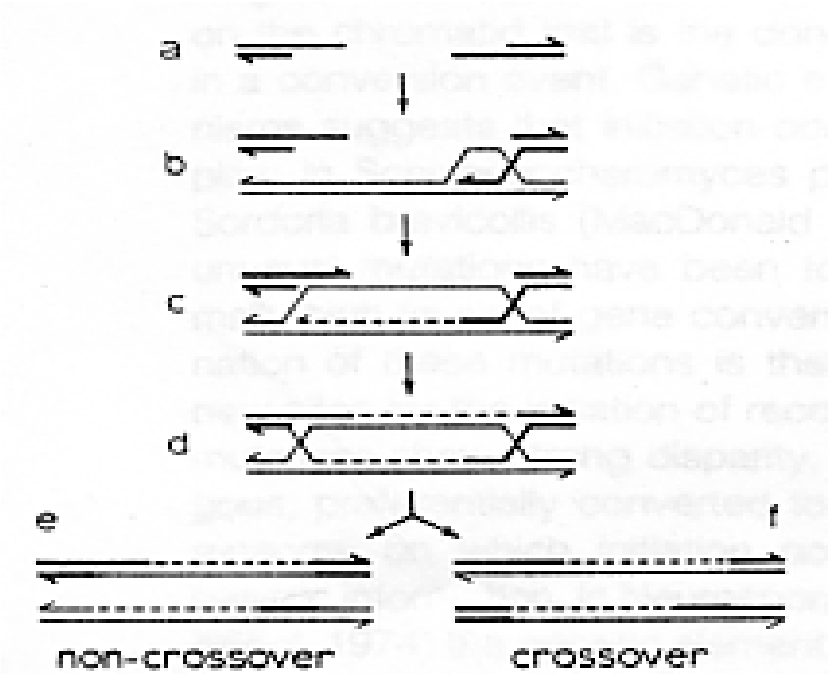


Figure 1.3[13]:

The double-stranded break repair model (DSBR) for homologous recombination:

A) A double-stranded cut in one homolog is converted to 3' single-stranded ends by RecBCD. **B**) One of the 3' single-strands invades the other homolog and displaces a D-loop in it. **C**) Repair synthesis of invading strand extends the D-loop till it can anneal to the other 3' single-strand. **D**) Repair synthesis of the second 3' single-strand leads to formation of heteroduplex with two joints. **E & F**) Resolution of joint yields recombinants.

1.1-2 DNA Replication and the genetics of recombination [1; 9]: The newest addition to the list of models and proposed mechanisms comes through studies on the replication-recombination interdependence. Until recently recombination was thought to play a role mainly for repair, however recent studies show the importance of the process to replication, as also the interdependence of the two processes for maintenance of genomic integrity. The need for recombination arises very frequently during replication, whenever the replication fork encounters discontinuities in the template. These discontinuities (single-stranded gaps/double-stranded breaks) may arise due to external factors or naturally due to release of free radicals during metabolism, as stated above, and the frequency of encountering such gaps or break is estimated to be almost one per cell division in *E. coli* [9]. The proposed model (figure 1.4) for repair of such a gap encountered in the leading strand again starts with the initial processing of the terminated double-strand, to produce a 3' single-stranded overhang. This is followed by D-loop formation, strand invasion and extension (of the leading strand). The lagging strand is then synthesized using the displaced strand as template. Nicks in lagging strand synthesis are repaired in a similar manner, except that they do not need the initial processing, as these nicks lead to a terminated strand already bearing a 3' single-stranded overhang. Recombination during transduction and conjugation also proceeds through initial processing of the DNA to be integrated, followed by strand invasion and D-loop formation. However formation of the final recombinant requires replication of the invading DNA, by the replication machinery. Hence this kind of recombination is called "replication-dependent-recombination".

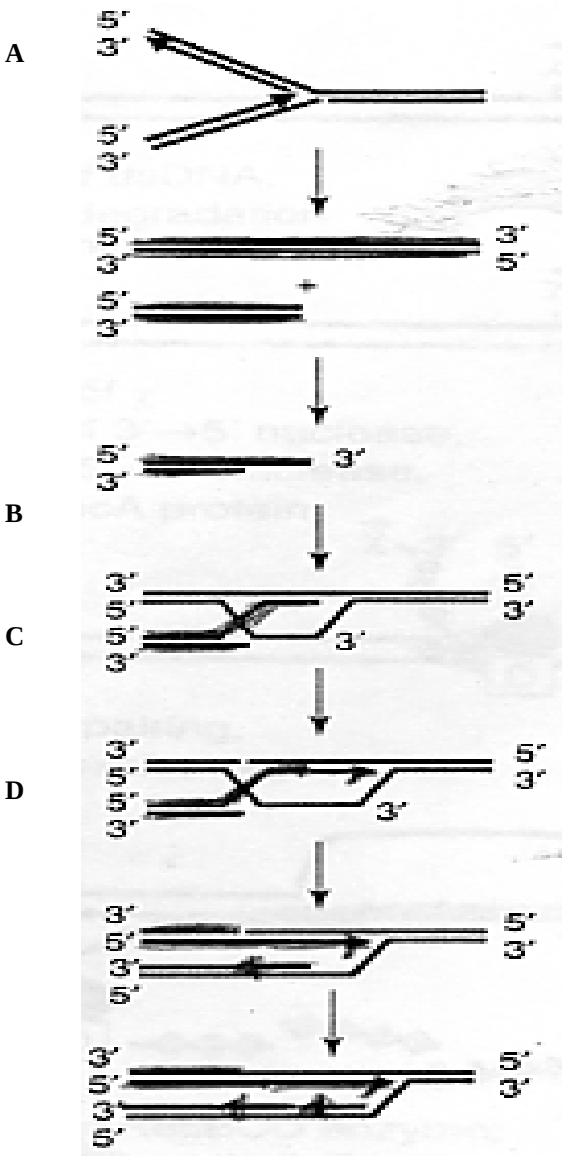


Figure 1.4:

Recombination dependent replication model:

A) A nick in the leading strand produces a doubles stranded break, which is processed to form a 3' single stranded end. **B)** Invasion of homolog by the 3' single strand to displace a D-loop. **C)** Synthesis of the invading strand. **D)** Resolution of the Holliday intermediate, to reform the replication fork and resume replication.

1.1-3 The biochemistry of recombination: The above models explain the genetics of the process of recombination. The biochemical details of the phenomenon, including the various proteins and enzymes that are involved in the various steps are outlined next.

A) Initiation: This refers to processing of double-stranded ends to generate a stretch of 3' ended single-strand, which is then used by RecA to bring about strand invasion. The primary protein machinery carrying out this process is the RecBCD enzyme [21; 22], which is responsible for 99% of the recombination events in *E. coli.* The enzyme is a trimer with helicase,[23] ATPase [24], exonuclease (SS and DS) [25; 26] and endonuclease (SS circles) activities [25; 27], which are regulated by an octameric regulatory sequence called Chi [28; 29]. The helicase activity of the enzyme has a preference for blunt or nearly blunt ends, and unwinds the duplex from the end (point of damage), at the rate of 1000 bp/sec, while consuming 2-3 ATP/bp unwound [30]. The unwinding is accompanied by degradation of the unwound strands, until a Chi sequence is encountered. Encounter with this sequence leads to an attenuation in its 3'-5' exonuclease function, without affecting the helicase action of the enzyme, which is directly responsible for generating the 3'ended single-strand that RecA needs for strand-invasion [31]. The details of this process are outlined in the next section on RecBCD. The next step in the mechanism is usually the loading of RecA, the protein that actually brings about strand invasion.

B) Strand-invasion and homologous pairing: As explained above, strand invasion refers to the "invasion" of one of the strands of a homologous chromosome, by the 3'ended single-strand of the damaged homologue. This kind of invasion leads to displacement of one of the strands of the invaded homologue, which gives rise to the "D-loop." The invading 3' ended single-strand first base pairs with its region of homology in

the invaded strand (homologous pairing) in the process displacing a D-loop. The primary enzyme involved in this process is the RecA protein [32], a 38-kDa monomer with ATPase [33], co-protease [5;34], and DNA renaturation and strand-exchange activities [3]. The first step in the RecA catalyzed strand invasion process is the formation of a "presynaptic" filament composed of several RecA monomers bound to the invading 3' single strand [35]. The formation of this filament is brought about by the loading of RecA by RecBCD or RecFOR [1; 36] after displacement of single-stranded binding protein (SSB), which is stabilizing the single- strand. In the case of RecBCD catalyzed RecA loading, the RecA is directly loaded onto the Chi bearing single-strand after ejection of SSB by RecBCD (another activity RecBCD displays after Chi-encounter) [1; 37].In cells functioning through alternative pathways the RecQ protein carries out the function of loading RecA (accompanied by RecFOR) to form the presynaptic filament [1]. RecO in these cells functions as a DNA-binding protein with ss DNA annealing and weak D-loop formation activity. Along with RecF (weak ATPase) and RecR it can bring about homologous exchange, and is responsible for displacing the SSB to enable formation of the presynaptic RecA filament. Once formed the RecA filament can scan the homologous chromosome for regions of homology, the exact mechanism of which is not clearly understood, but which eventually leads to "synapsis" or crossing-over, followed by pairing in the region of homology to give the heterologous 4-stranded Holliday intermediate.

C) Branch migration and heteroduplex extension: The above formed Holliday intermediate extends along the heteroduplex joint in a phenomenon called branch-migration which is accompanied by repair of the invading strand using the invaded

homologue as template. The process is powered by ATP hydrolysis and is catalyzed by members of the Ruv family of protein-the RuvAB proteins. RuvA, a tetramer facilitates loading of the hexameric RuvB at opposite arms of the Holliday junction, and powers extension in both direction by "pumping out" the DNA in both directions. [1]

D) Holliday structure resolution into recombinants: The last step of the process is brought about by the RuvC. The dimeric RuvC protein is a site-specific endonuclease that, in the presence of magnesium, cleaves at the Holliday junction, at either the strands involved in the cross-over, or those that are not, to give spliced/patched recombinants as explained above[38].

1.2 RecBCD: Functionality, biochemical properties and mechanism of action

The RecBCD enzyme is a 330 kDa heterotrimer composed of the RecB, RecC and RecD subunits, each with their distinct biochemical activities. As a holoenzyme it functions in the first step of homologous recombination, initiation of recombination by processing of blunt ended double-stranded breaks to produce a 3'ended single-strand. Its functionality and biochemical activities are regulated by the octameric regulatory sequence called "Chi", which plays a critical, but not clearly understood role in RecBCD mediated recombination. This section outlines the basic function of RecBCD in *E. coli*, followed by an overview of the properties of its three subunits, starting with their discovery and isolation. The biochemical activities of the holoenzyme are reviewed next, along with the mechanism of its action. Finally its interaction with the Chi sequence and the relevance of this regulatory element is discussed in detail in the last part of the introduction.

1.2-1 Function of RecBCD in *E. coli*: RecBCD is critical to the maintenance of chromosomal integrity in *E. coli*. In addition to its most obvious role in recombinatory processes, it also serves the purpose of protecting the *E. coli* chromosome from foreign invading DNA by degrading it. Its functions in maintenance of *E. coli* genome can be briefly summarized as follows.

A) Recombination: RecBCD functions during the recombination events that accompany the processes of transduction, conjugation, transformation, recombination of λ *red gam* phage and even during replication. In general, RecBCD is the first enzyme in any recombination pathway that starts with a linear duplex molecule [39; 40].

B) Repair: The RecBCD pathway is the primary pathway for recombinational-repair of double-stranded breaks and damage in *E. coli*, the kind that is induced by exposure to X-ray, UV or ionizing radiation and certain mutagenic agents. Recent studies have also elucidated the role of RecBCD in the repair of stalled replication forks and gaps or breaks that occur during replication as described above[1; 5].

C) Degradation of foreign DNA: Finally, RecBCD protects the host chromosome from invasions of phages and foreign DNA. There are several phage products, like the Gam protein of bacteriophage λ, and the gene 2 product of T4 phage, that are potential inhibitors of RecBCD activity [41; 42; 43]. If these genes are mutated the survival and replication of the phages in *E. coli* is severely affected. In addition to this, RecBCD also affects the rolling circle (σ) replication of plasmid DNA [44] (which produces infective concatamers). The double stranded end of this σ form can be bound by RecBCD which then proceeds to degrade the DNA.This is overcome if the plasmid has correctly oriented

Chi sequences, suggesting that the nuclease activity of RecBCD may be directly involved in the protection against foreign DNA.

1.2-2 The individual subunits: The RecBCD holoenzyme is composed of three individual subunits, which have been isolated, purified and characterized for their individual biochemical activities and contributions to the holoenzyme function. The *recB* and *recC* genes were the first ones to be identified on the basis of studies on recombination deficient *E. coli* mutants. RecD was identified later as the third essential subunit of the trimer. The studies on mutants, accompanied by physical characterization of the *recBCD* locus, sequence analysis of the genes and purification and biochemical characterization of the proteins, led to the implication of the 134 kDa RecB, 129 kDa RecC and 67 kDa RecD proteins in the first (initiation) step of recombination in *E. coli*.

A) Discovery and isolation of RecBCD: Studies on *E. coli* K-12 mutants showing reduced conjugational and transductional recombination, reduced cell viability and sensitivity to DNA damaging mutagens and radiation, led to the identification of *recB* and *recC* in the thyA-argA region of the *E. coli* chromosome [45; 46]. The discovery of these genes (1968) was followed by the initial isolation of RecBCD by Wright and Buttin (1971), and its identification as an ATP-dependent DNAse [26]. The enzyme was further purified to near homogeneity by Goldmark and Linn (1972), who characterized it as a DNA-dependent ATPase, ATP dependent exonuclease and endonuclease (on linear single-strands) [25], and later also by Lieberman and Oishi (1974) [25; 26; 46; 47]. Goldmark and Linn employed a multistep purification protocol involving a DEAE-cellulose column,

followed by gel filtration, DNA-cellulose chromatography and glycerol gradient sedimentation to purify RecBCD from *E. coli* K-12 [25].

At this point the RecD subunit was still not identified, and hence most preparations of RecBCD with exonuclease activity were initially thought to be RecBC. However, since RecBC by itself is not a good exonuclease, all these preparations had to be RecBCD. The actual discovery of the *recD* gene came much later, even after its initial isolation by Lieberman and Oishi. Since the RecD subunit was yet to be identified at the time of this purification, Lieberman and Oishi set out to purify what they thought was RecBC (but was actually RecBCD), and the identification of RecD was the accidental outcome of their purification procedure. The purification involved application of an inactivated (by use of high salt to separate the subunits) enzyme preparation on a DEAE-Sephadex column with its subsequent elution with a 0.15-0.45 M NaCl gradient. The enzyme, under these conditions dissociated into two inactive fractions. A low salt eluting fraction "α" (0.15 M NaCl) and a high salt eluting fraction "β" (0.28 M NaCl). Reconstitution of these two inactive fractions led to the restoration of wild type activity. As complementation studies on another class of mutants, the *recB* double dagger mutants later revealed, the high salt eluting fraction was the essential third subunit of the holoenzyme [48]. The *recB* double dagger mutants lacked the exonuclease activity, which reappeared when extracts of the mutants were reconstituted with purified β fraction but not the purified α fractions. These studies were carried out by employing a glycerol gradient to separate the α and β fractions obtained by Lieberman and Oishi. The faster sedimenting β fraction in this case was determined to be a 58-kDa polypeptide, which was not synthesized by the *recB* double dagger mutants.

The gene locus for this 58-kDa polypeptide was further mapped to the RecBCD locus (between *recB-argA* region of the chromosome), and the gene was designated as *recD*, the third essential subunit of RecBC, making it the RecBCD exonuclease. [48 ; 49; 50; 51] Characterization of the RecBCD locus (thyA-argA region) revealed that, while RecC was independently transcribed RecB and RecD constituted a single operon which was transcribed upstream of the *recB* gene. Complete sequencing of the *recB*, *recC* and *recD* genes following this study, led to the determination of molecular weights of the individual subunits which were estimated to be 134 kDa, 129 kDa and 67 kDa respectively.[48; 49; 50; 51]

B) RecBCD mutants: A large contributing factor in defining the function of RecBCD, as the primary enzyme involved in recombination initiation, was the complementary data available from studies on several recombination deficient mutant *E. coli* strains. These strains showed reduced cell viability, decreased phage recombination, transduction, conjugation, increased sensitivity to DNA-damaging agents, and reduced ATP-dependent exonuclease activity[5]. The mutants led to the identification of the RecBCD locus within the thyA-argA region of *E. coli* chromosome.

Further studies on mutants revealed that a large number mapped to either the *recB* or *recC* genes. These were classified as null, Class I, II and III mutants [5]. In addition the RecBC*D mutants and one RecBCD double dagger mutant also mapped to the *recC* gene. The *recD* gene was affected in the ΔrecD class of mutants and the majority of the RecBCD double dagger mutants. In general, most of the mutants studied so far map to defective *recB* or *recC* alleles and show considerable similarities in their phenotypes, which are briefly described as follows.

recB or *recC* mutants: Defective alleles for *recC* and *recB* are classified into either class I, II or III mutants. Mutants belonging to all three classes are recombination deficient and unable to form heteroduplex DNA *in vivo*. In addition, the Class I mutants ($recB^{2109}$) also show reduced helicase, ds and ss exonuclease and endonuclease and are defective in ATP-binding [52]. Further they do not display Chi-specific fragment formation, suggesting a defect in Chi recognition, or interaction. [53; 54] The Class II mutants (map to both *recB* and *recC*) display similar phenotypes as Class I in that they are defective in Chi recognition, recombination and heteroduplex formation. [52] Class III mutants (map to a single allele of *recB*) on the other hand can recognize Chi and are defective in recombination and heteroduplex formation *in vivo*. [52; 55] The *recC* gene is further found to be affected in the $RecBC^{*}D$ mutants. These are psuedorevertants of a missense mutation in *recC* that recover their recombination and heteroduplex formation ability. They are potent helicases and have wild-type exonuclease activity. The distinctive phenotype they have is that they are unable to stimulate recombination at Chi or Chi-dependent joint molecule formation, suggesting that Chi recognition may be affected in these mutants. [56] The *recC* gene is also affected in one mutant belonging to the RecBCD double dagger class of mutants. These mutants are defective exonucleases and once again, do not support Chi-dependent recombination [48].

recD mutants: Of the several mutants of RecBCD identified and studied, the *recD* gene is only affected in 2 classes of mutants. Most of the *recD* mutants are recombination proficient, UV and gamma radiation resistant and have normal cell viability, however they lack ds and ss DNA exonuclease activity and do not support Chi dependent recombination. A majority of the *recD* mutants belong to the RecBCD double dagger

class of mutants that show a defective exonuclease and are defective in Chi dependent heteroduplex formation [48; 57]. The other class of mutants that have a defective *recD* gene are the ΔrecD mutants, which also show the same defects as the RecBCD double dagger mutants [5].

Other mutants of RecBCD: Along with the above mentioned classes of spontaneously occurring or induced mutations, several mutants of *recB* and *recD* genes have also been constructed specifically for studying their effects and characterizing the activities of the individual subunits. One such mutant, a lysine to glutamine modification in the consensus nucleotide binding motif of *recB* (K29Q) led to the abolishment of ds DNA dependent exonuclease and ATPase activities, suggesting that RecB could be the primary helicase in RecBCD[58]. A similar lysine to glutamine mutant of the *recD* nucleotide binding motif (K177Q) showed reduced ds DNA dependent ATPase and exonuclease activities (ds and ss exonuclease) and most importantly, loss of processivity of unwinding, which reduced from 30kb (wild type) to approximately 1.5kb in the mutant [59]. Mutant constructs of the nuclease motif on the C-terminal 30 kDa domain of *recB,* the $recB^{D1080A}$, $recB^{D1067A}$ and $recB^{K1082Q}$ have been shown to abolish endonuclease and exonuclease activity of the enzyme, suggesting that RecB is the active nuclease. These nuclease site mutants are discussed further in the ensuing section on biochemical activities of RecBCD. Further studies on the $recB^{D1080A}$ mutant have also shown that it eliminates RecA loading by RecBCD, suggesting that RecB, along with being the primary nuclease, ATPase, and helicase, may also be involved in directing RecA onto the 3' Chi terminated strand [60].

1.2-3 Biochemical activities of RecBCD: Before purification and biochemical characterization of the individual subunits activities was achieved, the sequencing of the RecBCD locus (*thyA-argA* region of *E. coli* chromosome), coupled to sequence homology studies with ATPases, UvrB and myosin, revealed the presence of consensus nucleotide binding sites in the *recB* and *recD* genes. RecB further has the helix-turn-helix motif characteristic of some prokaryotic DNA binding proteins. Hence the sequence homology studies gave some indication about RecBCD functionality, which was later corroborated and enhanced by the biochemical characterization of its activities (both the holoenzyme, and the individual subunits) following purification. We now know RecBCD displays a host of biochemical activities, which are individually discussed and detailed next.

A) DNA dependent ATPase activity: Early studies on the characterization of the ATPase activity of RecBCD, demonstrated that ATP hydrolysis by RecBCD was intrinsically linked to its helicase activity. These studies carried out by Roman and Kowlczykowski showed that ATP hydrolysis by RecBCD followed a biphasic pattern that was composed of a fast component, and a slower component [24]. The fast component was demonstrated to coincide with DNA unwinding, with the slower round of hydrolysis being attributed to the accumulation of ss DNA in the reactions (following unwinding of the initial duplex substrate). Based on their results they estimated the K_m and k_{cat} values for ATP hydrolysis to be approximately 85 μM and 45 μM ATP sec^{-1}, with 2-3 ATP being hydrolyzed per base pair unwound. (This is relatively energy efficient, considering free energy of ATP hydrolysis is -11.3 Kcal/mol at 25°C, which is sufficient for opening

four AT base pairs or two GC base pairs (free energy of unwinding is 2.5 kcal/mol and 5 kcal/mol respectively))[24].

Following initial characterization of the ATPase, attempts were made to identify the specific subunit/subunits of RecBCD involved in this activity. Information available from the gene sequences of *recB* and *recD,* showing the presence of consensus nucleotide binding motifs suggested that one or both of these subunits could be the active helicase/ATPase of RecBCD. Actual evidence for this interaction followed, through the photoaffinity labeling experiments on RecBCD [61] and isolated RecB [62]. These experiments involving incubation of RecB/RecD with varying concentrations of the radioactive analog of ATP (α-^{32}P) 8-azido-ATP, in the presence and absence of oligonucleotides, positively identified the two subunits as ATP-binding sites within RecBCD. Further studies on mutants of the consensus nucleotide binding sequence of RecB and RecD (reconstituted into RecBCD) showed that while both subunits bind to ATP, RecB was probably the primary ATPase/helicase, with RecD playing a secondary role [59; 63; 64]. The RecB (K29Q) mutant within the consensus ATP binding site of RecB, resulted in essentially no ds DNA dependent ATPase (and nuclease) activity, and a greatly reduced (5-8-fold) ss DNA dependent ATPase. A similar mutation within the consensus ATP-binding site of RecD (K177Q) results in reduced affinity for ATP, and reduced processivity of unwinding (1.5 kb stretch of DNA vs 30 kb for wild type). However, it does not lead to the near total loss of ATPase or exonuclease function noticed for the RecB (K29Q) mutant [59; 63; 64]. Further studies by Boehmer and Emmerson on the isolated RecB subunits, confirmed its role as a ds DNA dependent ATPase and helicase, and studies on the isolated RecD subunit by Chen and Julin, characterized it as a

weak ss DNA dependent ATPase [62; 65]. Following identification of RecB as the primary ATPase/helicase, and RecD as the weaker ATPase, studies were also carried out to characterize the ATPase activity of RecBC. As expected RecBC showed reduced ATP-hydrolysis (10-fold reduction), leading to reduced unwinding and reduced processivity (in unwinding) as compared to RecBCD [66; 67].

B) ATP dependent helicase activity: The studies on ATP hydrolysis by RecBCD clearly showed that it was coupled to the DNA helicase activity of the enzyme. The earliest attempts to characterize this activity in RecBCD, were carried out by Taylor and Smith, using electron microscopy to visualize glutaraldehyde fixed reaction products of short (20-60 sec) unwinding reactions [30]. Their study showed the presence of partially unwound recombination intermediates, which enabled proposition of a model for RecBCD mediated unwinding of duplex substrates, and estimation of initial rates. The most common product observed on the micrographs (25-50% of the molecules) was a ”twin-loop” structure with two internally unwound loops of the substrate DNA flanked by base-paired DNA at either end (figure 1.5). 5-25% of the molecules seen were in the form of “loop-tail” structures bearing a single-stranded “tail” on one strand of the duplex, and a single-stranded loop on the other strand (figure 1.5). Based on calculation of the length and mean distance traveled by the twin-loop or loop-tails for individual time points they estimated an unwinding rate of 300 bp/sec. Further, the loops were found to enlarge at a rate of 100 bp/sec, leading to the proposition that the unwound substrate rewound at a rate of 200 bp/sec. On the basis of this study, Taylor and Smith proposed a model for RecBCD unwinding which is represented in figure 1.5. According to the model, RecBCD is bound primarily to one strand of the duplex, unwinding it faster than

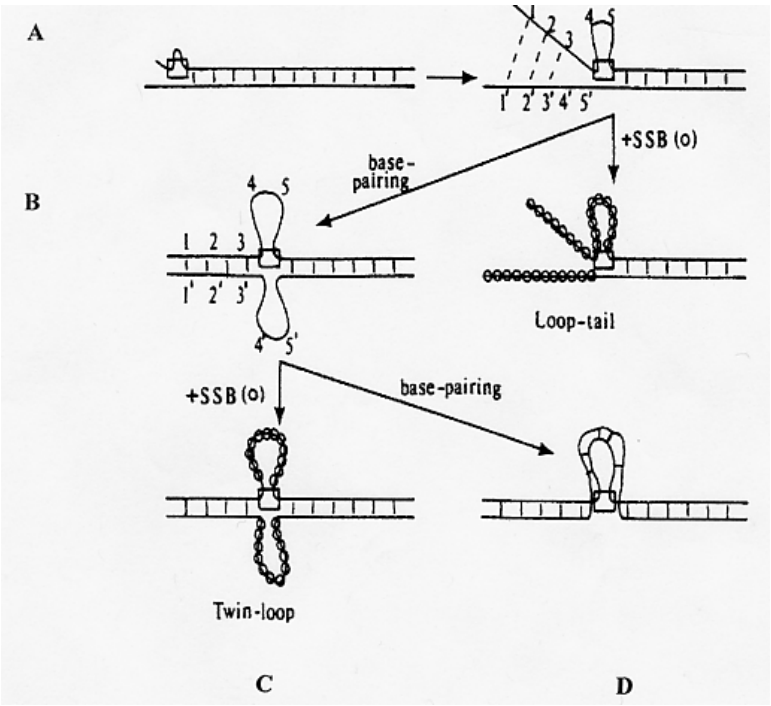


Figure 1.5

Intermediates of RecBCD unwinding: Twin-loops and Loop-tails

A. RecBCD is shown as a rectangle moving along the duplex unwinding it at unequal rates resulting in the displacement of a loop and formation of the "loop-tail intermediate"

B. The loop displaces a complementary region in the opposing strand, leading to formation of "twin-loops". Loop-tails are stabilized in the presence of SSB.

C. Twin-loops are also stabilized in presence of SSB.

D. In absence of SSB, the twin loops re-basepair. (Sourced from [30])

it gets rewound, leading to the displacement of a short loop in the strand, and the subsequent production of a tail in the other strand. These are further stabilized by SSB, preventing them from base pairing again. Under these circumstances, the single-stranded loop displaces its complementary region in the opposing strand leading to the formation of twin-loops.

Roman and Kowalczykowski further characterized the helicase activity of RecBCD using a novel assay, based on the quenching of intrinsic fluorescence of SSB on binding to DNA unwound by RecBCD[23]. Their assays, performed at varying temperatures (16 °C-37 °C), varying RecBCD, DNA, ATP, and salt concentrations, and in the presence of either linear dsDNA or super-coiled RF M13 DNA as substrate, showed that RecBCD preferred linear dsDNA as substrate for unwinding. Taylor and Smith, further characterized the substrate specificity[39]. Their studies on RecBCD showed that it preferred flush-ended, or nearly flush-ended linear duplexes for unwinding substrates. 3' or 5' flanks greater than 25 nucleotides long, were poorly unwound. Further, several circular gapped duplexes tested were also not unwound by RecBCD. Based on these results, it was concluded that RecBCD needs to interact with both strands of the DNA in order to facilitate unwinding, which is probably why it cannot unwind the gapped duplexes or the 3' and 5' flanked duplex, as it cannot bind to both strands at the same time. Confirmation of this result was provided by the results from Ganesan and Smith's UV-cross linking experiments which showed that while RecB bound to the 3' end of the duplex, RecC and RecD bound the 5'end [40] . Based on their results, they reinterpreted Taylor and Smith's model, in terms of unequal translocation of RecBCD along the two strands. According to their model, the RecCD subunits translocate along

the 5’ strand at a faster rate of 350 bp/sec, while the RecB subunit translocates the 3’ strand at a slower 250 bp/sec, leading to the formation of a loop-tail structure with the loop moving at 100 bp/sec.

This hypothesis is also supported by recent studies by Taylor and Smith. Their studies involved visualization of the unwinding intermediates of RecBCD, RecBC, $RecB^{K29Q}CD$ (RecB helicase mutant) and $RecBCD^{K177Q}$ (RecD helicase mutant) by electron microscopy. They observed similar rates of unwinding with wild-type RecBCD and $RecB^{K29Q}CD$, but only 20% unwinding (as compared to wild type) by $RecBCD^{K177Q}$ and RecBC. This led them to conclude that RecD was the faster translocating helicase. Examination of the nature of loop-tails of the reaction intermediates further demonstrated that the 5' end was being unwound faster than the 3' end. These experiments taken together with previous evidence that shows RecB binds to 3' end and RecD to 5' end lead to the conclusion that RecD is a fast 5’-3’ and RecB is a slower 3-5’ translocator.[68] Dillingham et al have further characterized this 5'-3' helicase activity of RecD as being ATP and magnesium dependent [69]. These studies taken together characterize RecBCD as a ‘bipolar” helicase with opposing helicase motors RecB and RecD, translocating unequally along the two strands (leading to the formation of the loop-tail structures visualized in earlier electron micrographs).

Finally, recent studies involving visualization of RecBCD translocating on an immobilized fluorescent DNA substrate have lead to the most recent estimation of rates for unwinding, to be approximately 1000 bp/sec, with as many as 42,300 bp unwound per RecBCD molecule, and a processivity of 30,000 bp[70].

C) Exonuclease activity: This was the first biochemical activity attributed to RecBCD following its purification by Wright and Buttin in 1971[26]. Wright and Buttin purified this activity using hydroxyapatite chromatography and glycerol gradient sedimentation, and coined the term exonuclease V to define it. They characterized it as acting on both linear ds DNA and linear ss DNA, being ATP and magnesium dependent, and acting over a broad pH range with optimum of pH 9.2. Their study also showed that this was a 3'-5' exonuclease producing 3'-hydroxy and 5'-phosphate terminated oligonucleotides with the average length of these products decreasing as the reaction proceeded.

Shortly after this initial characterization of ExoV, Mackay and Linn carried out a study on the mechanism of duplex DNA degradation by RecBCD [71]. They used multiple approaches of sedimentation, isopycnic centrifugation and electron microscopy to examine intermediates of exonuclease action in an attempt to explain its mechanism. Their study showed duplex intermediates with both 3' and 5' single-stranded tails, and single-stranded fragments several hundred nucleotides in length were produced during the reaction. They concluded from their studies that RecBCD was both a 3'-5' and 5'-3' exonuclease, and proposed that it unwound and degraded one strand at a time (explaining the presence of long-tailed intermediates), producing fragments 100-500 nucleotides long. These, on accumulation also act as substrates for RecBCD, and eventually are degraded to smaller oligonucleotides. After acting on one strand for a while, the enzyme switches polarity and degrades the opposing strand in a similar manner. An important aspect of the interaction that came into focus during the course of these studies was the significance of ATP: Mg^{2+} ratio. A higher concentration of Mg^{2+} than ATP (10 mM Mg^{2+}/25-40 μM ATP) was found to be optimal for exonuclease action,

resulting in the production of small oligonucleotides [25 72]. Lower Mg^{2+} (less than 0.5 mM), or higher ATP (millimolar amounts) resulted in decreased exonuclease activity, and partially unwound duplexes, and long single-stranded tails were obtained as products [71]. Further, like the helicase activity, the exonuclease activity was also found to be asymmetric with the 3' end being degraded faster than the 5' end. [29; 73]

The most significant development in the study of RecBCD exonuclease activity came with the identification of the RecB subunit as the exonuclease of RecBCD. Initial identification of the nuclease active site came through protease digestion studies on isolated RecB. These studies lead to the identification of two distinct domains in RecB: a 30 kDa C-terminal domain that seemed to resist any further digestion, and a 100 kDa N-terminal domain. The 100 kDa N-terminal domain was found to bind DNA and was shown to be active as a helicase when reconstituted with RecC and RecD but not as a nuclease (neither exonuclease, nor endonuclease) [74]. This suggested that the 30-kDa RecB domain might play an important role in the nuclease function of RecBCD. However characterization of its activities posed a problem as this domain did not bind to DNA and was found to flow through a ss DNA-agarose column during purification. This was overcome by constructing a fusion of this domain with the DNA-binding, (non-nucleolytic), T4 phage gene 32 protein (first 254 codons) [75]. The purified fusion protein was used in an endonuclease assay, and was found to be active as an ATP-independent endonuclease on single-stranded circular M13-phage DNA. Additionally, an Asp to Ala mutant of the C-terminal domain, $RecB^{D1080A}$ when reconstituted with RecC and RecD was found to be inactive as an exonuclease and endonuclease [75]. This mutation maps to a particular motif (pro-asp-X15-19-asp-X-lys) common to several nucleases like *Eco*RI and

*Eco*RV. The Asp residue in this motif is speculated to be involved in chelating magnesium. This mutant protein was found to be inactive both as an endonuclease (on M13 single-stranded circle) and as an exonuclease (on linear duplex and single-strands), even at 10 times higher concentrations than the wild type RecBCD [75]. However, the mutant retained its helicase activity, confirming that this site on the C-terminal domain is the primary nuclease and in fact, the sole nuclease in RecBCD.

This result was further corroborated by studies on two other "nuclease motif mutants" of the C-terminal domain. The Asp1067 and Lys1082 residues of the C-terminal domain, also a part of the motif common to the restriction endonucleases mentioned above, were mutated in this study [76]. The reconstituted, mutant proteins $RecB^{D1067A}CD$ and $RecB^{K1082Q}CD$ were both found to be inactive as endonucleases (on M13 circles) and exonucleases (on duplex substrate). They were also unable to produce Chi -specific fragments either by 3'-5'cleavage or 5-'3' cleavage, confirming that the same nuclease site in RecB is responsible for both these activities.

These studies have helped establish a clearer picture of RecBCD exonuclease function, defining it as an ATP-and magnesium dependent 3'-5' and 5-3' exonuclease on single and double-stranded linear DNA. It is also an ATP independent endonuclease on single-stranded circles, and the RecB subunit is the exclusive nuclease that hosts both these nuclease functions.

D) Endonuclease activity: RecBCD, though primarily an ATP-depended exonuclease, with a preference for duplex substrates, is also the only non-specific endonuclease in *E. coli*. This activity of RecBCD, unlike the exonuclease, does not need ATP, but is stimulated approximately sevenfold by ATP. [25; 53] The enzyme can catalyze cleavage of

single-stranded circles, [25; 27] single-stranded gaps between duplexes, [77] and D-loop structures. [78]

1.2-4 Chi-recognition activity

Probably the most intriguing feature of RecBCD's biochemistry is its recognition of, and interaction with the octameric Chi sequence. As mentioned above in the section on RecBCD's exonuclease activity, an interesting and unresolved feature of the exonuclease activity is its apparent attenuation on encountering a specific DNA sequence called Chi (χ). This is an octametric DNA element that stimulates recombination by RecBCD, and its encounter with the enzyme is absolutely critical for the initiation of recombination by RecBCD. The sequence 5' GCTGGTGG '3, [79] occurs with a very high frequency (one/5 kb of the genome, 1009 in the genome) on the *E. coli* chromosome. [80 81]

A) Discovery and early studies: The Chi sequence was first discovered in certain mutant λ bacteriophages, which were incapable of making the "gam" protein (product of the "red" genes, which is responsible for suppressing RecBCD nuclease activity) [82]. These mutants were incapable of forming infective concatamers and hence formed small sized plaques. However a set of extragenic suppressor mutations in these phages, resulted in a reversion to wild-type phenotype (formation of large plaques). It was revealed that these suppressor mutations lead to the generation of Chi sites on the phage genome, which enabled RecBCD mediated production and packaging of infective concatemers, leading to formation of larger plaques and reversion to wild type.

Early studies on the properties of Chi were done on Chi's role in the RecBCD-mediated recombination of these mutant λ phages. As mentioned above, it was observed that Chi stimulated recombination as far as 10 kb from the site, though with decreasing magnitude. The strongest stimulatory effect was obtained within the immediate vicinity of the sequence. Further, the stimulation was mainly to the left (5' end) of the Chi sequence, [83] and was dependent on the orientation of the Chi site relative to the *cos* site on the λ genome (from which sticky ends are generated by λ terminase for packaging and injection) [31]. Genetic evidence also indicated that Chi was a specific recognition sequence for RecBCD and that RecBCD nuclease activity is needed for Chi recognition[57].

Ponticelli and Smith provided the biochemical evidence that corroborated the genetic analysis of Chi [31]. Their experiments showed that RecBCD when presented with a Chi-bearing linear duplex substrate labeled at the 3' end, released a nucleolytic fragment whose size was consistent with RecBCD cutting the substrate at, or just before the Chi sequence and undergoing an attenuation in its nuclease function thereafter. Further, their experiments also showed that the strand bearing the Chi sequence (5' GCTGGTGG 3') was cut, and not its complement, and that this Chi dependent cleavage occurred only on duplex substrates, suggesting that Chi recognition occurs before or during unwinding of the substrate, and not after it.

Following closely on the heels of Ponticelli and Smith's biochemical characterization of Chi, Taylor and Smith's studies provided further insight into orientation and polarity dependence for Chi cleavage [84]. Their study showed that RecBCD cleaved the Chi-bearing strand of a duplex substrate 4-6 nucleotides from the 3' end of the Chi, leaving a product with a 3'-OH group and a 5' PO_4. They employed

multiple approaches to study the polar nature of RecBCD-Chi interaction. In one set of studies, they examined the time-dependent appearance of Chi cleavage product in substrates with Chi placed at either the 5' or 3' end. They observed a greater time lag (15 sec) in appearance of cleavage products with the 5' ended Chi substrate, as compared to the 3' Chi ended substrate (5 sec). They proposed that this was a result of RecBCD unwinding the substrate from the 3' end and producing the Chi cleavage product on Chi encounter. This result also indicated that approaches from the 5' end did not lead to recognition of Chi. They confirmed this further using substrates that RecBCD could enter from only the 5' end, or substrates that could be entered from both ends, and found the Chi dependent cleavage product only in the latter case. The combined results of their study confirmed the initial finding that Chi has to be approached from its 3' end for RecBCD to recognize it.

B) Effect of Chi on RecBCD:

Several studies showed that RecBCD is altered in some way following Chi encounter. Taylor and Smith studied the action of RecBCD on substrates bearing multiple (two) Chi sites [85]. Their study showed that RecBCD could only cut at one or the other of the Chi sites within the same molecule, but not at both. The enzyme showed reduced unwinding and Chi cutting when presented with a second different DNA molecule after it had a chance to react with a Chi-containing DNA. This clearly suggests some kind of alteration in enzyme structure post Chi-encounter.

In the most current study of Chi-RecBCD interaction by Spies *et al* a slowing of unwinding rate from 1000 bp/sec to 500 bp/sec post Chi encounter was observed [86]. Their study, involving visualization of single molecules of RecBCD

traversing a fluorescent-labeled Chi bearing duplex substrate, showed that the enzyme paused for about 5 seconds at the Chi sequence, following which its rate of movement and unwinding along the rest of the duplex slowed down to half its initial rate of 1000 bp/sec. Hence, it seems clear from these studies, that the enzyme undergoes some kind of alteration or modification in its structure following Chi encounter.

The exact nature of this alteration is still under considerable debate and discussion. There is some biochemical evidence that suggests that the Chi induced alteration of RecBCD is the ejection of the RecD subunit from it. This was proposed by Dixon and Kolwaczykowski, based on the reversible inactivation of RecBCD helicase activity on Chi-bearing substrates.[87] They saw that unwinding of Chi-bearing substrates by RecBCD was inhibited in low magnesium concentration, but the inhibition was overcome by addition of excess magnesium. Since this behavior of RecBCD was very similar to that of RecBC, without the RecD subunit, it was proposed that Chi encounter caused the functional or physical loss of RecD.

However, this suggestion was contradicted by later evidence, on RecBCD's nuclease activity following Chi encounter. Anderson and Kowlaczykowski showed that under some conditions (low magnesium concentrations) the slower 5'-3' exonuclease activity of RecBCD is activated following Chi encounter.[88; 89] The enzyme switched polarity of degradation, and started degrading the non-Chi-bearing strand preferentially. If this is an effect of ejection of the RecD subunit, or its inactivation, RecBC should also show the 5'-3 exonuclease activity. Since RecBC does not exhibit any 5'-3' exonuclease activity under these conditions, they concluded RecBC was not

biochemically analogous to RecBCD post-Chi, and hence the alteration in RecBCD was not the simple ejection of the RecD subunit.

The most recent proposal by Taylor and Smith supports the theory of disassembly of RecBCD into individual subunits, post-Chi encounter [90]. Their work involved the incubation of RecBCD with either a 345-bp duplex with 3 tandem Chi sites, or a similar 321-bp duplex without the Chi sites, and then testing the enzyme for DNA unwinding, exonuclease activity and Chi cutting. They observed much greater attenuation of all RecBCD activities by the Chi duplex, as compared to the non-Chi duplex suggesting that the enzyme was being inactivated by Chi (greater than 30 fold inhibition of exonuclease activity was seen by the Chi duplex, but less than 20 fold with the non-Chi). Further, they found that this inactivation by Chi was reversible and activity could be regained after addition of excess magnesium (about 50 % of the exonuclease activity regained in 15 minutes of addition of magnesium to 10 mM). In an attempt to determine the basis of this inactivation they carried out glycerol gradient sedimentation on the preincubated enzyme aliquots and observed a significant increase in the amount of free subunits obtained with Chi incubated RecBCD (66-68%) as compared to RecBCD incubated with the non-Chi DNA or RecBCD alone (5-13%). Their results led them to conclude that the basis for the inactivation of RecBCD is the disassembly of the subunits of RecBCD following Chi encounter. They also proposed that the subunits could be reassembled in presence of excess magnesium to form an active holoenzyme again.

C) Model for Chi regulated recombination by RecBCD: Based on our current knowledge and understanding of the properties of Chi, and RecBCD, a model that encompasses the key features of their interaction, and demonstrates the RecBCD-Chi

mediated initiation of recombination is outlined as follows (figure 1.6). RecBCD binds the duplex substrate at the point of a double-stranded break, (RecB is proposed to bind the 3' end of the duplex, while RecC and RecD are bound to the 5' end [40]) and its processive helicase starts unwinding the substrate, while the nuclease formation of a 3 'Chi-ended stretch of single-stranded DNA, for homologous recombination by RecA activity degrades the unwound single-strand. This process continues until RecBCD encounters a correctly oriented (with its 3' end towards RecBCD) Chi sequence. Encounter with such a sequence leads to nicking of the Chi containing strand 4-6 nucleotides on the 3' side of Chi, which is followed by an attenuation of the 3'-5' exonuclease activity. (Under certain magnesium concentrations the enzyme is proposed to switch polarity of degradation at this point and function primarily as a 5'-3' exonuclease on the non-Chi strand [89]). The result of this attenuation is that RecBCD now travels the remaining stretch of DNA, unwinding it without degrading the Chi bearing strand, leading to the production of a 3' Chi-ended single-stranded overhang which is then used by RecA and SSB for D-loop formation.

1.2-5 Structural basis for the effect of Chi on RecBCD function.

As discussed above, the nature of Chi-induced modification of RecBCD is still under considerable debate and speculation. One possibility is that there is a specific site on RecBCD that binds Chi and prevents it from binding or acting on the substrate. Such a Chi-specific site could be present on one of the subunits or may be shared between them. Binding of Chi could also induce a conformational change in RecBCD, which renders it catalytically inactive. Studies on *recB* and *recC* mutants suggest that

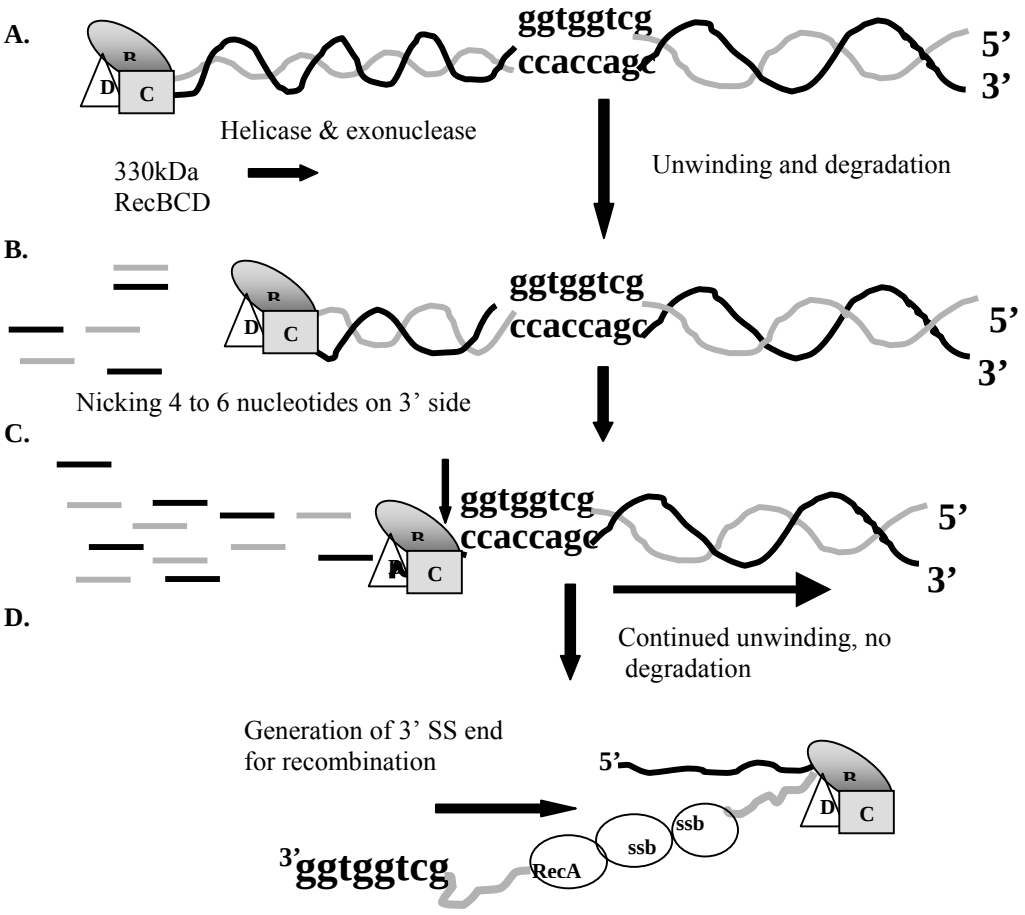


Figure 1.6

Model for Chi-mediated recombination by RecBCD:

A) RecBCD binds the double-stranded ends at the site of break or damage. **B)** It unwinds (helicase) and degrades (exonuclease) the duplex as it moves along it. **C)** On encountering Chi, RecBCD nicks the Chi bearing strand 4-6 nucleotides before the Chi sequence. There is an attenuation of its 3'-5' exonuclease activity following this encounter (it now functions mainly as a 5'-3' exonuclease on the strand that does not have Chi). The enzyme can however, still function as a helicase. **D)** The result of this process is the formation of a 3' single stranded end for RecA loading.

these subunits may have some role to play in Chi recognition. As outlined above, some of these mutants are defective in Chi recognition. The *recB*2109 mutant [53] and *recC** class of mutants are especially significant in this regard, as they seem to have all other RecBCD activities like helicase and exonuclease, and are only defective in Chi recognition. The *recC** mutants (bearing a defective *recC* allele, $recC^{1004}$) further have been shown to recognize an 11 nucleotide variant of Chi. Both the wild type and mutant proteins have been shown to stimulate RecA dependent joint molecule formation with this Chi variant. [91] These findings suggest that either of these subunits may have a Chi specific site on them, the locus of which may be altered in the mutant.

In terms of biochemical evidence however, RecB seems more likely to contain a Chi-specific binding site as it has been shown to bind the 3' end of the duplex, the end from which Chi is recognized [40]. The RecB subunit by itself has been shown to bind DNA (while RecC does not seem to bind DNA by itself [92]). RecB being the primary helicase/ATPase and nuclease of RecBCD may also be the primary mediator of Chi recognition.

In addition to these studies, there is also some evidence that suggests RecBCD may recognize and cut some "Chi-like sequences", to varying degrees of efficiency. Several single-nucleotide variants of Chi have been tested in that regard and show from 38% to 2% genetic activity relative to the wild type Chi [93]. Chi-like sequences recognized by specific exonuclease/helicase proteins have also been detected in unrelated bacterial species, like *Bacillus subtilis* (5' AGCGG 3' recognized by the DNA helicase and nuclease AddAB) and *Lactococcus lactis* (5' GCGCGTC 3' recognized by exonuclease/helicase RexAB).[94; 95; 96] The *Bacillus subtilis* AddAB and *Lactococcus*

lactis RexAB enzyme are functional analogs of RecBCD, involved in homologous recombination, and exhibit a similar kind of specificity for their respective Chi sequences. Analysis of the structural features of these related sequences might reveal certain similarities in base compositions that may also be responsible for the observed changes they induce in the biochemistry of their target proteins. This suggests that Chi may belong to a family of sequences with related structural features. Hence it is possible that the observed changes of RecBCD function stem out of it encountering an area of complex DNA topology at the Chi site, forcing a slight rearrangement of its subunits. Such a modified enzyme could lose one or all of its functions and activities, processivity, and optimum output to varying extents, explaining the observed specificity for the sequence.

1.2-6 Research significance

As seen from the above discussion**,** there are still several unexplained aspects of the Chi-RecBCD interaction that merit considerable further research and study. The only definitive conclusions that can be drawn from the study of this interactions, is that firstly, RecBCD seems to have some specificity for the Chi sequence, and that encounter with it somehow alters or modifies RecBCD (resulting in the observed changes in its activities).

As stated in the introduction, most of the work carried out so far on Chi-RecBCD has employed Chi-bearing duplex substrates. Chi-bearing single strands have never been tested for their efficacy as recognition substrates for Chi and we do not know if Chi can be recognized (leading to the ensuing changes in RecBCD's biochemical properties), when on single stranded DNA. One reason that this avenue has not been

explored is probably because it is known that RecBCD prefers duplex DNA as substrates. It has been known to bind very poorly to 3' or 5' flanks larger than 25 nucleotides in length. However, any study of the Chi-RecBCD interaction would be incomplete without a complete understanding of its effects on single stranded DNA as well.

This is especially important, as Chi is known to be recognized as a single stranded sequence in an otherwise duplex susbtrate[97] . Bianco and Kowlaczykowski have shown that Chi can be recognized by RecBCD when part of a single-stranded region of an otherwise duplex. They employed heteroduplexes containing either the Chi sequence (8 nucleotides) or a 22 nucleotide stretch of DNA containing the Chi within it, mismatched with non-complementary bases in the opposing strand. This kind of internal mismatch, in an otherwise duplex molecule, led to the formation of a single-stranded "bubble" region within the duplex. They found that not only did RecBCD travel through the mismatch bubble successfully, it was able to recognize the Chi within this region and produce the Chi specific cleavage product. This showed that Chi could be recognized as part of a single strand. However, even in this case, the susbtrate itself was a double stranded DNA, giving RecBCD the duplex ends it prefers for binding. Hence the interaction has never been studied on exclusively single-stranded substrates.

It would be interesting to examine this interaction when the enzyme has not been presented with the duplex end before it approaches a Chi. Under these circumstances, RecBCD may encounter Chi in a systematic approach from one or the other end of the ssDNA or it may be binding directly to the Chi sequence, (this is not possible with a duplex substrate, as the enzyme will always approach Chi from one end or the other). Hence the biochemical effects of this interaction might be different from

those reported with duplexes. If Chi can be specifically recognized on single stranded DNA, further examination of the size, structure of this DNA, or location of the Chi within it, might provide valuable information about the susbtrate specificity for Chi recognition, and contribute to a better understanding of the nature and mechanism of the interaction itself. The use of small sized oligomers for such a study would further enable a direct and straightforward study of the structural changes that occur post Chi encounter, and also detection of a Chi-specific site on RecBCD.

In this study, we have employed this approach in an attempt to understand and define better the RecBCD-Chi interaction. The study aims to test Chi recognition on exclusively single-stranded substrates and if detected, examine the effects of this recognition on RecBCD's biochemical activities and compare them to effects observed with duplex Chi bearing substrates. One of the key issues the study attempts to address through this approach, is that of specificity. Is there a specific Chi-RecBCD interaction on single stranded substrates leading to inhibition of RecBCD activity as observed before, or is the specificity abolished or reduced? This would also provide some information about the manner in which the two might interact. In addition to the biochemical changes resulting from the Chi interaction, the study initiates an attempt to identify a specific Chi binding site on RecBCD, by use of photoreactive nucleotide analogs to cross link single-stranded Chi substrates to RecBCD.

The approach employed involves the use of single-stranded oligonucleotides of varying lengths (8,14,20 nucleotides long) bearing either the Chi sequence (Chi^{+}) or a single nucleotide variant of the Chi sequence (Chi^{0}) in various reactions to test their effect on the biochemical activities of RecBCD. Results obtained

from this study show that in the presence of the oligonucleotides there is inhibition of the exonuclease, Chi-recognition and helicase activities of RecBCD.

The study also shows that in certain constructs, the Chi^+ oligomer inhibits the activities significantly better than the corresponding Chi^0 mutant construct of the same length and sequence, suggesting that it is being specifically recognized (and distinguished from the Chi^0) by RecBCD. These constructs are the ones in which the Chi^+ or Chi^0 is placed in the middle of the construct, flanked by some sequence at either end of it. This kind of specific recognition of the Chi^+ and its distinction from the Chi^0 is absent in the Chi-ended constructs and 8 mers (Chi sequence alone), in which, the attenuation of activities is very similar with either the Chi^+ or Chi^0 of a pair.

This is by far the most interesting result obtained in the study, which brings into focus a distinct and so far unreported property of Chi, that of its location-specific recognition. Chi seems to require a flank to be specifically recognized. The other interesting result obtained was the photocrosslinking of the RecC subunit to the oligomers, corroborating mutant studies that indicate RecC may be the primary subunit involved in Chi recognition.

Taken together, these studies provide some insight into the manner by which Chi (in these constructs) may be interacting with RecBCD. Based on these results a model that describes the location-specific interaction of Chi with RecBCD is proposed which also attempts to identify the RecBCD subunit that may be involved in this interaction. Though considerable further research and study is required to completely resolve the several contradictions and intricacies that define this complex interaction, this study hopes to have taken a small step in that direction.

CHAPTER II

EFFECT OF CHI ON THE BIOCHEMICAL ACTIVITIES OF RECBCD

2.1 Introduction

The following study examines the recognition of Chi on single-stranded substrates by RecBCD and characterize the related effects. A comparison of the results obtained here, with already known effects on Chi bearing duplexes, would serve to establish and confirm any substrate preference for recognition, and also provide additional insight into the mechanism of RecBCD-Chi interaction.

The broader aim of this study is however to validate RecBCD's specificity for the Chi sequence, as already observed with duplex substrates, and analyze the underlying basis for the specificity. The study attempts to detect this specificity by examining if the effects of RecBCD's interaction with the Chi sequence can be qualitatively or quantitatively distinguished from its effects with any other random DNA sequence. The approach employed is one of using single-stranded oligonucleotides, of varying lengths, with variably positioned Chi sequences in biochemical assays to test and characterize their effects on the RecBCD function. Another set of similarly designed single-stranded oligonucleotides containing a mutant Chi sequence are also employed in a set of assays done in parallel. The study aims to compare and analyze any differences in the effects observed with the two sets of constructs on RecBCD activities, and interpret the result in terms of specificity for the Chi sequence.

2.2 Materials and Methods

Materials

DNA oligonucleotides (Invitrogen.Corp.)

RecBCD enzyme (purified by Misook Yu)[75]

Shrimp alkaline phosphatase (United States Biochemicals)

*Hin*dIII Restriction endonuclease (Gibco BRL Life Technologies)

T4 Polynucleotide kinase (New England Biolabs)

γ-^{32}PATP (Amersham Pharmacia)

ATP 100 mM solution (Amersham Pharmacia)

Cellulose PEI-F TLC plates (JT Baker Incorporated)

Bovine serum albumin (New England Biolabs)

DNA grade agarose (Sigma)

Trichloroacetic acid (Fisher)

Minimal media (M9 media)[98]

Ampicillin (United States Biochemicals)

Vitamin B1 (Thiamine) (Sigma)

Tip-500 plasmid purification kit (Qiagen)

Methyl-^{3}H thymidine (Amersham Pharmacia Biotech)

Methods:

2.2-1 Design of oligonucleotides: Single-stranded oligonucleotides of varying lengths were designed with the Chi sequence at one or the other end (3' end or 5' end) and also in the middle of the construct with a flanking sequence at either end. [93]. For each Chi containing oligonucleotide, a corresponding "non-Chi" oligonucleotide was designed which has the exact same sequence except for a T to A modification within the Chi sequence, which renders it a non-Chi (akin to any random sequence) [93]. The Chi bearing oligonucleotides are denoted as Chi^{+} and the non-Chi ones as Chi^{0}. The oligonucleotides are listed in table 2.1.

Reconstitution and storage of oligonucleotides: The oligonucleotides were dissolved in approximately 200 μl of distilled water at room temperature for reconstitution and stored at -20°C. The Chi-centered 20 mers and 3' Chi-ended 20 mers were desalted by the manufacturer and used without further purification. Some batches of HPLC purified Chi-centered 14 mers and 20 mers did not inhibit RecBCD exonuclease activity, even at very high concentrations (50 μM 14 mer/ 20 μM 20 mer). These were further purified using a nucleotide exclusion kit (Qiagen), which restored inhibition, by these constructs (results not included here).

2.2-2) Preparation of tritiated chromosomal *E. coli* DNA:

A) Cell growth: A single colony of *E. coli* strain K-12 was grown in 2 ml M9 media[98] overnight at 37 °C with shaking. This was used to inoculate 200 ml of fresh minimal

media. When O.D.600 reached 0.1, 40 mg uridine and 2 ml of Methyl-^{3}H thymidine (1 mCi/ml) was added, and the culture was grown to saturation, at 37 ℃ for 8 hrs with constant shaking.

B) Isolation of chromosomal DNA: The cells were harvested by centrifugation at 5000 rpm for 10 min in a JA14 rotor (Beckman). The pellet was re-suspended in 20 ml of 50 mM Tris-HCl (pH 8.0) and incubated with 20 mg of lysozyme on ice for 45 min. The mixture was further incubated for 30 min, at 50 ℃, with 1mg/ml proteinase K in 4 ml of 50 mM Tris-HCl (pH 7.5) buffer containing 0.5% SDS and 0.4 M EDTA. The DNA obtained was then sheared by passing it successively through 18 G, 20 G and 25 G needles 3-4 times. The sheared DNA was subjected to repeated rounds of extraction in 1:1 phenol: chloroform and then centrifuged at 7000 rpm for 15 min, till the upper aqueous layer was clear and devoid of all the white material at the aqueous/organic interface. The DNA was then precipitated by addition of two volumes of ethanol and 6 ml 3 M sodium acetate and centrifuging for 30 min at 10,000 rpm.

C) Purification of isolated DNA: The pellet obtained was redissolved in 50 ml of 50 mM Tris-HCl (pH 8.5), 1 mM EDTA and 0.2 mg/ml RNAse A, and incubated at 37 ℃ for 30 min. The DNA was precipitated from the aqueous phase of a 1:1 phenol: chloroform treatment, by addition of twice the volume of 100% ethanol as described above. The pellet obtained was washed with 70% ice-cold ethanol, air-dried and dissolved in approximately 3 ml of TE (pH 8.0). This DNA was further sheared through a 25-gauge needle, and re-purified using the Qiagen tip 500 and the protocol given in the Qiagen Maxi-prep handbook. The re-purified DNA was reconstituted in 10 mM Tris-HCl (pH 8.5) buffer. Approximately 1 ml of 697 μM DNA (estimated from absorbance at 260

nM) was obtained. The specific radioactivity was estimated from the counts per minute (cpm) obtained from the scintillation counter as: specific radioactivity (cpm/ nmol nucleotides) = cpm/μl DNA÷ μmol DNA / μl x 1000 nmol/ μmol = 96866 cpm/ nmol nucleotides.

2.2-3) Preparation of tritiated Plasmid DNA: A single colony of *E. coli* strain HB101 containing the pTZ18R (2860 bp) plasmid was grown in 500 ml minimal media and the cells harvested as described above. The DNA was isolated using the Qiagen tip-500, and the protocol described in the Qiagen maxi-prep handbook. The final DNA obtained was dissolved in 10 mM Tris-HCl (pH 8.5) and linearized by cleavage with *Sma*I. The concentration and specific radioactivity were estimated to be 947 μM and 122,300 cpm/ nmol as described above.

2.2-4) Exonuclease assay: The standard assay was performed in 50 mM Tris-HCl, pH 8.5, 40 μM ATP, 10 mM $MgCl_2$, 0.67 mM DTT, 0.2 mg/ml BSA, 40 μM (nucleotides) *E. coli* chromosomal [^{3}H] DNA, or 5 μM linearized pTZ18R plasmid [^{3}H] DNA, with varied concentrations of oligonucleotides. The reactions were started by adding 0.31 nM RecBCD (for reactions employing chromosomal DNA) or 0.03 nM RecBCD (for reactions employing plasmid DNA) and were incubated at 37 °C. At pre-determined time intervals, 20 μl aliquots were removed, quenched in 100 μl ice-cold 10% trichloroacetic acid (TCA) plus 5 μl calf thymus DNA (0.5 mg/ml). Following quenching, the aliquots were incubated on ice for 15 minutes, and centrifuged at 14000 rpm for 10 minutes at 4 °C. During this procedure, the larger undegraded DNA is precipitated, leaving the smaller

acid-soluble nucleotides in the supernatant. The radioactivity in the supernatant was counted using a scintillation counter and the amount of acid soluble nucleotides estimated according to the equation: μM acid soluble nucleotides= (cpm-BG)/specific radioactivity of substrate (cpm/ nmol) x 125 μl /110 μl x 1/ 20 μl x 10^3, which abbreviates to (cpm-BG)/ specific radioactivity x 56.3 (BG = Background; cpm obtained for a zero time point aliquot quenched before addition of enzyme to start the reaction.)

A) Reaction with pre-incubated RecBCD- oligonucleotide mixture: A set of exonuclease reactions were also carried out by first incubating RecBCD with the oligonucleotide to be tested as an inhibitor, and then adding the preincubate to reaction mixtures that contained the [^{3}H] DNA substrate. The basic pre-incubation conditions were those used by Taylor and Smith [90] and contained 20 mM MOPS-KOH, pH 7.0, 20 mM DTT, 0.01 mg/ml BSA, 3 mM magnesium acetate, 5 mM ATP, 50 nM RecBCD, and varying concentrations of oligonucleotide. An aliquot of the pre-incubation mixture was added to an exonuclease reaction mixture containing the reaction components given above and the acid-soluble products obtained were measured. A number of variations were made, including different ATP: Mg^{2+} ratios and incubation times and temperatures. A list of these variations is as shown in table 2.2.

2.2-5) Preparation of [5' ^{32}P] end-labeled linearized pBR322$\chi^{\pm}$F DNA: pBR322χ^{+}F plasmid DNA (substrate for the Chi-dependent cleavage reactions described next) was purified using the Qiagen maxiprep tip-500 from a 500 ml culture of *E. coli* strain HB101 grown in LB media. The DNA obtained was linearized by treatment with *Hind* III restriction endonuclease for approximately 2.5 hrs at 37 °C. Following inactivation of

Hind III at 65°C for 20 minutes, the 5' terminal phosphate was removed with shrimp alkaline phosphatase, and the DNA was purified using the Qiagen plasmid purification kit (special applications protocol). The linearized plasmid was then 5'[^{32}P] end labeled using polynucleotide kinase and [γ-^{32}P] ATP (Amersham Pharmacia, 3000 Ci/mmol). [99] The reaction products were repurified (Qiagen miniprep kit) and the amount of labeled DNA obtained was estimated by comparison of the band intensity to a DNA standard (High DNA Mass Ladder, Life Technologies Corp.) on a 1% agarose gel stained with ethidium bromide.

2.2-6) Thin layer chromatography: TLC was used to analyze the ^{32}P labeled DNA during the course of its purification. Cellulose PEI-F TLC plates were pre-treated by soaking in 10% sodium chloride solution for 1 hour followed by rinsing thoroughly with distilled water and drying overnight at room temperature. Samples were spotted approximately 1 cm from the bottom of the plate, air-dried and plates were developed in 0.3 M or 1 M phosphate buffer (pH 3.5). Plates were dried and the images visualized using a Phosphorimager (Molecular Dynamics).

2.2-7) Chi-recognition reactions: Chi-recognition reactions were performed in 25 mM Tris-acetate buffer (pH 7.5), 1 mM magnesium acetate, 1 mM ATP, 1 mM DTT, with 1.15 nM labeled DNA molecules, 0.31nM RecBCD enzyme and varying concentration of the oligonucleotides (as indicated in the figure legends). The reactions were started by adding the enzyme, incubated at 37 °C, and 10 μl aliquots were quenched at predetermined time intervals in 2.5 μl of quench solution (30 % glycerol, 90 mM EDTA,

0.2 % bromophenol blue, and 1.3 % SDS). The samples were run on a 1 % agarose gel at 130 volts for 2 hrs or at 35 volts overnight, in 1x TBE buffer. The gel was dried and the radioactivity was detected using a Phosphorimager.

2.2-8) Preparation of ^{32}P labeled oligonucleotides: The oligonucleotide 5'-ATGTCGATGGATGTCGATGTGCATACTACGGC '3 to be used in helicase assay, was 5' end-labeled using [^{32}P] ATP and polynucleotide kinase as described[99]. The products were purified (Qiagen Nucleotide Purification kit), and analyzed by thin layer chromatography on polyethyleneimine-cellulose plates in 1 M potassium phosphate buffer (pH 3.5) to ensure separation of [^{32}P] ATP from labeled oligonucleotide. The concentration of the purified-labeled oligonucleotide was estimated from the amount of radioactivity (determined by scintillation counting) of samples taken before (counts correspond to 0.5 μM DNA) and after purification.

2.2-9) Preparation of ^{32}P labeled duplex oligonucleotides: The substrate for the helicase assay was made by annealing two complementary DNA oligonucleotides (32 mers, from Invitrogen Corp): oligonucletide1: 5' GCCGTAGTATGCACATCGACATCCATCGACAT, and oligonucleotide2: 5'-ATGTCGATGGATGTCGATGTGCATACTACGGC. The double-stranded helicase substrate was prepared by mixing [^{32}P] oligonucleotide 1 (5 nM) with 95 nM of unlabeled oligonucleotide1 and 102 nM of its unlabeled complementary strand (oligonucleotide 2) in 0.5 M NaCl, 0.2 M Tris-acetate, pH 7.5, and 10 mM MgCl. The mixture was heated to 95-100 °C for 2 min, cooled gradually to 28 °C in the same water bath, placed on ice for

10-15 min, and stored at 4 ℃ or used in the helicase assay. The formation of double stranded substrate was confirmed by running the product obtained on a 15% polyacrylamide gel, alongside labeled single strand marker.

2.2-10) Helicase assay: The standard reaction was carried out in 25 mM Tris-acetate buffer, pH 7.5, 1 mM ATP, 1 mM DTT, 1 mM magnesium acetate, 0.31 nM RecBCD enzyme, 10 μM oligonucleotide, and 3 nM [^{32}P] dsDNA substrate. The control reaction (no oligonucleotide) had 0.1 nM RecBCD. The reactions were incubated at 37 ℃ and 5 μl aliquots were added to 2 μl of a quench solution containing 10% glycerol, 25 mM EDTA, 0.6 % SDS, 0.03 % bromophenol blue and a 5- fold excess of unlabeled oligonucleotide 1. The quenched samples were run on 15 % polyacrylamide gels. The gels were pre-run for 30 min in 1x TBE at 15 mAmp before loading. The samples were loaded and run at 15 mAmp for 2 hrs. The gels were dried and analyzed using a Phosphorimager.

2.2-11) Enzyme-substrate binding reactions: A variation of the above "Chi-dependent cleavage assay" was carried out to test if the oligonucleotides affect binding of RecBCD to the DNA substrate. RecBCD was mixed with the [^{32}P] plasmid DNA and other reaction components except for ATP to allow the enzyme to bind the dsDNA. After a 2 min incubation at 37 ℃, a zero time point aliquot was quenched as above, and ATP alone or a mixture of ATP and the oligonucleotide was added to start the reaction. The final reaction mixture contained 3 nM RecBCD, 2.3 nM [^{32}P] DNA molecules, and other

reactants as above. Samples were removed and quenched within the first 30 seconds after initiation of the reaction and analyzed on agarose gels as above.

2.2-12.Quantitations for Chi-cleavage and helicase assays: The percentage of the dsDNA unwound in these assays was quantitated using the ImageQuant software (Molecular Dynamics) on the Phosphorimager. Objects were drawn to encircle the bands to be quantitated, and the volume of the band was integrated using the software. Background was subtracted from the values obtained for each band. Percentage values were calculated relative to the volume of the same band in the zero time point or control reaction lane. The percent Chi fragment was quantitated in a similar manner, by comparison of the volume of the Chi band to the volume of dsDNA at zero time in that reaction.

2.3 Results

2.3-1) Design of oligonucleotides: Chi containing oligonucleotides (Chi^+) 14 and 20 nucleotides in length, were designed with the Chi sequence at either the 3' end, the 5' end or in the middle of the construct (with an equal flank at either end). Corresponding 14 and 20 mer oligonucleotides with a single base mutation within the Chi sequence (Chi^0) were also designed. This set of oligonucleotides was designed to be used in parallel with the Chi constructs to determine if RecBCD distinguished Chi from its mutant, which would serve as an indicator of its specificity for Chi. The mutant sequence selected for this purpose bears a single nucleotide changed from a T to an A within the Chi. This mutant 5' GCaGGTGG 3' was selected based on previous evidence, showing it to be least active as a Chi sequence [93]. These studies, carried out by Cheng and Smith, examined the extent of Chi dependent cutting (4-6 nucleotides from the 3' end of Chi) and Chi dependent □ crosses obtained when Chi was substituted by a single nucleotide variant. Of the 5 variants tested, the GCaGGTGG variant selected for this study was seen to be the least "Chi-like," showing less than 2% Chi-dependent genetic activity and cutting. The Chi^+ and Chi^0 oligonucleotide sequences hence differ only at that one position within the Chi sequence. In addition to the 14 mers and 20 mers, a set of 8 mers were also designed, which were merely the Chi sequence itself (5' GCTGGTGG 3') or its mutant (5' GCaGGTGG 3'). A complete list of the oligonucleotides designed and their sequences is as presented in table 2.1.

2.1 Table of oligonucleotides

oligomer	Length (Nucleotides)	Sequence	Abs*	Mol.Wt (Daltons)
8mer^{+}	8	3'**ggtggtcg** 5'	33.2	2481.66
8mer^{0}	8	3'**ggtggacg** 5'	31.1	2490.68
14mer^{+}	14	3'tag**ggtggtcg**gat 5'	31.8	4374.88
14mer^{0}	14	3'tag**ggtggacg**gat 5'	30.7	4383.9
3'14mer^{+}	14	3'**ggtggtcg**gattag 5'	31.6	4374.88
3'14mer^{0}	14	3'**ggtggacg**gattag 5'	30.5	4383.9
5'14mer^{+}	14	3'gattag**ggtggtcg**5'	31.6	4374.88
5'14mer^{0}	14	3'gattag**ggtggacg**5'	30.5	4383.9
20mer^{+}	20	3'tactag**ggtggtcg**gatcat 5'	31.7	6188.06
20mer^{0}	20	3'tactag**ggtggacg**gatcat 5'	30.9	6197.08
3'20mer^{+}	20	3'**ggtggtcg**gatcattactag 5'	31.7	6188.06
3'20mer^{0}	20	3'**ggtggacg**gatcattactag 5'	30.9	6197.08
5' 20mer^{+}	20	3' tactaggatcat**ggtggtcg** 5'	31.9	6198.0
5' 20mer^{0}	20	3' tactaggatcat**ggtggacg** 5'	30.9	6198.0
Scramble14	14	3' gcgagtgagtggtgg 5'	31.4	4374.9

CHI+AND CHI- SEQUENCES SHOWN IN BOLD.

Other nomenclature used in text:

14mer^{+} and 14mer^{0} are collectively referred to as Chi-centered 14 mers.
20mer^{+} and 20mer^{0} : Chi-centered 20 mers
3'14mer^{+} and 3'14mer^{0} : 3' Chi-ended 14 mers.
3'20mer^{+} and 3'20mer^{0} : 3' Chi-ended 20mers.
5'14mer^{+} and 5'14mer^{0} : 5' Chi-ended 14 mers.
5'20mer^{+} and 5'20mer^{0} : 5' Chi-ended 20 mers.

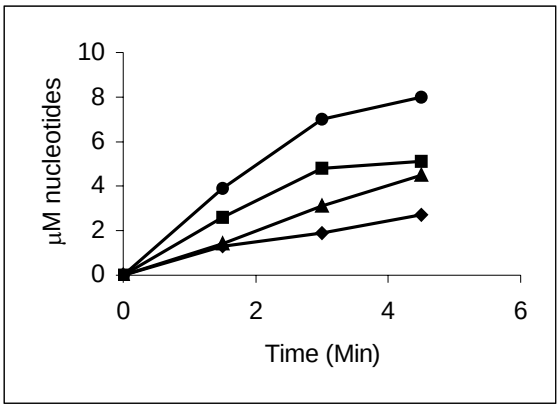
*Absorption coefficients (Abs): Calculated by the nearest-neighbor method using the Bench Mate program (http://biochem.roche.com/benchmate) and parameters given in Fasman [100]. Units are OD/ μM.

2.3-2 Exonuclease reactions: The simplest exonuclease reactions involved addition of the oligonucleotides to an exonuclease reaction to test their efficacy as inhibitors of RecBCD exonuclease. The oligonucleotides in this case were directly added to the exonuclease reaction mixture and the reaction was started by addition of RecBCD and incubation at 37 °C for predetermined time periods. If the oligonucleotides modify RecBCD in some way through their interaction, it would result in decreased exonuclease activity. Comparison of the time dependent inhibition of this activity by the Chi^+ and Chi^0 constructs would enable prediction of specificity.

Three sets of oligonucleotides were used for this set of exonuclease assays. These were the 8 mers, the Chi-centered 14 mers and the 3' Chi-ended 20 mers. The standard exonuclease reaction conditions were employed which used 10 mM magnesium chloride and 40 μM ATP, and 40 μM trititated chromosomal DNA (4040 cpm/nmol).

For each oligonucleotide set, there was concentration dependent inhibition of RecBCD exonuclease observed. Figure 2.1a shows time courses for the $8\ mer^0$ at 5, 30 and 50 μM concentrations. As seen from the figure there is an increase in inhibition of reaction with the increase in concentration. Similar results were obtained for the $8\ mer^+$ at various concentrations, from which reaction rates were estimated to illustrate the effect of concentration on inhibition. Figure 2.1b shows a plot of these reaction rates for the $8\ mer^+$ and $8\ mer^0$ reactions at the above concentrations. The plot also allows a comparison between the extents of inhibition by the $8\ mer^+$ and $8\ mer^0$, which, as seen from the figure, are very similar in their effect on exonuclease inhibition. A similar set of data was

A.



B.

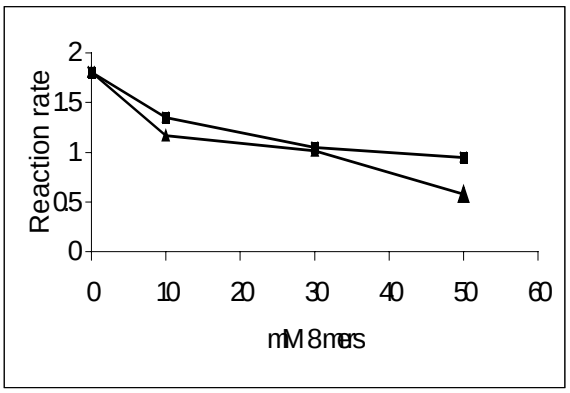


Figure 2.1

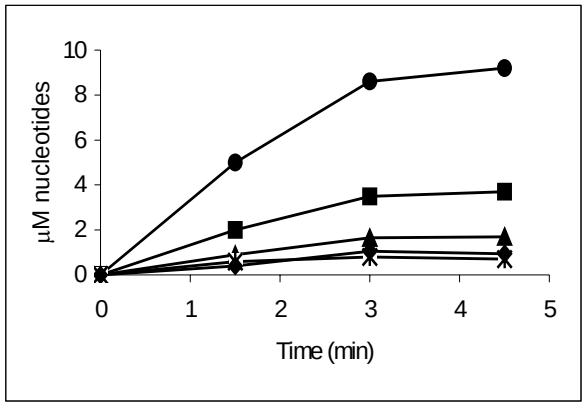
Exonuclease activity as a function of concentration of 8 mers

A) Time course at variable concentrations of 8 mer^0: Reactions contained 10mM magnesium chloride, 40 µM ATP, 40 µM tritiated *E. coli* chromosomal DNA substrate (4040 cpm/ nmol), no oligonucleotides (●), 5 µM (■), 30 µM (▲), or 50 µM (◆) 8 mer^0 and 0.31 nM RecBCD, and were carried out as described in the methods section. The µM acid soluble nucleotides were calculated from the counts per minute obtained as outlined in methods. **B)** Reactions rates estimated from the slopes of the individual time courses for varying concentrations of the 8 mer^0 (▲), and a similar set of time courses performed for 8 mer^+ (■), were plotted vs the oligonucleotide concentration.

obtained for the 14 mers and 3' Chi-ended 20 mers, which are shown in figures 2.2a-b and 2.3a-b respectively. As seen from figures 2.2a and 2.3a, the 14 mer and 20 mer oligonucleotides also show greater inhibition with increasing concentration. A comparison of the Chi^+ and Chi^0 14 mers (figure 2.2b) shows that there is a noticeable difference between their extents of inhibition with the Chi^+ 14 mer inhibiting more than the Chi^0 14 mer. This kind of quantitative difference in reaction rates is absent between the Chi^+ and Chi^0 8 mers and the 3' Chi ended 20 mers (as seen from figures 2.1b and 2.3b). These results suggest that there is something specific about the recognition and interaction of the Chi sequence with RecBCD, in the case of the 14 mers, but not in case of the 20 mers or 8 mers. Since the only difference between the design of the 14 mers and the other two sets of constructs is that in the 14 mers, Chi is flanked at either end by some sequence, it may mean that this feature of the construct is responsible for the observed effect. (In case of the 8 mers or 3' 20 mers, the flank is missing at one or both ends). Consequently, it is likely that Chi needs to be flanked at both ends to be specifically recognized by RecBCD. An overall comparison of the extents of inhibition by the different length construct also shows that the inhibition is length dependent. The smaller 8 mers need to be at a much higher concentration to inhibit than the 14 mers, which inhibit at a higher concentration than the 20 mers (figure 2.4).

To verify the observed effect of a flank and the hypothesis that it might be the mediator of specific recognition of Chi, the standard exonuclease reactions were repeated with a set of redesigned 14 mers and 20 mers. The 14 mers were redesigned to bear the Chi^+ or Chi^0 at either the 5' end or the 3' end, and the 20 mers were redesigned to bear

A.



B.

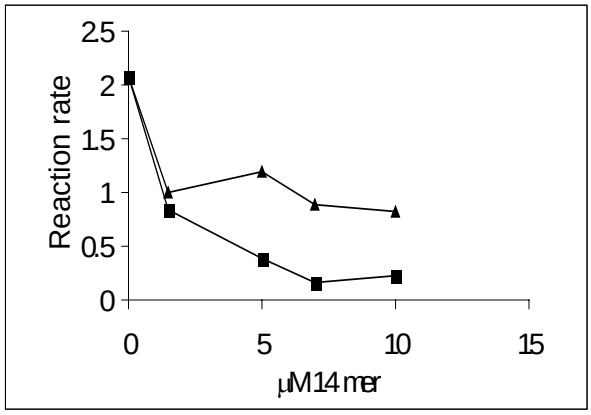


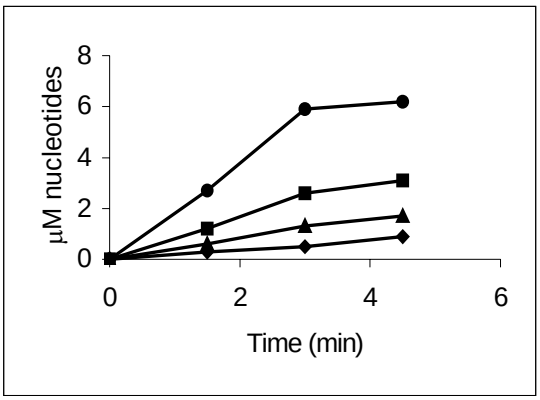
Figure 2.2

Exonuclease activity as a function of concentration of 14 mers

A) Time courses at variable concentrations of 14 mer^{+}: Reactions carried out as described in figure legend 2.1 with 0 µM (●), 1.5 µM (■), 5 µM (▲), 7 µM (◆) or 10 µM (✷) 14 mer^{+} and 0.31 nM RecBCD.

B) Slopes for above time-courses with 14 mers Chi^{+} (■) and 14 mer Chi^{0} (▲) calculated and plotted vs the corresponding concentration of the 14 mers.

A.



B.

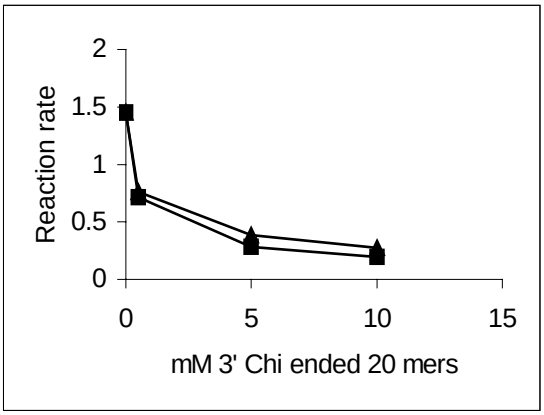


Figure 2.3

Exonuclease activity as a function of concentration of 3' Chi-ended 20 mers

A) Time courses at variable concentrations of 20 mer^{+}: Reactions contained 0 μM, (●), 0.5 μM (■), 5 μM (▲) or 10 μM (◆) 3' $20mer^{+}$, and were carried out as described above.

B) Slopes for above time-courses with 3' Chi ended 20 mer^{+} (■) and 20 mer^{0} (▲) calculated and plotted vs the corresponding concentration.

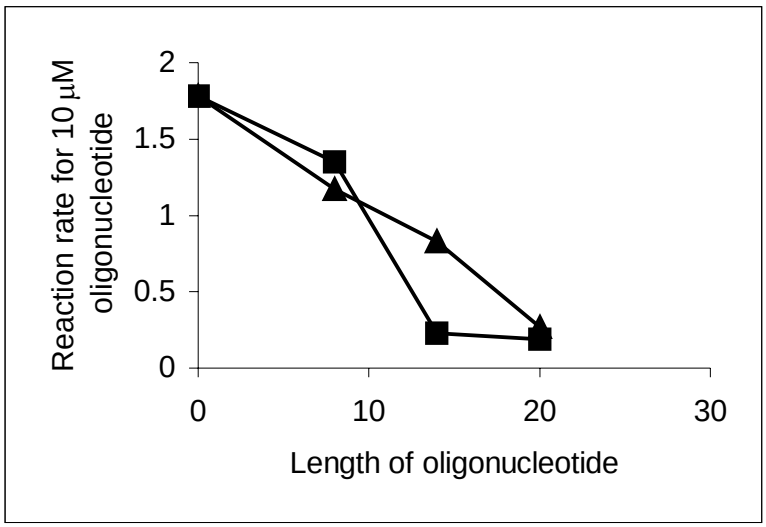


Figure 2.4

Inhibition of exonuclease as a function of oligonucleotide length:

Comparison of extent of inhibition by 10μM oligonucleotides of varying lengths. Slopes for the time-course with 10 μM 8 mers, Chi-centered 14 mers and 3' Chi ended 20 mers (Chi^+ ■ and Chi^0▲) plotted vs the length. The control reaction (with no oligonucleotide) is plotted as 0 length.

Chi^+ or Chi^0 at the 5' end or in the middle of the construct, with equal flanking length at either end. These newly designed constructs have the exact same base composition as the earlier set, and differ only in the placement of the Chi^+/Chi^0 (refer to table 2.1). If the above assumption that a flank is necessary for specific recognition is true, then the newly designed 14 mers with Chi at one or the other end should now show similar effects with Chi^+ or Chi^0 constructs. This would show that RecBCD was no longer able to distinguish between the two sequences, and was not specific for Chi. Similarly, the newly designed 20 mers with Chi in the middle, should now show a noticeable difference between inhibition by the Chi^+ and Chi^0 construct, with the Chi^+ construct inhibiting exonuclease function noticeably better than the Chi^0. Further, the 5' 20 mer^+ and 5' 20 mer^0 should show more or less similar effects with respect to inhibition of exonuclease. The standard reactions were hence repeated with the new and old set of constructs of both lengths and the 8 mers (no flanking sequence at either end of Chi^+ or Chi^0) to enable comparison of these effects and validation of the requirement of flanks for recognition.

The results obtained from the assays are consistent with the proposition made above, regarding the requirement of flanking sequences at either end of Chi, mediating its specific interaction with RecBCD. The 8 mers as in earlier assays do not show any specific recognition of the Chi sequence. Both the 8 mer Chi^+ and Chi^0 oligomers inhibit the activity to similar extents (figure 2.5).

Time courses with the 3 sets of 14 mers are shown in figure 2.6a-d. As seen from the plots, specific recognition of Chi is seen only for the Chi-centered 14 mer, where the Chi is in the middle flanked by sequence at either end. This is seen from the

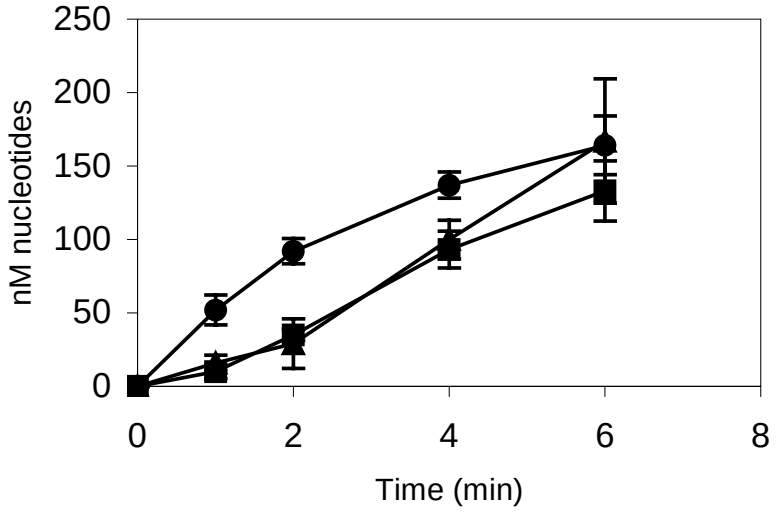


Figure 2.5

Inhibition of exonuclease activity by 8 mers: The reactions contained 10 mM magnesium chloride, 5 μM tritiated pTZ18R plasmid DNA, 1 mM ATP, 50 μM 8 mer Chi^{+}(■) or Chi^{0} (▲), 0.03 nM RecBCD and were performed as described in the methods section. The control reaction (●) does not have any oligonucleotides. The plots show averages from three sets of repeats, and error bars represent the corresponding standard deviations.

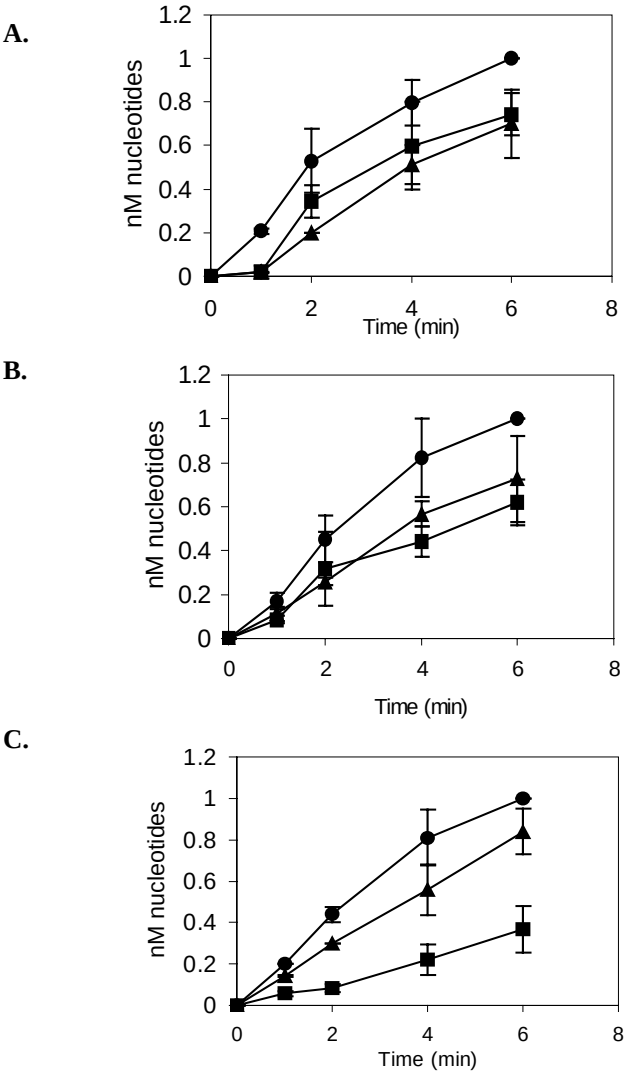


Figure 2.6:

Comparison of exonuclease inhibition by Chi-ended and Chi-centered 14 mers: The Reactions are carried out as described above in figure 2.5. The control reaction (●) for all three sets is without any oligonucleotides. Averages of three repeats (normalized as for figure 2.5) are plotted along with standard deviations.

15 μM 3' 14 mer^{+}(■) or 3' 14 mer^{0} (▲)

15 μM 5' 14 mer^{+}(■) or 5'14 mer^{0} (▲)

15 μM 14 mer^{+}(■) or 14 mer^{0} (▲).

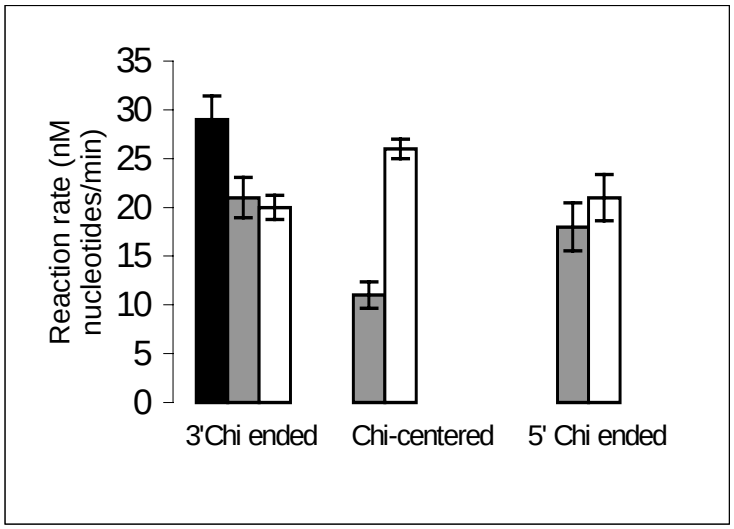


Figure 2.6:

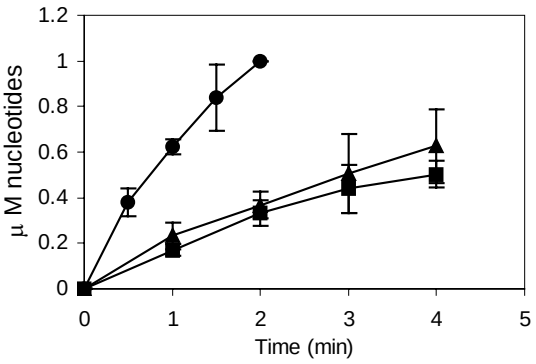
D) Comparison of exonuclease inhibition by Chi-ended and Chi-centered 14 mers

Reaction rates were calculated for Chi^+ (□) and Chi^0 oligomers (❑) as the average of the slopes obtained from each time course (three repeats). Standard deviations were also calculated from slopes of the individual time courses. The control (■) value plotted is an overall average of slopes from 9 time courses.

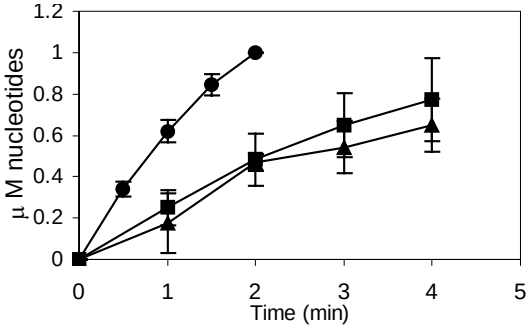
difference in extents of inhibition of exonuclease by the Chi^+ and Chi^0 in this set of assays. This difference is not observed in the case of the Chi ended 14 mers. Similar results were obtained for the 20 mers as well, which show specific recognition of the Chi sequence only in the Chi-centered 20 mer (figures 2.7a-d), confirming the proposed requirement for flanks.

A comparison of these results shows that there is a difference in overall extent of reaction (even control reaction) between the sets of reactions presented in figures 2.5-2.7 and the ones presented in figures 2.1-2.3. This difference can be attributed to the use of different substrates for the reaction. The pTZ18r plasmid DNA substrate used in the reactions with the 8 mers and 14 mers, has a definite number of double-stranded flush ends for RecBCD action, which may be quite different from the number available in a sample of linearized *E. coli* chromosomal DNA. In case of the chromosomal DNA substrates, the method of preparation (as described in the methods sections) is such that each batch of substrate processed is different from the previous one in two respects: firstly, with respect to the number of double stranded ends available for RecBCD binding, and secondly, with respect to how many of the duplex ends that are available are blunt ended. Each preparation of chromosomal DNA is a heterogeneous mixture of blunt-ended and sticky–ended double-strands, and is different from other batches. Since RecBCD prefers flush-ended DNA for substrates [39] and the number of these available for initial processing by it differs from one batch to the next, the overall amount of reaction obtained can also be different with substrates from separate batches. This is not true for the linearized plasmid substrate, which owing to its linearization with restriction enzymes has blunt ended DNA. Hence, in this case, the amount of substrate

A.



B.



C.

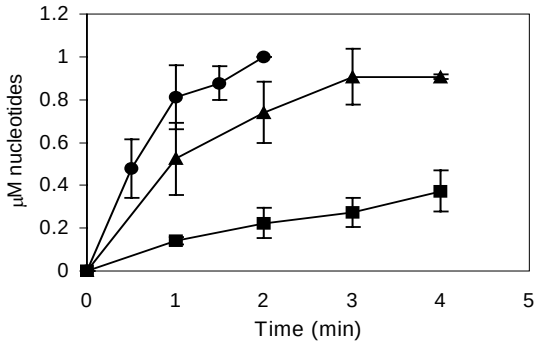


Figure 2.7:

Comparison of exonuclease inhibition by Chi-ended and Chi-centered 20 mers: The reactions were carried out as described above. The control reaction (●) for each figure A-C is without any oligonucleotides. Averages of three repeats and their standard deviations are plotted.

2 μM 3' 20mer^{+}(■) or 3' 20mer^{0} (▲)

2 μM 5' 20 mer^{+}(■) or 5'20 mer^{0} (▲)

2 μM 20 mer^{+}(■) or 20 mer^{0} (▲).

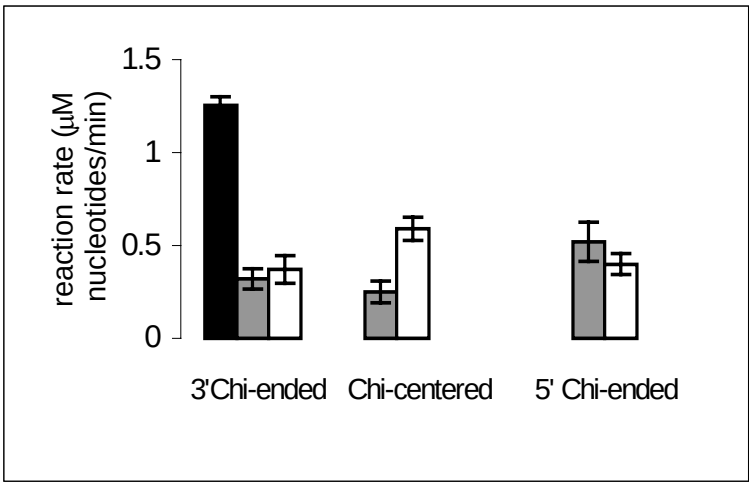


Figure 2.7:

D) Comparison of exonuclease inhibition by Chi-ended and Chi-centered 20 mers

Reaction rates (μM nucleotides produced/min) were determined as described in figure legend 2.6d, for the control (■), Chi^+ (□) and Chi^0 oligomers (❑) reactions and plotted against the corresponding set of 20 mers.

used for the reaction had to be adjusted to observe inhibition and Chi recognition. However, since the study aims to compare relative differences between inhibition by the Chi^+ and Chi^0 sequences, the overall extent of inhibition being different did not significantly alter the interpretation of results.

Irrespective of variations in this aspect, the overall pattern of inhibition observed was the same, whereby only the Chi-centered constructs showed any specific recognition of the Chi sequence. Within the non-flanked 8 mers and Chi-ended pairs of constructs, the Chi^+ and Chi^0 always inhibited the reaction to very similar extents. This is especially interesting when one focuses on the fact that the only difference in the sequences of any given length is the placement of the Chi-site, and within a pair of constructs, the only difference in the base composition is one single nucleotide. Thus, the positioning of the Chi site seems to make all the difference to its recognition, leading to an initial proposal, that in order to be recognized specifically by RecBCD, the Chi sequence needs to be flanked by some DNA at either end of it.

A set of reactions with the Chi-centered 14 mers were also carried out to test the effect of a change in the ratio of magnesium to ATP. The standard reactions described above with higher magnesium (10 mM) than ATP (40 μM or 1 mM) are favorable for exonuclease activity. A ratio of higher ATP (1 mM) to magnesium (2 mM) concentration has been reported to be favorable for Chi recognition [89]. The assay carried out under these conditions as expected, shows a reduction in overall reaction. The $14mer^+$ and $14\ mer^0$ yield similar extents of inhibition (figure 2.8).

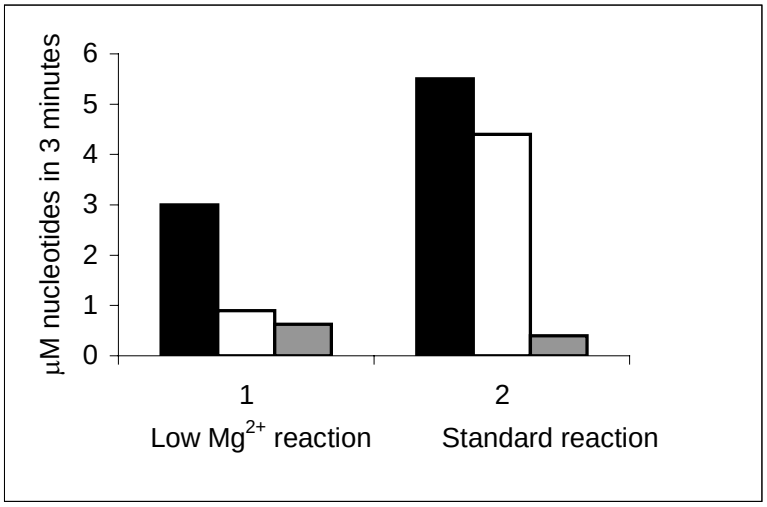


Figure 2.8

Exonuclease assay under limiting Mg^{2+} conditions

The standard reaction was carried out as described above with 40 µM chromosomal DNA, 40 µM ATP and 10 mM Mg^{2+}. The low Mg^{2+} reaction had 2 mM $Mg^{2+}$and 1 mM ATP. control (■), Chi^{+} (□) and Chi^{0} oligomers (❑) reactions.

In addition, a completely scrambled sequence of the 14 mer^{+} (such that it does not bear a Chi sequence) was also tested in an exonuclease reaction as an additional test for specificity toward Chi. The scrambled 14 mer ("Scramble14 – see table 2.1) was found to behave similarly to the 14 mer^{0} in terms of inhibition (Figure 2.9).

A) Reactions with Pre-incubated RecBCD-oligonucleotide mixture: A set of exonuclease assays was also carried out in parallel, using pre-incubated oligonucleotide-RecBCD mixtures to start the reaction to determine if interaction with these constructs causes structural changes in the enzyme as noticed earlier by Taylor and Smith. Taylor and Smith used a similar approach to show that preincubation of double-stranded Chi-bearing substrates with RecBCD led to inhibition of its activities and disassembly of its subunits in a glycerol gradient as described earlier (Introduction)). The pre-incubations themselves were carried out for varying periods of time, at variable temperatures, with variable oligomer concentrations. A complete list of the preincubation conditions for the oligomers tested is presented in table 2.2. Pre-incubation were carried out with either the 8 mers, 3' Chi-ended 20 mer, or Chi-centered 14 mers to see if this increases the overall inhibition or the Chi-specific inhibition observed in the standard reactions carried out above. The results obtained with all three sets of oligomers shows very little inhibition by any of them. This can be attributed to the fact that the concentration of the oligomers in the final exonuclease reaction was very low, (compared to the concentration in the pre-incubating mixture). A representative result for this set of assays, using a 30 minute preincubate at room temperature, is as shown in figure 2.10a (8 mers) and 2.10b (3' Chi ended 20 mers) and figure 2.10c (14 mers).

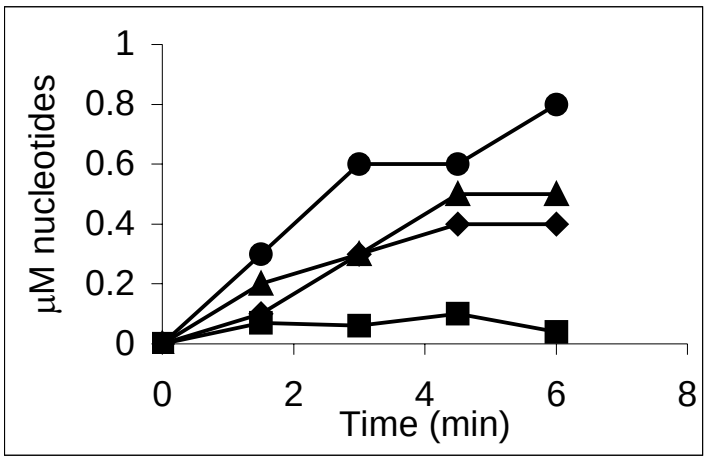


Figure 2.9

Inhibition of exonuclease activity by a random 14 mer sequence.

Exonuclease reactions with 5 µM 14 mer^{+} (■), 14 mer^{0} (▲), a scrambled 14 mer (◆) or RecBCD alone (●), contained 40 µM *E. coli* chromosomal DNA, 40 µM ATP and 0.3 nM RecBCD and were carried out as described in methods.

Table 2.2: Pre-incubations for Exonuclease Assay:

Oligo	*Enzyme: Oligos (nM: μM)	Mg-acetate (3mM)	ATP (5mM)	ATP-γ–S (5mM)	Inc Time	Temp (°C)
14mers	50: 0.5	✓	✓	✖	5min-ATP 5min+ATP	RT
14mers	50: 0.5	✓	✓	✖	as above Overnight	RT -20
14mers	50: 0.5	✖	✖	✖	30min/3hrs	RT
14mers	50: 1	✖	✖	✖	30min/3hrs	RT
14mers	50:2.5	✖	✖	✖	30min/3hrs	RT
14mers*	0.6: 20	✖	✖	✖	10min	RT
14mers*	0.6: 20	✖	✖	✖	10min	0
14mers*	0.6: 20	✖	✖	✖	10min	37
8mers[A]	50:2.5	✖	✖	✖	30min	RT
8mers[A]	50:2.5	✖	✖	✓	30min	RT
Chi-ended 20mers[A]	50:2.5	✖	✖	✖	30min	RT
Chi-ended 20mers[A]	50:2.5	✖	✖	✓	30min	RT
Chi-ended 20mers*	0.6:20	✖	✖	✖	10min	RT
Chi-ended 20mers*	0.6:20	✖	✖	✖	10min	0
Chi-ended 20mers*	0.6:20	✖	✖	✖	10min	37

Final concentration of oligomer in most exonuclease reactions was between 1-15 nM except for the following reactions where the concentrations were as indicated below.
*: 0.3nM: 10μM (enzyme: oligomer)
A: 0.3nM: 15nM (enzyme: oligomer)
(The values for enzyme: oligomer in the table are their concentrations in the preincubation mixture. Checks denote presence of reactant, crosses denote absence of the reactant)

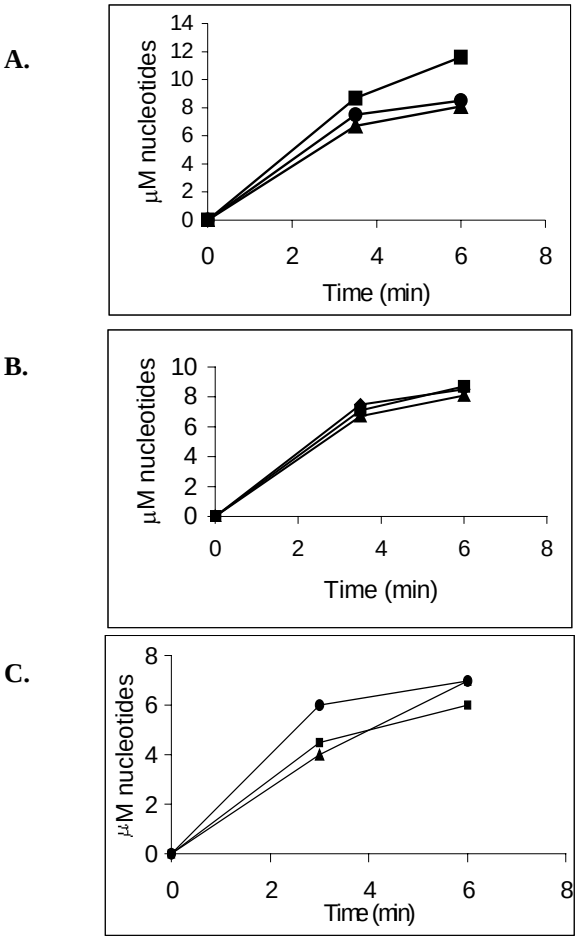


Figure 2.10

Exonuclease assay with pre-incubated oligonucleotide- RecBCD mixtures:

2.5 µM 8 mer, 14 mer, or 3'20 mer was incubated with 50 nM RecBCD in 10% glycerol, 50 ng BSA, 10 mM DTT, 10 mM MOPS-KOH (pH 7.0) at 25 °C for 30 minutes. Reactions were started by addition of an aliquot of the mixture to exonuclease reaction mixtures containing 40 µM tritiated *E. coli* chromosomal DNA, 10 mM magnesium acetate and 40 µM ATP. Final concentration of RecBCD and oligonucleotides in the reaction mixture is 0.3 nM and 15 nM respectively. Control (●) reaction is without any oligonucleotides. **A)** 8 mer $^{+}$(■) or 8 mer^{0} (▲), **B)** 3'20 mer $^{+}$(■) or 3'20 mer^{0} (▲), **C)** 14 mer $^{+}$(■) or 14 mer^{0} (▲).

Further, results obtained with higher (μM) concentration of oligomers in the final exonuclease reaction were similar and comparable to those obtained in the direct addition reactions. Thus, a reaction containing 0.3 nM RecBCD and 10 μM 14 mers preincubated together first gives similar effects to a reaction where the same amount of RecBCD and 14 mers are directly added. Hence, preincubation of RecBCD with the oligomers did not lead to significantly enhanced inhibition. Figure 2.11 shows this comparison between extent of exonuclease obtained with the direct addition reactions and 3 sets of pre-incubated reactions (at different temperatures) for the 14 mers. As seen from the figure, there is only a very slight increase in the overall extent of inhibition by either the 14 mer^{+} or 14 mer^{0} on preincubation. The preincubated 14 mers also retain the pattern of inhibition observed with the direct addition reactions, with the 14 mer^{+} inhibiting the reaction noticeably better than 14 mer^{0}.

2.3-3 Chi recognition reactions: The oligonucleotides were next tested for their ability to inhibit the Chi recognition activity of RecBCD itself. This was assayed by monitoring production of a Chi-specific fragment that arises from attenuation of nuclease activity, following encounter with and recognition of a Chi site. The substrate used in this reaction, pBR322χF^{+}, is a 4.3 kb plasmid which after cleavage with *Hind* III bears a Chi site 1463 bp from the 5' end.[31] (figure 2.12). Recognition of this site from its 3' end by RecBCD followed by attenuation of its nuclease activity at that point, leads to the generation of a 1463 nt 5' ^{32}P-labeled fragment of ssDNA, called the downstream Chi fragment (figure 2.13). This cleavage at the Chi sequence, resulting in the formation

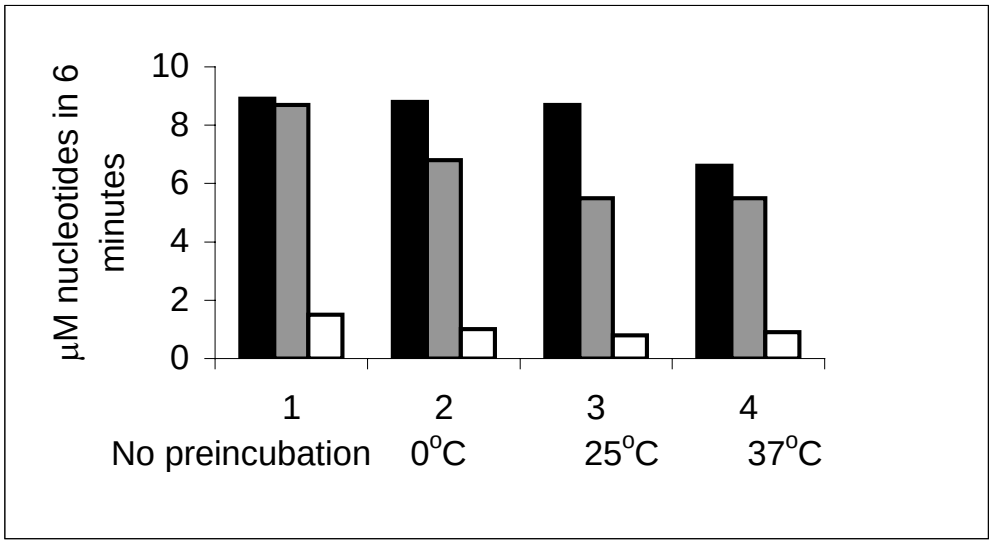


Figure 2.11

Comparison of exonuclease inhibition by 14 mers with and without pre-incubation:

1) No preincubation: Direct addition of 0.3 nM RecBCD to an exonuclease reaction mixture containing either no oligomer (■), 10 μM 14 mer ^{0} (❑) or 10 μM 14 mer^{+} (❑).

2-4) Preincubation at varying temperatures: 0.6 nM RecBCD was preincubated in RecBCD storage buffer for 10 minutes at the indicated temperatures in the absence (■) or presence of 20 μM 14 mers^{+} (❑) or 14 mer^{0} (❑). An aliquot of this mixture was used to start the reaction. Final concentration of RecBCD and oligonucleotides in exonuclease reaction was 0.3 nM and 10 μM respectively.

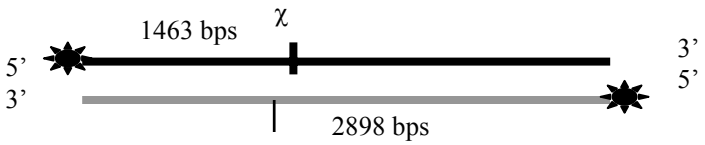


Figure 2.12

*Hind*III linearized PB322χF$^{\pm}$ plasmid DNA used as substrate in "Chi-dependent cleavage" reactions: The 3'-5' exonuclease activity of RecBCD is proposed to degrade the upper strand till it encounters Chi. Recognition of Chi from the 3' end followed by nicking at the site and attenuation of the 3' -5' exonuclease thereafter results in release of the 1.46-kb fragment. Under some conditions, RecBCD switches polarity of cutting after Chi encounter and cuts the bottom strand instead resulting in a 2.89-kb fragment.[89]

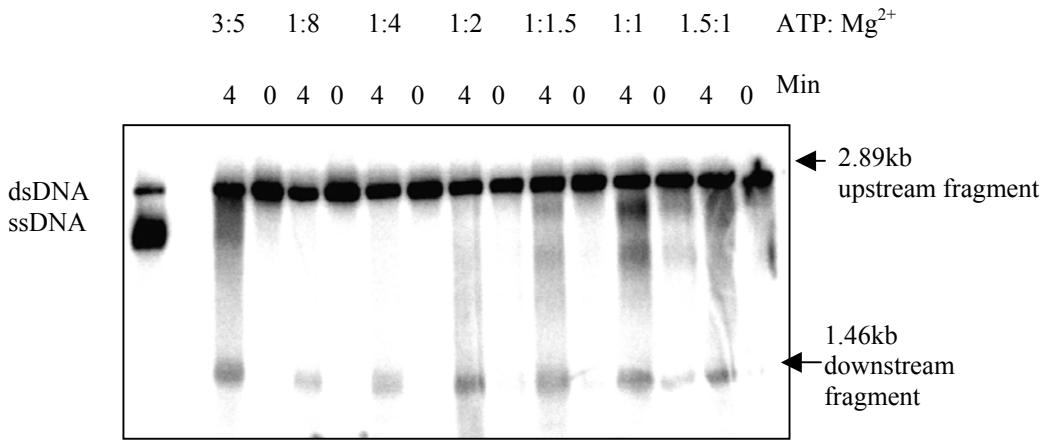


Figure 2.13:

Chi-cleavage assay at varying Mg^{2+}: ATP ratios:

Reactions contain 1.15 nM molecules of labeled plasmid substrate, 1 mM DTT, 25 mM Tris-acetate (pH 7.5) and the indicated concentrations (mM) of ATP and magnesium acetate. ssDNA refers to single-stranded (heat denatured) ^{32}P DNA. Reactions were started by addition of 0.31 nM RecBCD and incubated at 37 ºC for 4 minutes. Products were resolved on a 1 % agarose gel and visualized by phosphorimaging.

the downstream Chi fragment is referred to as "Chi-dependent cleavage" and the downstream fragment is also called the Chi-specific fragment.

The downstream Chi fragment is the most commonly obtained product of a Chi encounter, and has been used as a marker in almost all studies on Chi-RecBCD interaction. However, under certain experimental conditions, a switch of polarity of degradation to the non-Chi (5'-3') bearing strand has been proposed to occur[89] resulting in an additional product. This kind of switch leads to preferential degradation of the 5-3' bottom strand following Chi encounter, and is especially seen under lower Mg^{2+}: ATP ratios. The product of this switch is a 2.89 kb "upstream fragment" as shown in figure 2.12.

The experiments carried out here attempt to test the effect of the Chi-containing oligonucleotides on Chi recognition by RecBCD, on competing substrates. A specific interaction of RecBCD with Chi^{+} oligonucleotides, leading to an alteration or modification in the enzyme, might result in reduced recognition of Chi on a competing duplex. The outcome of such an interaction with Chi, could result in the reaction being altered in two ways: qualitatively (e.g., appearance of an upstream Chi fragments as shown in figure 2.12) or quantitatively (less overall reaction of the dsDNA leading to less production of the downstream Chi fragment).

Initial reactions were carried out using different ATP: Mg^{2+} ratios to determine the optimum conditions for the assay. The best results were obtained for a 1:1 ratio of ATP: Mg^{2+}, where, in addition to the downstream band, ssDNA, and the upstream band were also visible to some extent. (Under the ATP and Mg^{2+} concentrations used for these experiments, and because of the absence of single-strand stabilizing SSB

protein, [101] the upstream Chi fragment and uncleaved full-length ssDNA cannot be distinctly visualized). Figure 2.12 shows the reaction products obtained under varying ATP: Mg^{2+} ratios.

Further reactions to test for the effect of oligonucleotides were mostly carried out at a 1:1 ratio of ATP: Mg^{2+}. The results obtained (figure 2.14-2.15) showed a decrease in the extent of unwinding of substrate. Consequently the amount of downstream Chi fragment obtained was also decreased. As in the exonuclease reactions, there was a length and concentration based inhibition of the reaction by the oligomers. Further, the extent of reaction obtained was noticeably different between the Chi^{+} and Chi^{0} constructs, only in the case of the Chi-centered 14 mers and Chi-centered 20 mers. The Chi-ended 14 mers and 20 mers, and the 8 mers showed similar effects with either the Chi^{+} or Chi^{0} construct, thus confirming the location dependent recognition of the Chi sequence.

Figure 2.14a shows the results for the Chi-centered 14 mers. The % double-strand left unwound was calculated from the band intensities of the dsDNA band for the time course, and plotted against the time as shown in figure 2.13b. Figure 2.14c shows the corresponding percent Chi band obtained. As seen from figure 2.14b, while all of the DNA is unwound and degraded within 2 min in the control reaction, and 85 % has disappeared in 3 min in the presence of the 14 mer^{o}, less than 40 % of the dsDNA has reacted in 3 min in the 14 mer^{+} reaction. The total amount of downstream Chi fragment obtained is also much less when the 14 mer^{+} is present presumably because of the inhibition of the unwinding reaction illustrated by Fig. 2.14b. Thus, there is a quantitative difference in the degree of inhibition by the Chi^{+} vs. Chi^{o} oligonucleotides.

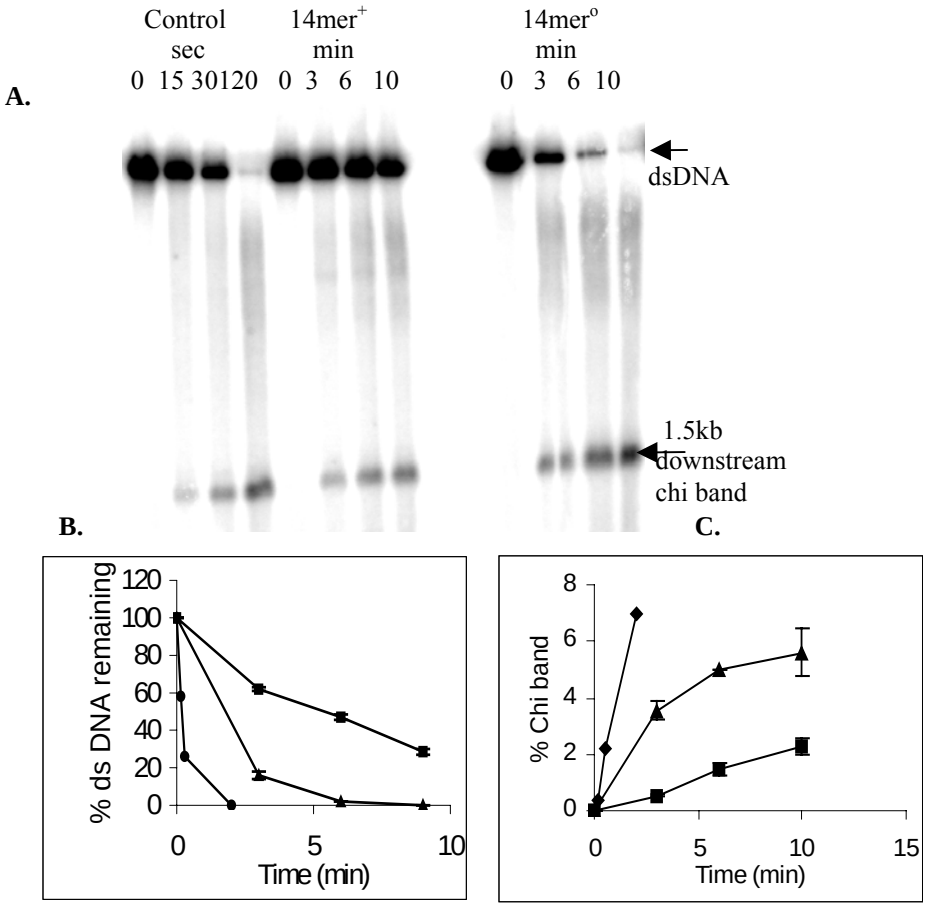


Figure 2.14:

Inhibition of Chi recognition by Chi-centered 14 mers:

A) Reactions were carried out as described above with 1 mM ATP, 1 mM magnesium acetate, 0.3 nM RecBCD and 10 μM 14 mer^{+} or 14 mer^{o}. **B)** The % dsDNA remaining at each time point for reactions containing 0.3 nM RecBCD (●) or 0.3 nM RecBCD with 10 μM 14 mer^{+} (■) or 14 mer^{o} (▲) was quantitated as described in experimental Procedures. Plot shows averages of duplicate runs, and the error bars show the range of the individual determinations. **C)** The corresponding Chi band was quantitated as a percentage of the total DNA (double strand and Chi band) signal for each time point

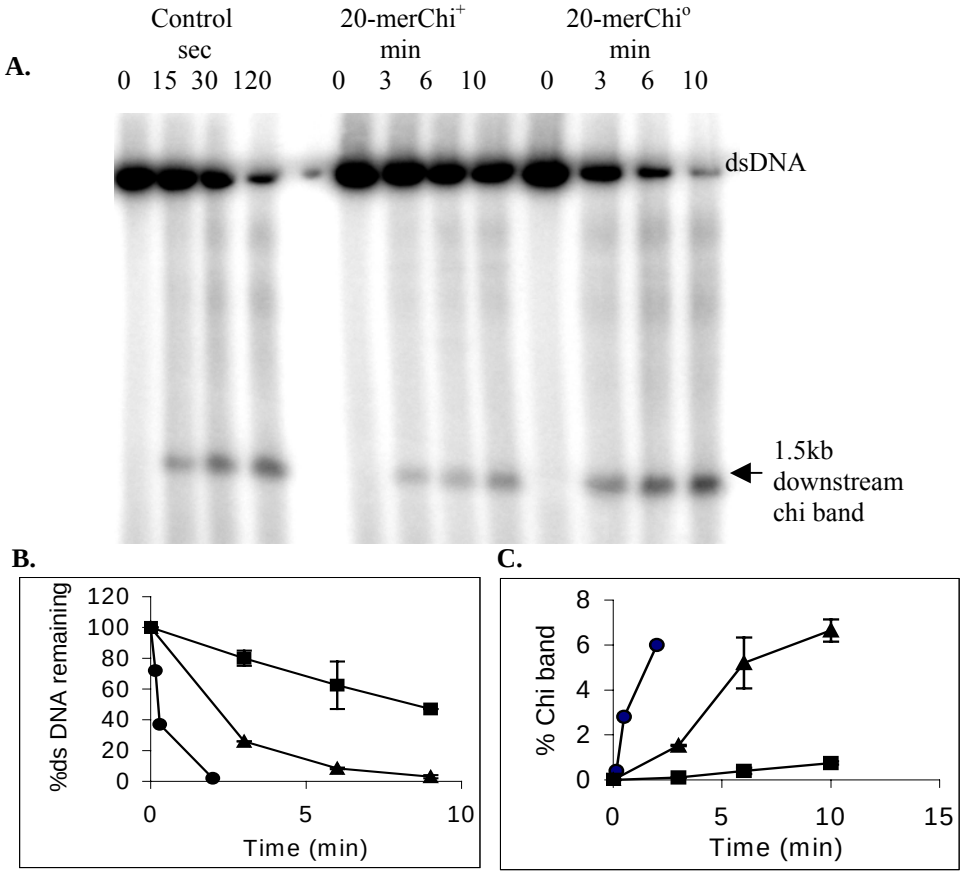


Figure 2.15:

Inhibition of Chi recognition by Chi-centered 20 mers:

A) Reactions were carried out as described in figure 2.14 with 0.3 nM RecBCD and 2 μM 20 mer^{+} or 20 mer^{o}. **B)** Inhibition of reaction by Chi-centered 20 mers: Plot shows average values of the % double strand remaining from duplicate time courses, for reactions with 2 μM 20 mer^{+} (■) or 20 mer ^{o} (▲). Control reaction has RecBCD alone (●). **C)** Percent Chi-band quantitated as described in figure legend 2.14c plotted vs. time for 20 mer^{+} (■) or 20 mer ^{o} (▲) and control reaction (●).

Figure 2.15a shows the Chi-centered 20-mer reaction. Again, the 20 mer^{+} inhibits the helicase activity much better than the 20 mer^{o}. After 3 min 70 % is unwound in the presence of the 20 mer^{o} whereas only about 15 % of the dsDNA substrate is unwound in the presence of the 20 mer^{+} (figure 2.15b), and consequently the amount of Chi fragment obtained is also reduced (figure 2.15c).

Results with the 8-mer are shown in figure 2.16. As observed with the exonuclease assay, there is little difference between the reactions containing the 8 mer^{+} and 8 mer^{o} oligonucleotide. Similarly there is little difference in the inhibition by the Chi^{+} and Chi^{0} of any Chi-ended oligomer pair. A representative result for the Chi-ended constructs is shown in figure 2.17. As seen from the figure, the net reaction with either the Chi^{+} or Chi^{0} of the 3' Chi-ended 14 mers or 20 mers is almost the same confirming the proposition that Chi-recognition is a location specific effect dependent upon the presence of flanks at either end.

2.3-4 Helicase reactions:

The helicase reactions were primarily carried out to confirm the inhibition of helicase activity by the 14mer^{+} and 20 mer^{+} observed in the Chi recognition assays. Additionally the assay was also designed to examine if the observed inhibition arises out of the processivity of RecBCD helicase being affected, or if it is a direct consequence of changes in the helicase function itself. In order to establish this, a much smaller 32 bp dsDNA substrate was employed in this set of reactions. Since the Chi recognition assay

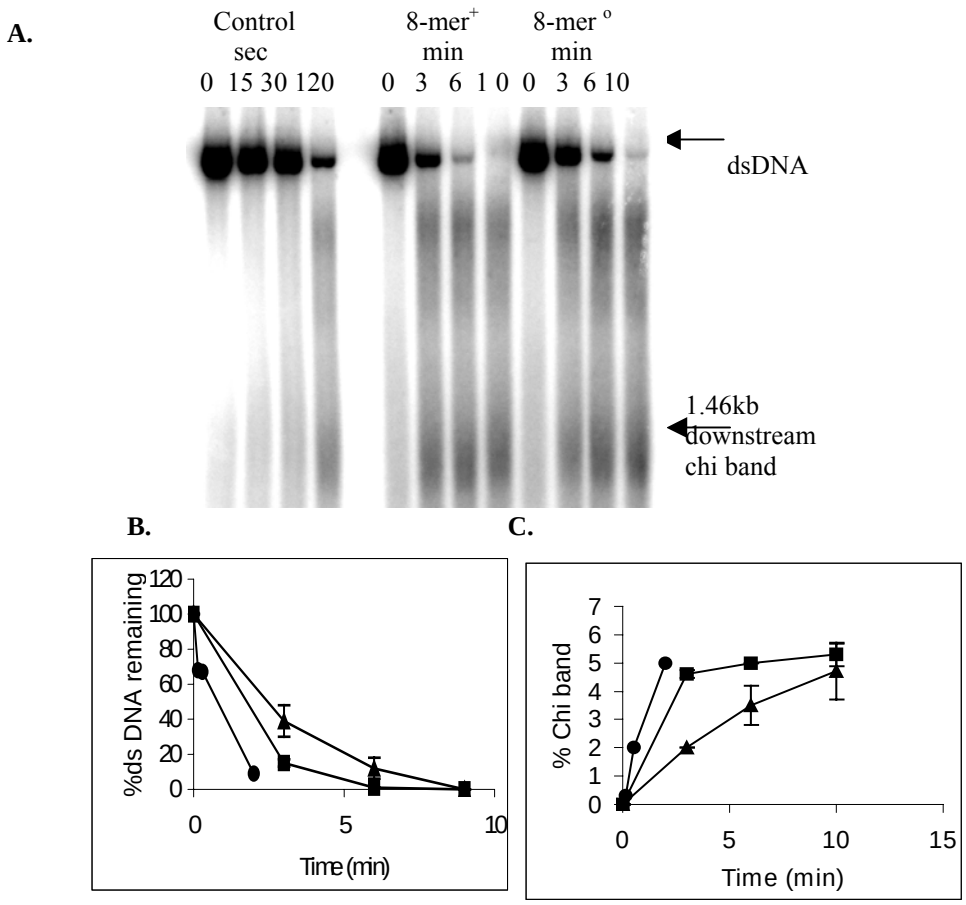


Figure 2.16:

Inhibition of Chi recognition by 8 mers:

A) Reactions were carried out as described in figure legend 2.14 with 0.3nM RecBCD and 25 μM 8 mer^{+} or 8 mer^{o}. **B)** Percent double strand vs. time for 25 μM 8mer^{+} (■) or 8 mer ^{o} (▲). Control reaction has RecBCD alone (●). Averages of duplicate runs are plotted. **C)** Percent Chi band vs. time for 8mer^{+} (■) or 8 mer ^{o} (▲) and control reactions (●) respectively.

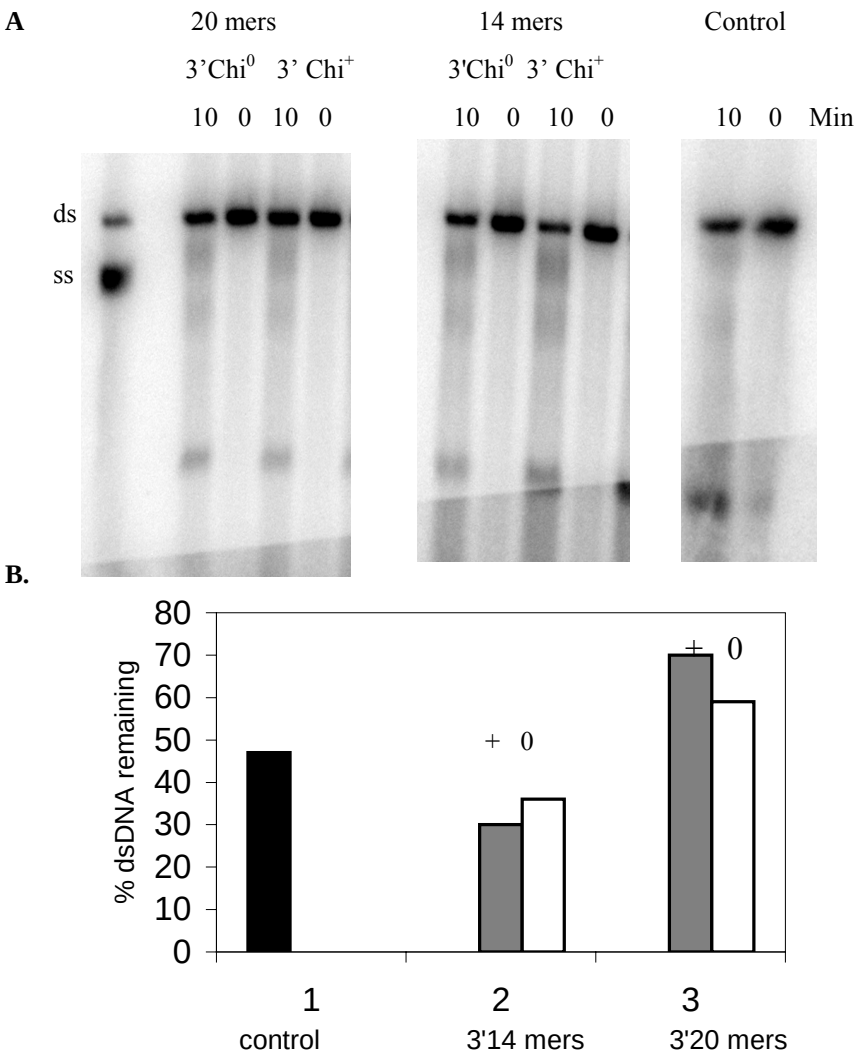


Figure 2.17:

Inhibition of Chi recognition by Chi-ended oligomers:

A) Reactions were carried out under the standard conditions described above, with 0.3 nM RecBCD and 5 μM 3' Chi ended 14 mers or 3' Chi ended 20 mers.

B) Percent dsDNA remaining after 10 minutes.

involved the use of a much longer plasmid substrate, it is possible that hampered processivity due to interaction of RecBCD with the Chi^+ 14 mer or 20 mer, would lead to the observed inhibition of unwinding. However, if processivity is the only thing that is affected, then these oligomers should have no effect on the unwinding of a smaller duplex. Inhibition of unwinding of this duplex would then indicate an actual loss of helicase function itself, and show that processivity is not the only thing affected due to the interaction.

Results obtained from this set of assays confirmed the decreased unwinding of substrate observed in the Chi-cleavage assays, which also indicates that interaction of RecBCD with the Chi site has affected the helicase function directly, and not only the processivity.

Results for the Chi-centered 14-mers are as shown in figure 2.18, which illustrates the difference in effects caused by the 14 mer^+ and 14 mer^0. While almost all the substrate is unwound in 3 min in the presence of the 14 mer^o, only about 20 % is unwound in the 14 mer^+ reaction. Similar results were obtained with the 20 mers, (figure 2.19) with almost 70% of the DNA unwound in the 20 mer^0 reaction, and only about 20% of it unwound in the 20 mer^+ reaction. These results clearly indicate that there is something very specific about the interaction of the Chi sequence with RecBCD, which has led to an overall inhibition of its biochemical activities.

2.3-6 Enzyme-substrate binding reactions: The observed inhibition of the exonuclease and helicase activities might arise if oligonucleotides interfere with binding of RecBCD

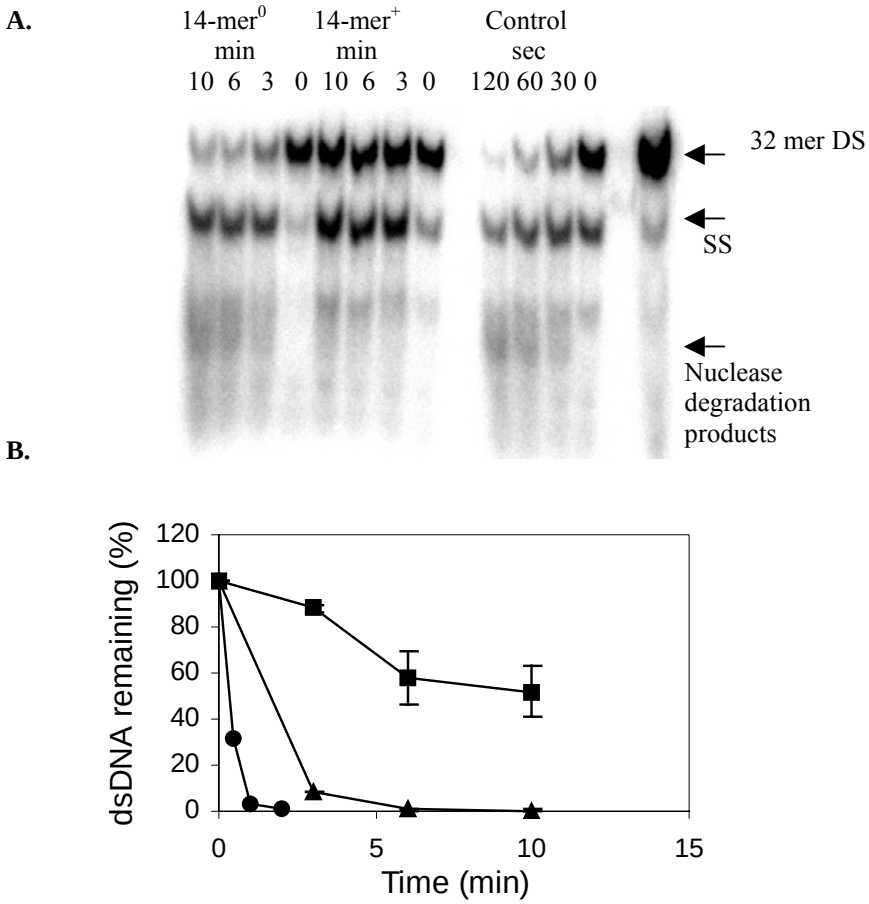


Figure 2.18:

Inhibition of helicase activity by Chi-centered 14 mers:

A) Reactions contained 25 mM Tris acetate, pH 7.5, 1 mM magnesium acetate, 1 mM ATP, 1 mM DTT, 3 nM of 5' ^{32}P-labeled double-stranded 32 bp substrate, 0.3 nM RecBCD and 15 μM 14-mer^{+} or 14 mer^{o}. The control reaction contained 0.1 nM RecBCD and no oligonucleotide.

B) Percent dsDNA remaining as a function of time for control (●), 14 mer^{+}(■) and 14mer^{0} (▲) reactions. Error bars represent standard deviation from duplicate runs.

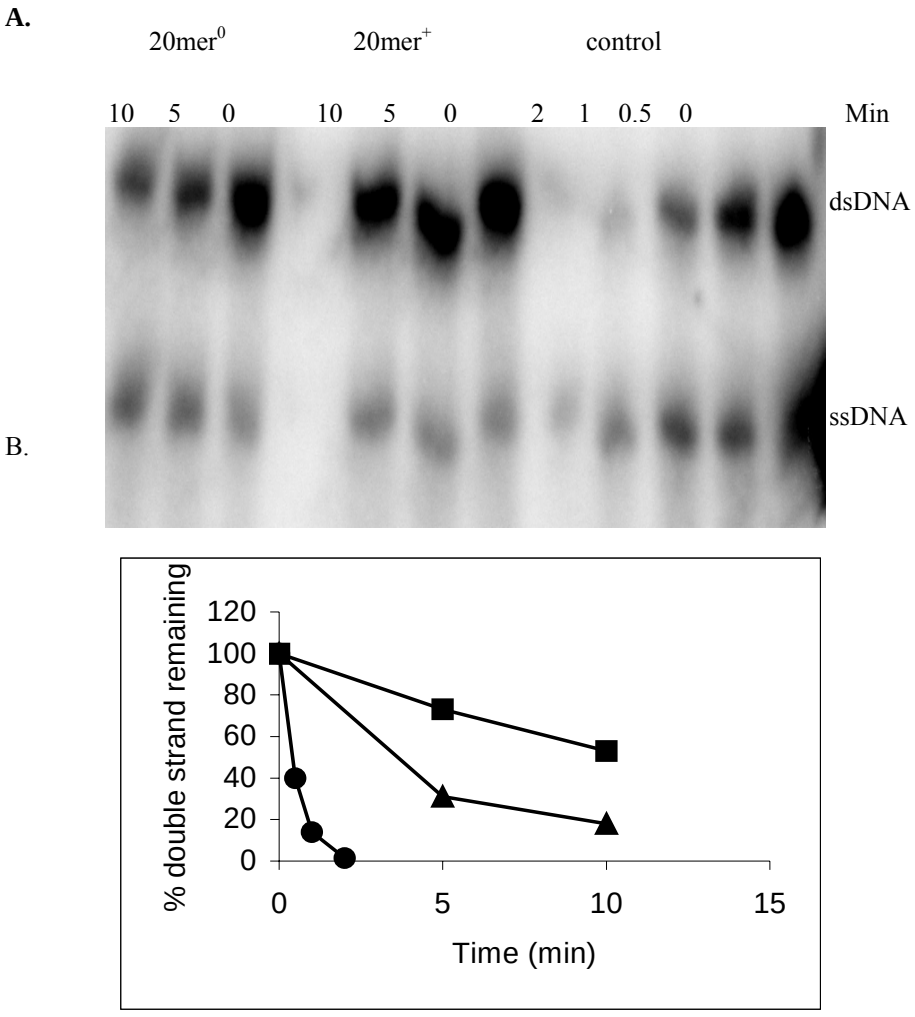


Figure 2.19:

Inhibition of helicase activity by Chi-centered 20mers:

A) Reactions were performed as described in Figure 2.18 legend with 10 μM 20 mer^{+} or 20 mer^{0} and 0.3nM RecBCD. Control reaction was performed with 0.1nM RecBCD and no oligonucleotides.

B) Percent dsDNA remaining as a function of time for control (●), 20 mer^{+}(■) and 20mer^{0} (▲) reactions

to the dsDNA substrate. In order to test if this is true, the Chi-cleavage assay was repeated with an initial pre-incubation of the enzyme and the 4.3 kb plasmid substrate for 2 min at 37 °C. This pre-incubation would allow the enzyme to bind the duplex ends first without interference by the competing oligonucleotides [64]. The reactions were started by adding either ATP alone (to the control reaction) or an ATP-oligonucleotide mixture and samples were removed within the first 30 seconds. A higher enzyme: substrate ratio was used for this set of reactions than for those shown above, to ensure that most of the duplex ends have an enzyme molecule bound to them. The concentrations used and other details are as described in the experimental procedure section.

Qualitatively distinct results were obtained with the non-incubated and pre-incubated enzyme, indicating the oligomers were preventing substrate-enzyme binding. Figure 2.20a shows the result obtained with the 8 mers used as the inhibitors. The oligomers inhibit very strongly when the reaction is carried out without preincubation of the [^{32}P] DNA substrate with RecBCD. While all of the linear substrate is consumed within 20 seconds in the control reaction (no oligomers), very little substrate has been consumed in the first 20 seconds in presence of the oligomers. In contrast, the enzyme that is allowed to bind first to the dsDNA substrate is impervious to inhibition by the oligonucleotides, and most of the substrate is consumed by the first time point whether or not an oligonucleotide is present in the mixture. This shows that allowing the enzyme to bind the [^{32}P] dsDNA substrate first ensures that oligonucleotides, even those with the Chi sequence, are unable to affect the enzyme once it initiates its helicase/nuclease activities on the dsDNA. To verify the result obtained, the reactions were also repeated with one more set of oligomers, the Chi-centered 20 mers and the

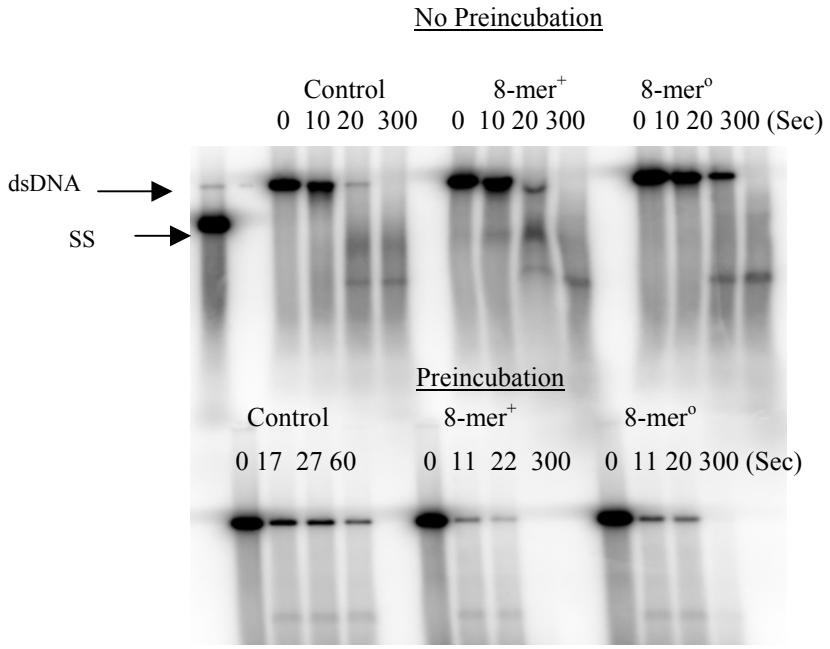


Figure 2.20

A) Absence of inhibitory effect of oligomers by pre-incubation of enzyme-substrate mixture:

Reactions done without preincubation were started by adding RecBCD enzyme to mixtures that contained all other reaction components. The final reaction mixture contained *Hind* III-linearized [5' ^{32}P] pBR322χF^+ (2.3 nM molecules), 3 nM RecBCD, 25 μM 8 mer$^+$ or 8 mer^o, and other components as in the Methods section. The control reaction contained no oligonucleotide.

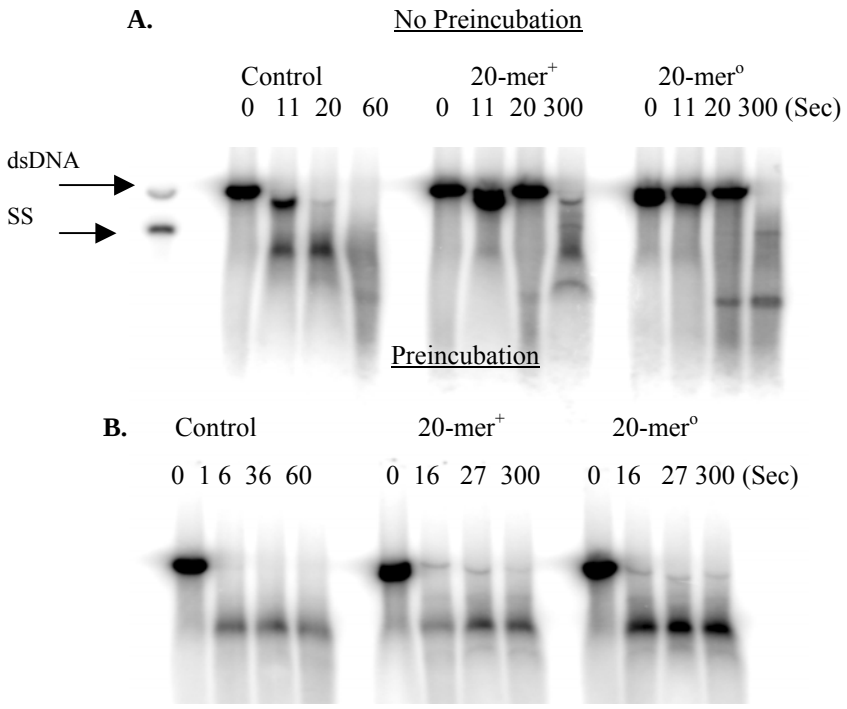


Figure 2.20

B) Absence of inhibitory effect of oligomers by pre-incubation of enzyme-substrate mixture:

Substrate-enzyme preincubation reaction as described in figure 2.20a legend was carried out using 2 μM Chi centered 20 mers.

same result was obtained (figure 2.20b), confirming that the oligomers are effective inhibitors of substrate binding to enzyme.

2.4 Discussion

2.4-1 Design of oligonucleotides: The study of the interaction between RecBCD and the Chi sequence has been addressed earlier through examination of the effects of the Chi sequence on the various biochemical activities of RecBCD. Since most of these studies have involved the use of duplex Chi bearing substrates, recognition of Chi on single-stranded DNA, and the related effects of the recognition have not yet been ascertained. The attempt here is to test the same kind of recognition and interference by the Chi sequence, but by single-stranded Chi-bearing substrates and to characterize the related effects. More importantly, the study aims to determine if the Chi sequence is specifically recognized by RecBCD when present on a single stranded DNA, and if this is true, to explain the underlying basis for the observed specificity.

The approach used to address all the above questions is to use single-stranded oligonucleotides bearing the Chi sequence in reactions to test their effects on the various biochemical activities of RecBCD. The simplest oligonucleotide designed for this purpose, the 8 mer, is merely the Chi sequence itself. Since the study attempts to examine specificity for the Chi sequence by RecBCD, it is also necessary to distinguish any effects on RecBCD caused by the Chi containing 8 mer from the effects caused by the presence of any other random 8 mer sequence. Hence, the 8 mer^0 was designed to be used alongside the 8 mer^+ when testing for the various biochemical activities of RecBCD. The 8 mer^0 is a single nucleotide variant of the Chi sequence (T to A, refer to table 1). This

sequence has been studied earlier and has been shown to be the least active as a Chi site, given a number of other single nucleotide variants of Chi that were also tested in the same study[93]. Hence it was selected as the comparative control for this study to define specificity for Chi.

The 14 mer and 20-mer length were arbitrarily selected to represent a medium or long oligomer. For each of the lengths, the location of the Chi sequence was varied to be at either the 3' or 5' end of the construct, or in the middle of the construct flanked by equal length at either end, keeping the rest of the sequence the same. Hence these oligomers differ only in the position of the Chi sequence and are exactly the same in terms of the base composition (i.e.the three sets of 14 mers are exactly the same, except for the positioning of Chi, and the same is true of the 20 mers). Further, for each of these 14 mers and 20 mers a corresponding Chi^0 construct was also designed bearing the single nucleotide variant of Chi. Each of these constructs is used in biochemical assays for RecBCD.

2.4-2 Exonuclease reactions: As outlined in the introduction, the 3'-5'exonuclease activity of RecBCD has been shown to be affected by Chi encounter and recognition. Combining data from several studies we now know that the enzyme undergoes an as yet unexplained attenuation of its 3'-5'-exonuclease activity, and possibly a switch in polarity of degradation to 5'-3' exonuclease following Chi encounter [89]. Since all these studies examined effects of Chi-bearing duplex on RecBCD biochemistry, a similar reaction with Chi bearing single-stranded oligonucleotides was selected as the starting point for this study of Chi-RecBCD interaction, and to establish the efficacy of the single-stranded

DNA as a Chi recognition substrate for RecBCD. The standard assay condition employed higher free Mg^{2+} than ATP concentration, which has been found to be favorable for exonuclease activity of RecBCD [87; 88; 89; 102]. These reactions involved the direct addition of the oligomers to the rest of the components of the reaction, followed by quenching of a zero time point, addition of RecBCD and incubation at 37 ºC for the selected times. The assays showed that all the oligonucleotides were inhibitors of the exonuclease activity, with the longer (20 mers) constructs being more effective as inhibitors than the shorter (8 mers) oligomers. The inhibition also shows concentration dependence, with the oligomers inhibiting better at higher concentrations. However, the inhibition is seen to plateau off after a certain concentration of the oligomer is added, such that addition of any further oligomer to the reaction does not significantly increase the inhibition. Even at a very high concentrations (50 μM 8 mers, 4 μM 20 mers, 15 μM 14 mers) the oligomers don't completely inhibit the reaction.

The most interesting results of this assay however, was the greater inhibition seen by the Chi^+ constructs than the Chi^0 construct, but only in those oligomers where the Chi was in the middle, flanked by sequence at either end (Chi centered 14 mers and 20 mers). This can be seen by comparing figures 2.5 through 2.7 where the amount of inhibition obtained by Chi^+ and Chi^0 of any Chi-ended construct or the 8 mers is very similar, as compared to the Chi-centered constructs where the Chi^+ construct inhibits much better than the corresponding Chi^0. In general, for any given length, the Chi-centered Chi^+ oligomer shows the best overall inhibition of the reaction, again suggesting specific recognition of Chi.

A) Reactions with preincubated RecBCD-oligonucleotide mixtures: Taylor and Smith in their study on Chi-RecBCD interactions, had observed a loss of all biochemical activities of RecBCD following its preincubation with a duplex containing multiple Chi sites [90]. They further observed almost 65% disassembly of this "Chi-reacted" RecBCD into its individual subunits. This led them to propose that this RecBCD disassembly on Chi encounter was the cause for the observed loss of RecBCD function.

The rationale behind carrying out preincubations was to see if we could mimic this effect observed earlier by Taylor and Smith, and see a greater loss of RecBCD activity than when oligonucleotides are added directly to the exonuclease reactions (as described above). The preincubation might serve to optimize the interaction between RecBCD and the Chi-bearing oligomers, and with it the ensuing structural changes that may occur as a result of Chi encounter.

The results obtained with all the oligonucleotides tested under different pre-incubation conditions (refer to table 2) show that there is no significant increase or change in the pattern of inhibition by any oligomer due to pre-incubation. When the preincubate is diluted into an exonuclease reaction to yield a low (nM) concentration of the oligomers, there is little or no inhibition observed by any of them, suggesting that there has been no significant distortion of enzyme structure or function. At higher oligomer concentrations (20 μM) the effects seen are in a proportion and pattern similar to those obtained if the oligomers were directly added to the reaction without pre-incubation (figure 2.11). Hence, these results do not indicate any additional changes in enzyme structure due to preincubation.

One possible explanation for this could be that the oligomers do not bind to RecBCD under the pre incubation conditions. However, this does not seem to be the likely explanation going by previously measured K_ds of 70 nM, 15 nM and < 1 nM for a 12 mer, 15 mer, and a 25-30 mer respectively.[103] The other, more likely explanation is that the interaction between the Chi containing oligomers and RecBCD is a reversible one with very fast exchange between the bound and unbound states. The two states would then be in rapid equilibrium with each other, and hence at any given time, the overall RecBCD in contact with any oligomer is the same as when there is direct addition of the oligomer to the reaction.

2.4-3 Chi-recognition reactions: This set of reactions was carried out to determine if, like the exonuclease activity, the Chi-recognition and the subsequent Chi-cleavage activity of RecBCD was also inhibited by single-stranded Chi substrates. The rationale behind this set of reactions is that, if Chi in any of the single-stranded constructs is specifically recognized by RecBCD, resulting in its specific interaction with RecBCD, that would hinder RecBCD from interacting optimally with a competing duplex. Given that the competing duplex also bears a Chi site, this would result in reduced interaction with this competing Chi site, and hence, reduced production of the downstream Chi band. As stated earlier, another possible outcome of this assay is the enhanced production of the upstream Chi band, due to stimulation of the 5'-3' exonuclease activity of RecBCD following Chi encounter in dsDNA (as seen by Anderson and Kowlaczykowski [89]).
An interesting result of this set of assays, was that the inhibition obtained was not just in the expected production of the downstream Chi band, but of the unwinding of the duplex

substrate itself (which would also lead to reduction in the amount of Chi-specific fragment being produced). Consequently, it is possible that the observed inhibition of exonuclease activity is an artifact of this reduced unwinding of substrate rather than a reduction in actual exonuclease function itself. This set of assays hence reveals a slowing of overall reaction due to the presence of Chi.

2.4-4 Helicase reactions: Since the inhibition of unwinding observed in the Chi-cleavage assays was not an expected result, in order to confirm this effect, the oligomers were tested in an assay that directly measures the helicase activity of RecBCD. A smaller 32 mer duplex substrate was specifically designed for this assay, to determine if the processivity of helicase activity was solely affected. Since RecBCD is a highly processive helicase, any interaction that affects processivity would result in decreased unwinding as observed in the Chi recognition assays which employed a longer 4.3 kb substrate. However a smaller substrate would be efficiently unwound if the only factor affected was processivity. Inhibition of unwinding of this substrate would indicate changes in the overall helicase function.

The results obtained confirm and consolidate both the inhibition of helicase by the oligomers, the fact that the helicase function as a whole is affected by the Chi sequence. Even in this set of assays, the inhibition by the Chi^{+} is greatest when it is in the center of the construct. The corresponding Chi^{0} construct shows very little inhibition of the reaction, indicating that there is something very specific about the Chi sequence itself. Since the $14mer^{+}$ and $20\ mer^{+}$ were both effective in inhibiting unwinding of a much smaller 32 mer duplex used for this assay, this result also shows that interaction of

RecBCD with Chi within these constructs has a direct effect on the helicase activity of the enzyme itself, and not just the processivity of the helicase.

2.4-5 Enzyme-substrate binding reactions: This set of enzyme-substrate pre-incubation reactions were carried out to test whether the inhibitory effects exhibited by the oligomers arise partly out of their interference with substrate enzyme binding. The results obtained (figure 2.20a or 2.20b) corroborate this hypothesis. The fact that pre-incubation of RecBCD with the substrate results in a complete lack of inhibition by the oligomers suggests that an exclusive Chi-specific pocket, if present, may exist adjacent to a non-specific DNA binding site, such that pre-incubation with substrate DNA prevents Chi from accessing that site. This would also mean that the interaction between the substrate and RecBCD is strong and not easily reversible, so that the Chi sequence cannot displace the substrate once it is bound. RecBCD is known to have a high affinity for a duplex substrate. [39]

CHAPTER III

PHOTOCROSSLINKING OF RECBCD TO CHI

3.1 Introduction

The most obvious question now is what are the structural changes that underlie the observed inhibition of helicase, exonuclease and Chi-recognition activities, and how the location of the sequence and presence of flanks matters in Chi recognition. Especially in case of the Chi centered constructs, the observed effects suggest that there is something very specific about the Chi-RecBCD interaction in these constructs, leading to a significant reduction in all biochemical activities tested. However it is not certain at this point, what this specificity stems from. One distinct possibility is that it may arise from the presence of a specific Chi binding site on RecBCD.

Assuming that such a specific site does exist, it would be interesting to know if it rests exclusively within one of the three subunits of RecBCD, or if it is a feature of the holoenzyme and is shared between the subunits. A large number of studies on Chi mutants of *E. coli* speculate on the role of RecC as the primary subunit of RecBCD involved in Chi recognition ([5; 91; 104]). However, given the fact that Chi is recognized from its 3' end, and RecB is the subunit that binds the 3' end[40] it is possible that RecB may hold a large part of the Chi binding site. RecB is also the primary nuclease and helicase in RecBCD[74; 75], hence it seems likely that it interacts with Chi and undergoes structural changes thereafter resulting in the observed effects. It may also be possible that Chi-specificity arises out of mutual co-operation between RecC and RecB, and not just the involvement of one subunit alone. RecC could in this case host a primary

site for Chi recognition, and its interaction with RecB's helicase could be critical to the optimum binding of Chi and its observed effects thereafter. The identification of the subunit/s that interact optimally with the Chi site could hence provide a good start towards understanding the structural changes within RecBCD that underlie the observed change in its activities.

We have attempted to identify the primary zone on RecBCD involved in Chi affinity, via a set of photocrosslinking reactions with the Chi -centered 14 mers and RecBCD. The approach employed involved the use of iodouracil (I-dU) substituted oligonucleotides (14 mers) to enable efficient photocrosslinking at wavelengths of 300 nm or more. The iodouracil substituent has an absorbance maximum of 250-310 nm allowing the use of longer wavelengths for the crosslinking, which causes less photodegradation of the protein as compared to the shorter wavelengths. The 5-position of iodouracil containing the iodide group has been shown to produce zero length cross links with the aromatic amino acids Tyr, Trp and Phe following irradiation at 250-310 nm. Excitation at these wavelengths results in homolysis of the carbon-iodide bond leading to formation of a reactive group for cross-linking[105; 106]. In addition, it is very similar in size to thymine (iodide: 2.15 Å, methyl group: 2.0 Å) [106] and hence was selected for use in this set of assays.

3.2 Materials and Methods

Materials

I-dU substituted oligonucleotides (Midland Certified Reagent Corp., Midland. Texas)

Chromato-vue transilluminator, model TM-36 (UVP Inc)

γ-^{32}P ATP (Amersham Pharmacia)

ATP 100 mM solution (Amersham Pharmacia)

Cellulose PEI-F TLC plates (JT Baker Inc.)

RecBCD enzyme (purified by FPLC, refer to appendix A)

Methods

3.2-1 ^{32}P labeling of I-dU substituted oligonucleotides: I-du substituted 14 mer $^{+}$ (5' TAGGCTGG (IdU) GGAT 3') and 14 mer ^{0} (5' TAGGCaGG (IdU) GGAT 3') were 5' end-labeled using [^{32}P] ATP and polynucleotide kinase as described[107]. The products were purified (Qiagen Nucleotide Purification kit), and analyzed by thin layer chromatography on polyethyleneimine-cellulose plates in 1 M potassium phosphate buffer (pH 3.5) to ensure separation of [^{32}P] ATP from labeled oligonucleotide.

3.2-2 UV cross-linking of RecBCD to I-dU oligonucleotides: Standard reactions contained 25 mM Tris acetate pH 7.5, 2 mM magnesium chloride, 0.1 mM DTT, and variable amounts of RecBCD and oligomer. A 2 µl aliquot was quenched in 3.3x SDS loading buffer (0.15 M Tris-HCl (pH 6.8), 30% glycerol, 3% SDS, 0.03% β–mercaptoethanol and 0.008% BPB) before exposure of the reaction mixture to UV light. Photocrosslinking was started by exposing the reaction as a droplet to UV light of 302 nm wavelength at 4 °C, using a UV transilluminator, held 2 cm away from the reaction droplet. The reaction droplet was exposed to the radiation for pre-determined time periods at the end of which 2 µl aliquots were quenched in 3.3x SDS loading buffer. The quenched samples were heated at 100 °C for 2 minutes before loading on a 7.5% SDS-PAGE. The gel was pre-run at 15 mAmp for 30 mins before loading the samples. The samples were run at 15 mAmp till the dye band reached the bottom. Gels were dried and subjected to phosphorimaging. A lane from the same gel was loaded with RecBCD protein, cut and stained separately with Coomassie blue for identification of the cross-linked band.

3.3 Results

RecBCD purified from 4 liters of *E. coli* strain HB101 [pDJ05] using a combination of affinity FPLC, anion exchange, and hydroxyapatite chromatography was used in these cross-linking reactions. Details of the procedure are provided in appendix A.

3.3-1 ^{32}P labeling of I-dU substituted oligonucleotides: Figure 3.1 shows the TLC of the labeled purified oligomers. The concentration of the oligonucleotides was estimated to be 92 nM (14 mer $^{+}$) and 122 nM (14 mer^{0}) from the amount of radioactivity (determined by scintillation counting) of samples taken before and after purification (the radioactivity of the labeling reaction before purification corresponds to 3 μM oligonucleotides). The amount of labeling achieved was almost the same for both the oligomers as estimated by the counts obtained from the purified labeled oligomers. (355394 cpm/min for 0.5 μl 14 mer^{+} and 284880 cpm/min for 14 mer^{0}).

3.3-2 UV cross-linking of RecBCD to I-dU oligonucleotides: The Chi-centered 14 mer oligonucleotides were used in cross-linking reactions as described above to examine the pattern of crosslinking obtained. The overall results show that the oligonucleotides both cross-link to the same site/sites on RecBCD giving the same pattern of bands on phosphorimaging. The crosslink signal is mainly concentrated to the lower of the two closely spaced bands of RecBCD, which corresponds closely to the position of RecC in RecBCD. Hence, these preliminary results seem to indicate that it is the RecC subunit

I-dU 14 mer^{+} I-dU 14 mer^{0}

Figure 3.1

Thin layer chromatogram of ^{32}P labeled I-dU 14 mers

0.3 µl of pure labeled oligomer was spotted on TLC plate, run, and developed as described in methods section.

that is primarily involved in cross-linking of both the I-dU substituted 14 mers (figure 3.2), with the 14 mer^{+} binding more efficiently. This is clearly visible in figure 3.2, which shows the results of a 10 minute cross-linking reaction carried out with varying concentrations of the oligonucleotides. The figure clearly shows a stronger signal for the cross-linking of the 14 mer^{+} at each of the indicated concentrations. Band volume integration shows that the extent of cross-linking for the 14 mer^{+} is approximately 76% higher than the 14 mer^{0} (averaging the percent cross-linking for each of the three concentrations of oligomers cross-linked).

The results obtained also show an overall increase in cross-linking for each oligomer with increase in time of radiation exposure (figure 3.3) and increase in concentration of the oligonucleotide (figure 3.2 and 3.4). However, these results also show that for either construct, under the given reaction conditions, the overall efficiency of cross-linking was not very high (approximately 0.08% as estimated from the large radioactive signal accumulated at the bottom of the phosphorimages from the uncross-linked oligonucleotides).

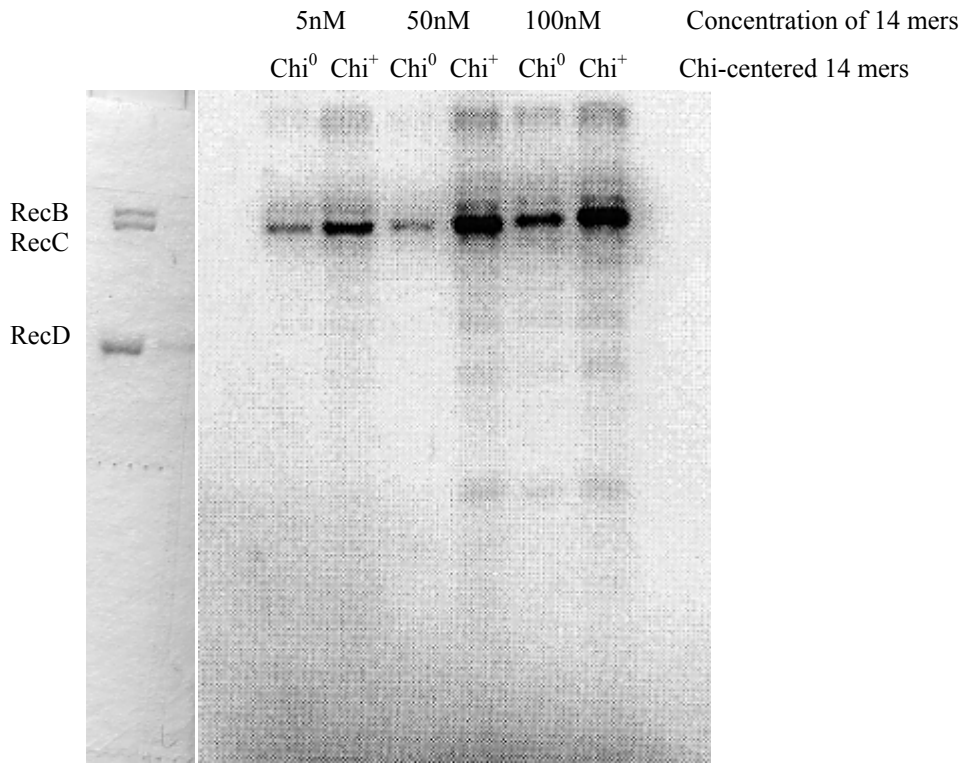


Figure 3.2

Cross-linking by 14 mer^{+} and 14 mer^{0} To RecC: The standard reactions were set up as described in the methods section. All reactions contained 50 nM RecBCD. The reactions with 50 nM oligomers contained a mixture of 40 nM unlabeled and 10 nM ^{32}P labeled oligomer. The reactions with 100 nM oligomers contained a mixture of 90 nM unlabeled and 10 nM ^{32}P labeled oligomer. The reactions with 5 nM oligomers contained only ^{32}P labeled oligomer. The cross-linking time for the reactions was 10 minutes at 4 °C.

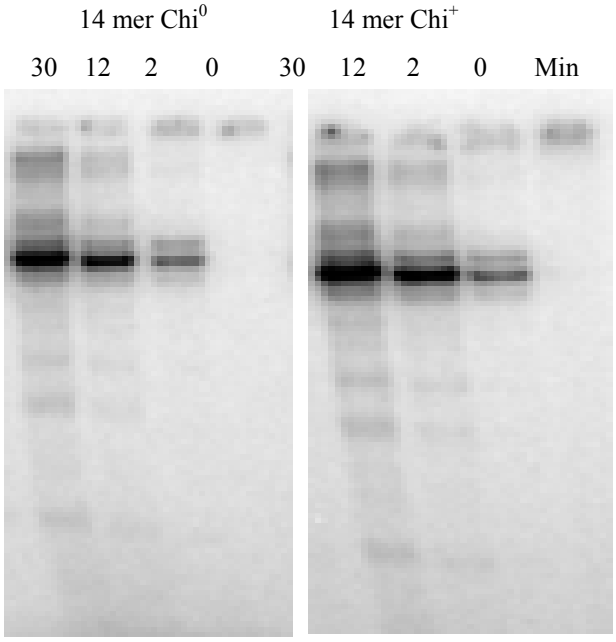


Figure 3.3

Increase in cross-linking with time: The standard reactions were set up as described in the methods section, containing 100 nM RecBCD and approximately 11 nM 14 mer^{0} or 15 nM 14mer^{+} and photocrosslinked for the indicated amount of time.

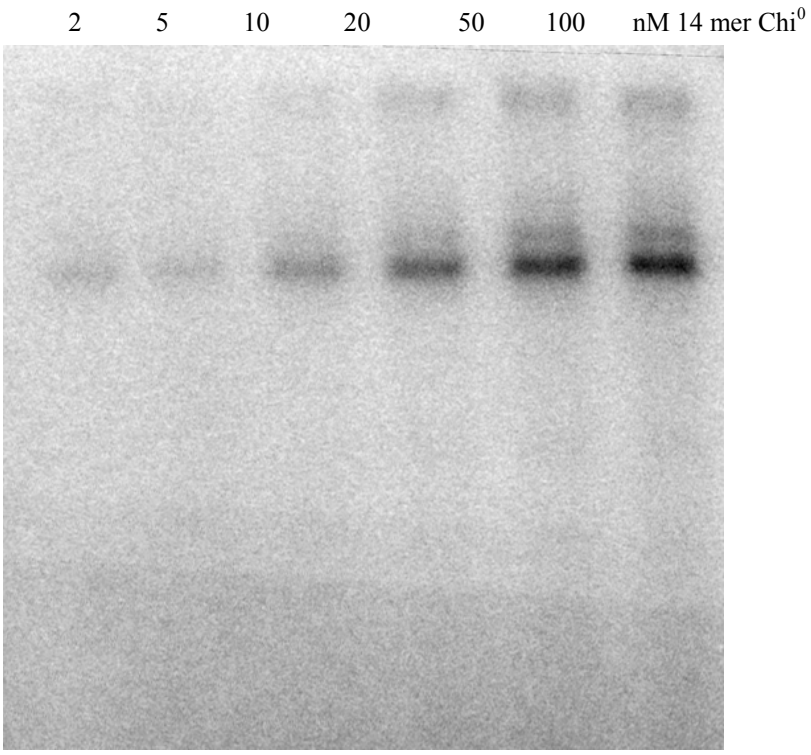


Figure 3.4

Increase in cross-linking with increasing oligomer concentration: The standard reaction, containing 10 nM RecBCD and indicted amounts of 14 mer^0 were set up as described in the methods section and photocrosslinked for 10 minutes.

3.4 Discussion

This section of the study only tested the 14 mers for their effectiveness in cross-linking to RecBCD. The rationale behind this set of assays was to obtain some preliminary and immediate data that might steer the course of future studies and provide direction to begin a more extensive and through investigation of the structural changes underlying RecBCD-Chi interaction.

3.4-1 UV cross-linking of RecBCD to I-dU oligonucleotides: The results showed a time and concentration dependent increase in overall crosslinking for both oligomers, with a greater extent of cross-linking by the 14mer^{+} oligomer. Assuming that TLC and radiation counts (which show the amount of signal for the two labeled oligomers to be very similar) are accurate indicators of concentration of the labeled oligomers, this result complements the results obtained so far, and confirms RecBCD's greater affinity for Chi. Since the results show a concentration of crosslinked signal at the approximate site where RecC would appear on the gel, if confirmed true, this would corroborate findings from studies on Chi defective mutants of *E. coli*, which map to the *recC* gene and suggest that RecC is the primary mediator of Chi activity in RecBCD. These mutants have been categorized into three different classes (null, class II and RecBC*D) based on common phenotypes, and loss of Chi dependent joint molecule formation is a common feature spanning all the classes [5]. The RecBC*D class of mutants encompassing 4 different alleles of *recC* (*recC*^{1001}-*recC*^{1004}) actually have wild type helicase, ATPase, and exonuclease activity, and are defective only in Chi dependent joint molecule formation. One member of this class of mutants is also known to recognize an 11 nucleotide variant

of Chi [91] indicting that Chi recognition may involve this locus in *recC.* Hence *recC* functioning, as the primary mediator of Chi recognition is a distinct possibility. In that light, the fact that most or all of the 14 mer signal cross-links to RecC makes an interesting result.

However, since these crosslinking reactions employ only nM concentrations of 14 mers, which are too low for inhibition of RecBCD function (□M concentrations are needed to observe exonuclease/helicase inhibition), the results obtained here may be only a partial indication of the binding specificity. At such low concentrations the oligomers might cross-link to a high affinity DNA binding site on RecBCD, that does not induce structural or functional changes in RecBCD. Use of μM concentrations in cross-linking, might be a better indication of binding events that leads to actual loss of RecBCD function. For instance, RecBCD could have more than one binding site, which get occupied only at higher concentrations of the oligomers, leading to their observed biochemical effects. Hence the results presented here are preliminary, and further study is needed to confirm specificity for the Chi sequence. A possible approach to this would be to attempt isolation and sequencing of a Chi-crosslinked peptide. This might be a potential Chi specific site, and mutants of this sequence can be tested for Chi binding/ Chi effect to confirm this.

Further studies on this set of assay, might help to confirm and implicate RecC as the Chi-recognition subunit, while also verifying if the greater extent of cross-linking seen with the $14mer^{+}$, is in fact a true indication of a specific recognition site for Chi, or a much greater affinity for the sequence. Either way, it would confirm the fact that RecBCD has specificity for the Chi sequence along with possibly clarifying some of

the complex and yet unseen structural changes that most surely constitute a major part of the Chi-RecBCD interaction.

CHAPTER IV

DISCUSSION AND CONCLUSION

Studies on RecBCD-Chi interaction have shown that RecBCD is specific for the Chi sequence and that interaction with this sequence results in an alteration or modification of RecBCD and hence a change in its biochemical activities. This is often referred to as the "Chi-effect" and though several studies have attempted to define its finer aspects, the overall picture is still very hazy. It could be the disassembly of the subunits, the ejection of the RecD subunit, or an overall change in conformation of the protein itself. Similarly, the basis for the specificity for this sequence is not known. It could arise from the presence of an exclusive site for Chi on RecBCD and a stronger affinity for this sequence as compared to any other sequence.

In this study, we attempted to examine the interaction of RecBCD with the single- stranded Chi sequence, to determine if effects observed were similar to those reported earlier with duplex DNA. The primary aim of the study is to address the issue of specificity for Chi on exclusively single stranded substrates and define the related biochemical effects of this interaction. We have employed the approach of using single-stranded oligonucleotides of varying lengths, bearing the Chi sequence either at one or the other end, or flanked by equal length of DNA at either end, to test their efficacy as inhibitors of various activities of RecBCD.

The results obtained showed that all the oligonucleotides were inhibitors of RecBCD activities. The activities inhibited included not just the exonuclease but also the helicase and the Chi-recognition activity itself. The overall inhibition was length

dependent, with the longer oligonucleotides being more effective inhibitors than the shorter ones.

The flank-dependent recognition of Chi revealed in this study brings into focus a distinct and so far undetected feature of this interaction, namely its “location-specificity”, and with it, significance of the related flanking sequences. The need for a flanking sequence can be explained if it is assumed that there is a specific Chi binding site on RecBCD, which is part of a larger, non-specific DNA binding site. The non-specific DNA binding site would then serve the purpose of binding the flanking sequences which would serve as a kind of "handle" to optimize contact with the substrate and position the Chi in its specific binding site on RecBCD.

This kind of a requirement for flanks has been observed in other sequence-specific proteins, like the restriction endonucleases, transcriptional repressor and activator proteins.[108; 109]. Alkylation interference and protection studies on the restriction enzyme *Eco*RI have shown that the enzyme interacts with two nucleotides to the 5' side of its hexameric target sequence, and alteration of the phosphate backbone at this flanking region impacts the enzyme's ability to bind its target sequence[110]. The estrogen receptor (specific for a 13 bp response element) and the bovine papillomavirus (BVP-I) replication protein E1 (specific for an 18 bp inverted repeat) are other examples of proteins whose sequence-specific interactions are subject to flank requirements. Binding of these proteins to their target elements has been shown to require additional sequence. Immunoprecipitation experiments employing BVP-E1 and single or double stranded oligonucleotides have shown that the protein did not bind the 18 mer target sequence, but a 31 mer encompassing the 18 mer was bound[109]. The general observation from these

studies suggests that longer flanks lead to formation of more stable DNA-protein complexes, suggesting that they contribute to anchorage of the enzyme on the substrate and to the thermodyamic stability of the complex. Hence it seems likely that flanks might be critical to the stabilization of the RecBCD-Chi interaction. Though at this point, it is not clear if the sequence of the flank is of any importance in recognition, earlier studies on RecBCD-Chi have demonstrated that RecBCD recognizes Chi with equal efficiency no matter what the base composition of the flanking sequences is. [80; 81; 91]

Based on the results obtained and the observed location-specificity for Chi recognition, we have proposed a model explaining the interaction of RecBCD with Chi, encompassing the location specificity and requirement for a flank. According to this model (figure 4.1) RecBCD needs a handle region to grip on before it can interact closely with and recognize any specific sequences or regulatory elements. The handle sequence is envisioned to serve as an anchor for RecBCD enabling optimal contact with the remaining stretch of DNA, such that any specific sequences that are part of the handle escape recognition in the process. This would explain the lack of distinction between the Chi^{+} and Chi^{0} oligomers in case of the Chi-ended constructs, or the Chi-sequence alone (8- mer). In this case part or all of the Chi sequence itself becomes part of the handle and hence escapes recognition (figure 4.1). The model further proposes that a specific site for Chi does exist on RecBCD, which binds Chi with stronger affinity than the non-Chi. The site though not exclusive for the Chi sequence, is proposed to be part of, or adjacent to, a larger non-specific DNA binding site, also encompassing the helicase active site (explaining the observed attenuation of helicase activity), having greater affinity for the Chi sequence. Once trapped, the Chi is specifically recognized by RecBCD leading to the

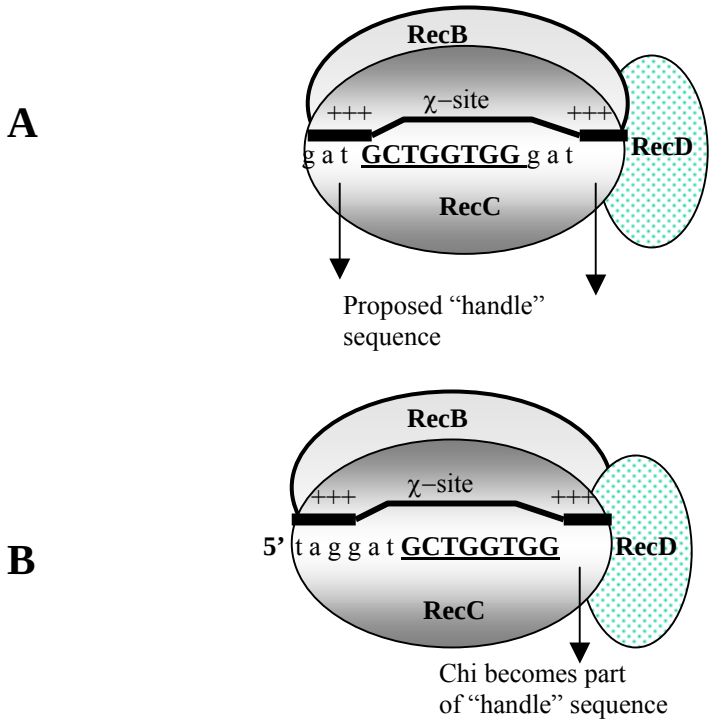


Figure 4.1

Model for "location-specific" recognition of the Chi sequence by RecBCD

A) Interaction of RecBCD with a Chi-centered oligonucleotide. The flanking regions on either side of the Chi act as the proposed "handle" regions that simply enables RecBCD to grip the substrate. Any specific sequences that are a part of this handle are proposed to go unrecognized. The Chi sequence in this case fits perfectly onto an adjacent site and is specifically recognized. The site is shown to be on RecC in view of the photocrosslinking results, but may be jointly shared by RecB and RecC (refer to discussion). **B) Interaction of RecBCD with a Chi-ended oligonucleotide or the 8-mer.** In this case the Chi sequence itself becomes part of the handle and hence goes unrecognized.

series of events that end in the production of a single-stranded overhang for recombination.

In terms of the location of this site on RecBCD, the data from photocrosslinking experiments indicate that RecC is the primary subunit involved in binding. Though, this result is consistent with the finding that some *recC* mutants are wild-type helicases, nucleases and ATPases, only defective in Chi recognition[52; 56; 91; 104], the fact that Chi is recognized only from its 3' end which is bound by RecB [40], seems likely that at least part of the Chi-effect is mediated by RecB. Further RecB is also the primary nuclease, ATPase and helicase of RecBCD and it can be safely concluded that it would have several non-specific DNA binding sites, that could potentially serve as Chi anchors [62; 75]. Isolated RecC on the other hand does not bind DNA [92], but it is known to stimulate the ATP hydrolysis and helicase activities of RecB [66; 103; 111]. However, considering the oligomers in this study photocrosslink primarily to RecC, and the corroborating evidence that supports its role in Chi recognition, it is possible to imagine a scenario whereby the Chi specific site is encompassed by the RecC and RecB subunits together. It is possible that within this joint venture, RecC has a larger contribution to make either in terms of forming the site itself, or in terms of initially contacting and binding the Chi, and presenting it to RecB. Eventually, the overall changes in exonuclease and helicase activities indicate some alteration or rearrangement of the RecB subunit. Hence, though figure 4.1 shows the site to be on RecC, it is equally possible that the site is not exclusively present on one subunit, but is molded out of mutual cooperation between the subunits of the holoenzyme.

Finally, some speculations can be made about the significance of these findings for the *in vivo* functioning of RecBCD. Given the observed need for flanks in this study, it is possible that if RecBCD were to encounter double-stranded DNA damage at a Chi sequence *in vivo*, it might not recognize it, and move along the DNA till it encounters the next Chi site (which would then be recognized). If this hypothesis is true, it might explain the large abundance of Chi sequences found on the *E. coli* genome[81] which is much more than what would be normally expected for any given sequence. Double-stranded damage that occurred at or just before a Chi site might render the Chi non-recognizable, which would prevent repair of the damage and threaten the survival of the cell itself. Similarly repair of collapsed replication forks will need a Chi site in the vicinity. Over-expression of the sequence in such cases would ensure that RecBCD encounters at least one functional recognizable Chi sequence before too long, and repair processes are not hampered.

In conclusion, it can be said that the RecBCD-Chi interaction is an intricate and complex mosaic of several distinctive features and characteristics, a large number of which are probably still undetected. Considerable further research and study needs to be directed towards the detection, analysis and comprehension of these aspects, before we are in a position to fit the pieces together and completely resolve the intriguing issue of Chi specificity and Chi-induced alteration of RecBCD.

APPENDIX A

Purification of RecBCD by FPLC

Materials and Methods

Materials

0.2 and 0.45 micron ultrafilters (Millipore)

10,000 molecular weight cut off snakeskin pleated dialysis tubing (Pierce)

Polyethylene tubes 15 ml and 50 ml (VWR)

18 and 20 gauge needles (Becton-Dickinson)

Syringes (Becton-Dickinson)

Luria Bertani media

Centrifuge bottles (500 ml, 250 ml, and 50 ml)

Glycerol (Fisher)

EDTA (Fisher)

DTT (Fisher)

Q-sepharose Hitrap Q-FF/5 ml FPLC column (Amersham Pharmacia)

Heparin FF 16/10 (20 ml) FPLC column (Amersham Pharmacia)

Mono-Q HR 5/5 FPLC column (Amersham Pharmacia)

Hydroxyapatite (Bio-Rad, DNA grade)

Ampicillin (United States Biochemicals)

Ammonium Sulfate (J.T. Baker)

Methods

1. Purification of RecBCD using FPLC:

A) Cell growth: Preparation of HB101 [pDJ05] plates: HB101 [pDJ05] cells from a -80 °C stock were grown in 2 ml Luria Bertani media (LB) (1% sodium chloride, 1% tryptone, 0.5% yeast extract) containing 100 μg/ml ampicillin at 37 °C for 6 hours and then streaked on an LB-plate (100 μg/ml ampicillin). The plates were incubated for 14 hrs at 37 °C. Colonies were obtained.

B) Growth of larger culture: A single colony from the streaked plates was grown in 2 ml LB medium containing 100 μg/ml ampicillin at 37 °C for 14 hours. Of this, 200 μl was used to inoculate a fresh culture of 40 ml LB media (100 μg/ml ampicillin). $O.D._{650}$ was monitored till it reached 0.09 and the culture was stored at 4 °C overnight. Approximately 20 ml of this culture was used to inoculate two separate 2 liter LB cultures. The 2 liter cultures were grown at 35-37 °C for 13 hrs 15 min. The cultures were harvested at 5000 RPM / 4 °C / 10 min using the JA-10 rotor in the Beckman centrifuge. 22.84 gm of cell pellet was obtained.

C) Sonication: The cell pellet was resuspended in 130 ml lysis buffer (50 mM TrisHCl 10% sucrose, 0.2 M sodium chloride, 10 mM mercaptoethanol, 1 mM EDTA pH 7.5), and sonicated in 40-45 ml aliquots, with a Branson Sonifier at output setting 60%, duty cycle 30%. Each aliquot was sonicated for a total time of 7 min, which includes 30 second pulses, and 30 second intervals between pulses. The supernatant was spun down

(Beckman centrifuge, JA-14 rotor, 4 ℃/ 14K RPM/ 30 min). The supernatant (fraction 1) was used for the next step (ammonium sulfate precipitation).

D) Ammonium sulfate precipitation and dialysis: 0.282 gm of ammonium sulfate/ml of fraction 1 was added slowly in small aliquots, with constant stirring at 4 ℃. After all the salt is added the solution is allowed to stir for an additional 40-45 min to ensure that all the salt is dissolved. Proteins precipitated were collected by centrifuging at 4 ℃/ 12K RPM/ 45 min. The pellet was dissolved in 15-20 ml Q-sepharose start buffer (20 mM Bis-Tris propane HCl, pH 6.9, 0.1 mM EDTA, 0.1 mM DTT, 10% glycerol), and dialyzed against 4 liters of the buffer overnight at 4 ℃ (two exchanges of 2 liters each). The dialyzed protein was filtered through a 0.45-micron filter and loaded onto the Q-sepharose column.

E) Q-sepharose anion exchange (Hitrap Q FF) chromatography: (Note: All buffers used for the FPLC column were prepared in de-ionized distilled water and sterilized by filtration through a 0.22 micron filter. All protein solutions were filtered through the 0.45 micron filter before loading onto the column)

Column equilibration: The column was washed successively with 5 column volumes (25 ml) of the Q-sepharose start buffer followed by 5 column volumes of distilled, deionized water, 5 column volumes of buffer B (Q-sepharose start buffer with 1M KCl) to ensure removal of any residual protein, followed by 2 volumes of Q-sepharose start buffer (all washes at 5 ml/min)).

Column loading and run: Following equilibration of the FPLC Pump A with Q-sepharose start buffer and pump B with the high salt buffer B, the bypass position was equilibrated with Q-sepharose start buffer. 18 ml of filtered protein was loaded onto the column using a superloop, at 3.5 ml/min. The column was washed with 40 ml Q-sepharose start buffer followed by 5 ml 15% buffer B at 3.5 ml/min. The protein was eluted with 10 column volumes of a gradient of 15% buffer B (0.15 M salt) to 60% buffer B (0.6 M salt). Following elution, the column was washed with an additional 5 column volumes of 100% buffer B to ensure removal of all traces of protein, followed by wash with 5 column volumes of FPLC grade water and 5 column volumes of 20% ethanol (for storage). 1µl of each peak fraction (10 ml fraction size) was tested for exonuclease activity with and without ATP, and 10.46 µl of each fraction was analyzed by 10% SDS-PAGE for detection of protein.

The RecBCD active fractions from the Q-sepharose column were pooled and precipitated with 0.39 gm/ml of ammonium sulfate as described above. The pellet was resuspended in 10-15 ml heparin buffer (20 mM potassium phosphate, 0.1 mM EDTA, 0.1mM DTT, 10% glycerol, pH 6.9) and dialyzed against a total of 4 liters of heparin buffer overnight at 4 °C (two exchanges of 2 liters each). The dialyzed protein was filtered through a 0.45-micron filter for loading on the next (heparin affinity) column.

F) Heparin affinity (FF 16/10 column) chromatography:

Column equilibration: As described for the Q-sepharose column, the column was first washed with 5 column volumes (100 ml) of high salt buffer (0.5 M KCl in heparin

buffer), and 5 column volumes of FPLC grade water (all washes at 3 ml/minute) till column is washed of all traces of proteins and impurities. The column was then equilibrated with 6 column volumes of heparin buffer (3 ml/min).

Column loading and run: 10 ml of the above-dialyzed protein was loaded onto the column at 0.15 ml/min flow rate. The column was washed with 4 column volumes heparin buffer at 0.25 ml/min. The protein was eluted with 10 column volumes of a gradient going from 0% heparin buffer (no KCl) to 35% heparin buffer (0.175 M KCl) at 0.25 ml/min flow rate. The column was washed of residual protein with an additional 3 column volumes of 100% heparin buffer (0.5 M salt), followed by washes with FPLC grade water and 20% alcohol as described above. Peak fractions were analyzed for protein and exonuclease activity as described above for the Q-sepharose column. Peak fractions (5 ml each) were pooled and dialyzed overnight against 4 liters of Q-sepharose start buffer as before (two exchanges of 2 liters each).

G) Mono-Q anion exchange (HR 5/5) chromatography:

Column equilibration: As described for the Q-sepharose column.

Column loading and run: The above dialyzed protein (27 ml) was filtered and loaded on the column through a superloop at the flow rate of 1 ml/min. The column was washed with 5 column volumes (5 ml) of the Q-sepharose start buffer, followed by elution with 10 column volumes of a gradient starting at Q-sepharose start buffer going to Q-sepharose start buffer with 0.6 M KCl. Residual protein was washed off as described above followed by wash with 20% ethanol. Peak fraction (1 ml each) were analyzed for protein and activity as described above. The active fractions (1 ml each) were pooled and

dialyzed overnight against 4 liters buffer C (10 mM potassium phosphate, 1 mM DTT, pH 6.9) as described above.

H) Hydroxyapatite chromatography:

Column preparation and equilibration: A slurry of hydroxyapatite resin was prepared by suspending 1 part of the dry powder in 6 parts of 10 mM potassium phosphate buffer containing 1 mM DTT (pH 6.9) at room temperature. The resin was allowed to settle for about 30 minutes following which the supernatant was decanted to remove fine suspended particles and impurities. The procedure was repeated three times till all fines were removed, and the slurry was slowly poured along the edges of a glass column to give a 20 ml hydroxyapatite column. The surface of the resin bed was further protected from particulate matter that might be present in the sample, by covering it with a piece of round Whatman filter paper cut to fit the inner diameter of the glass column. The column was equilibrated at 4 ℃ with 5 column volumes (100 ml) of the low salt (10 mM phosphate) buffer C, following a wash with 5 column volumes of the high salt buffer C (0.5 M phosphate buffer)

Column loading and run: 16 ml of the above dialyzed protein was loaded (under gravity at 4 ℃) on the column. Unbound protein was washed off with 3 column volumes (60 ml) of low salt buffer C, followed by elution with 10 column volumes of a gradient, of low salt buffer C to 0.5 M phosphate buffer. Fractions were collected at 75 drops/tube, and 43 elution fractions of approximately 6 ml were collected. Following elution, the column was washed of residual protein with additional high salt buffer and water as before. The peak fractions were analyzed for protein and activity as before. The active fractions

contained pure RecBCD and were pooled and dialyzed as before, against 4 liters of RecBCD storage buffer (10 mM potassium phosphate, 0.1 mM DTT, 0.1 mM EDTA, 50% glycerol, pH 6.9). Aliquots of 100 µl were stored at –80 °C

2. ^{3}(H) Exonuclease assay: 1µl of peak fractions from each column chromatography were assayed for presence of RecBCD by using them to start a 10 minute exonuclease reaction at 37 °C. The standard assay was performed in 50 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, 0.67 mM DTT, 0.2 mg/ml BSA, 40 µM (nucleotides) *E. coli* chromosomal [^{3}H] DNA. Following 10 minutes incubation, 10 µl of the reaction mixture was quenched in 50 µl of 10% TCA containing 0.5 mg/ml calf thymus DNA. The undegraded substrate was precipitated at 14000 rpm/10 minutes in a microcentrifuge at room temperature. 30 µl of the supernatant was added to 3 ml scintillation cocktail containing 90 µl 10% TCA and the amount of radioactivity was measured using a scintillation counter. The counts obtained are a measure of the exonuclease activity. As a control, a similar reaction was run with 0.3 nM of previously purified RecBCD.

3. SDS-PAGE electrophoresis: For all the above separations, the fractions were analyzed for RecBCD presence by SDS-PAGE. 10.46 µl of each fraction was heated at 100 °C for 2 min in 4.54 µl of 3.3x SDS loading dye. A total volume of 15 µl per sample was loaded in wells on a pre-run (15 mAmp/ 30 min) 10% 1 mm SDS polyacrylamide gel. The gel was run till the dye band reached the bottom, stained in Coomassie blue for 30 min, followed by destaining. The gels were dried under vacuum for 50 minutes at 80 ^{o}C for preservation.

Results

1.Purification of RecBCD using FPLC:

A) Q-sepharose anion exchange (Hitrap Q FF/ 5ml) chromatography

The first round of column chromatography with Q-sepharose anion exchanger shows multiple peaks in the elution profile. Figure A1 shows the chromatogram for this separation, which is a plot of the absorbance at 280 nm vs. the fraction number. The chromatogram shows several absorbance peaks. The first sharp peak, which elutes with 0.15 M salt, does not have the protein of interest (10% SDS-polyacrylamide gel analysis, figure A2). The second broader peak, encompassing two smaller peaks within it, has the protein eluting with the second of these two peaks, encompassing six 10 ml fractions (B4-B9) and corresponding to an elution concentration of 0.32 - 0.44 M salt.

In addition to polyacrylamide gel analysis, the peak fractions were also tested in exonuclease reactions to ensure that RecBCD was in fact localized to the gel analyzed fractions. Each of the fractions was used in both ATP-dependent and ATP-independent exonuclease reactions, to further select for those fractions that were relatively free of contaminating exonucleases that may have eluted with RecBCD. RecBCD being an ATP dependent exonuclease shows very slow activity in the absence of ATP. Hence a high yield of acid soluble nucleotides from the ATP reaction, with little or no reaction in the corresponding ATP-independent reaction, would suggest absence of any contaminating exonucleases that might interfere with later purification steps, allowing for the selection of those fractions which show this effect.

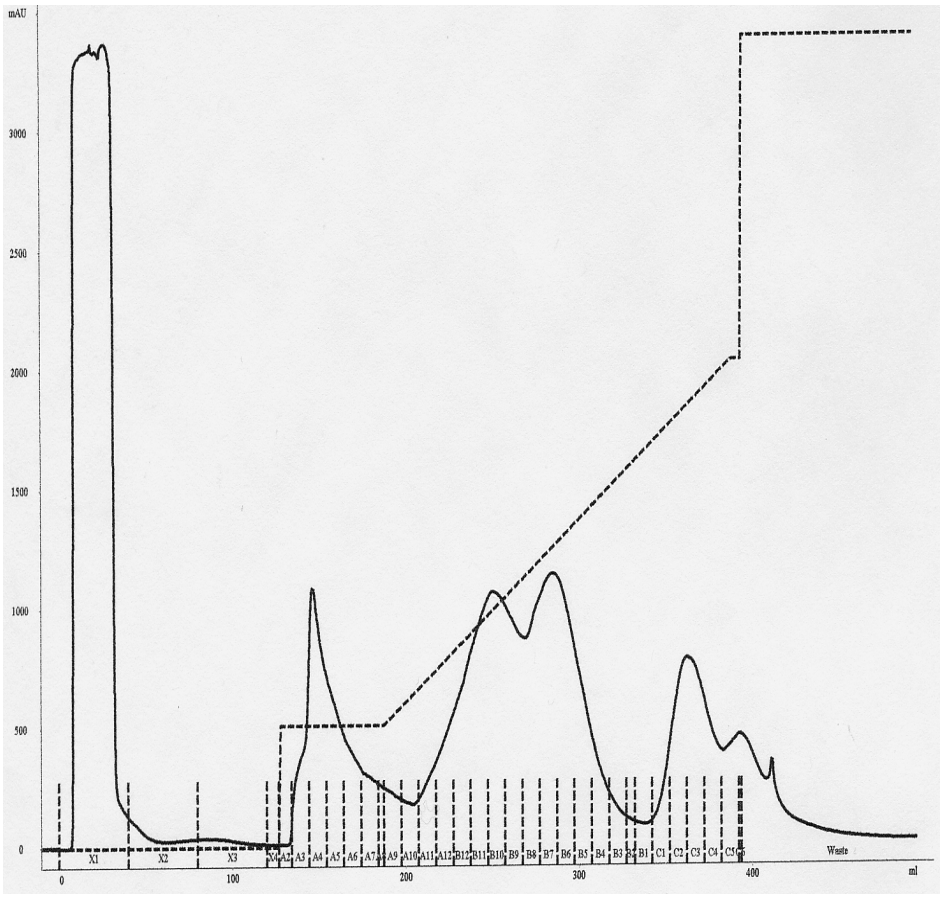


Figure A1

Chromatogram from anion exchange (Q-sepharose) column:

The fraction numbers and the total time (min) for the run, are plotted on the X-axis, and the corresponding absorbance is plotted on the Y-axis.The volume of the individual fractions is represented by hatch marks on the X-axis, between the fraction numbers. Fractions numbered X1-X4 represent the flow-through and wash fractions. Fractions numbered A3-C5 represent elution fractions. The solid line represents the absorbance (mAU) at 280 nm. The dashed line represents the buffer salt concentration.

C3 **B4 B5 B6 B7 B8 B9** B10B11B12A7 A6 A5 A4 A3 X2 X1 RecBCD

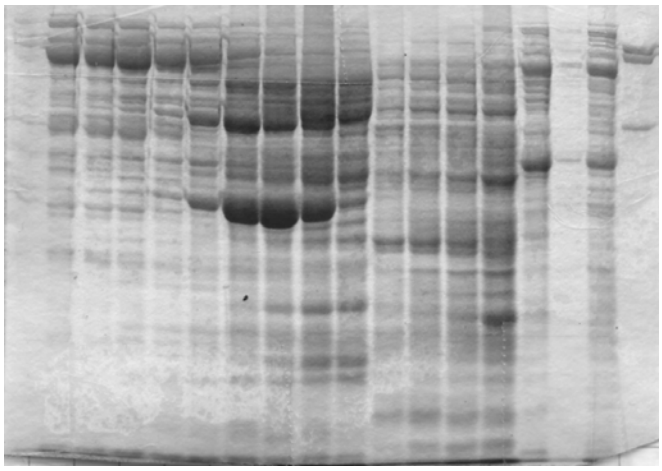


Figure A2:

Polyacrylamide gel electrophoresis of Q-sepharose chromatography fractions: The lanes are numbered according to the fraction numbers. X1 and X2 are flow through and wash fractions. A3-C3 are elution fractions. The fractions in bold are the ones confirmed for RecBCD presence by ATP-dependent exonuclease activity and pooled for further purification.

exonuclease activity analysis of the B4-B9 peak fractions, which also show the presence of RecBCD on SDS-PAGE (figure A2), confirmed the presence of RecBCD, and the absence of contaminating exonucleases. These fractions, which also showed little or no ATP-independent activity, were pooled and treated for the next round of chromatography as outlined in the methods section.

B) Heparin affinity (FF 16/10 column /20 ml) chromatography

The heparin column chromatography yielded 3 sharp absorbance peaks on the elution gradient. Figure A3 shows a chromatogram of the purification. All the fractions, encompassing the three peaks were subjected to polyacrylamide gel analysis (figure A4). The highest absorbance peak, encompassing two fractions (B9, B10) eluting with 0.07 M salt, did not show the presence of RecBCD bands.

Strong bands were obtained for fractions B8-B3, corresponding to 0.084 M to 0.11 M salt and a total volume of 30 ml. However, these fractions did not show too much ATP-dependent exonuclease activity. High ATP-dependent exonuclease activity was obtained for fractions B3-C5 instead, corresponding to 0.11 M to 0.14 M salt and a total volume of 40 ml. As seen from figures A3-A4, these fractions do not correspond to either of the three absorbance peaks obtained on the chromatogram, and though they show presence of RecBCD, it is less concentrated in these fractions, as compared to the fractions B8-B3 which eluted with lower salt. Further the amount of RecBCD decreases progressively going from fraction B3 to fraction C5, as seen from the gel, such that fraction C3 has much less RecBCD than fraction B8 or B7.

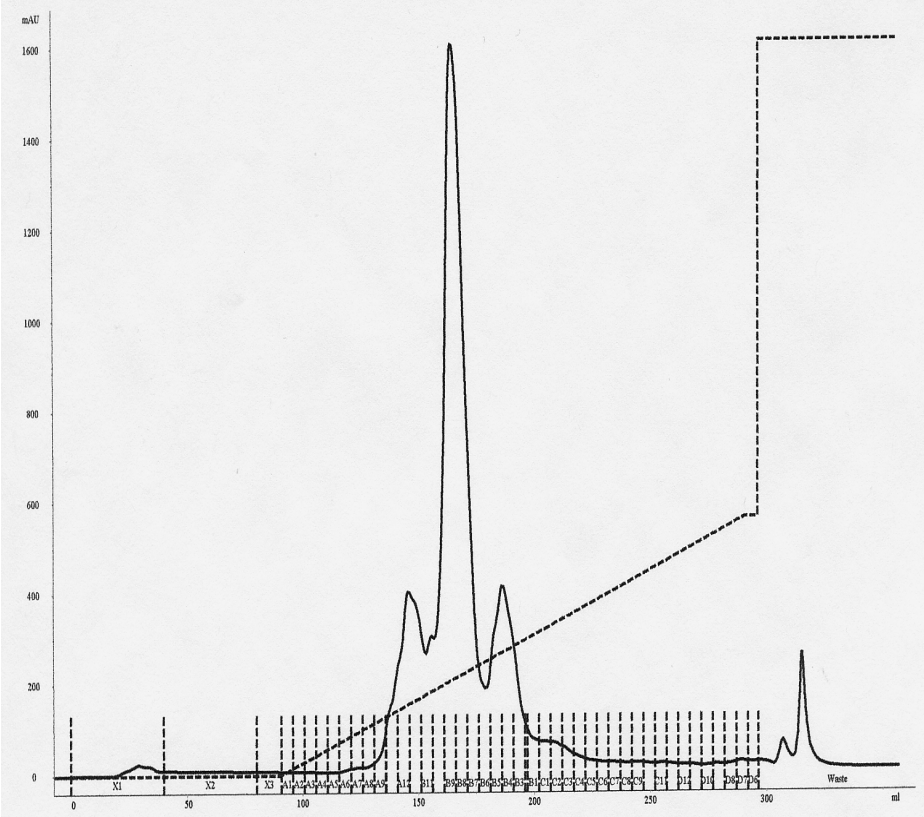


Figure A3.

Chromatogram of affinity (heparin) chromatography:

Y- and X-axis plot A_{280} (mAU) and fraction numbers respectively. X1-X3 represents flow-through and wash fractions. A1-D6 represents elution fractions. The solid line represents the absorbance (mAU) at 280 nm. The dashed line represents the salt concentration employed during the gradient.

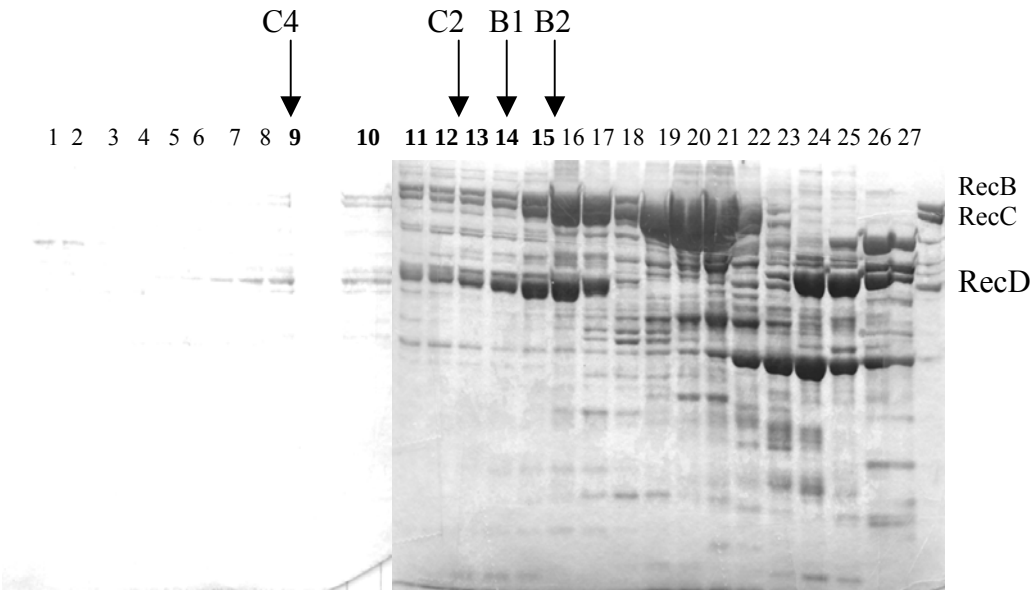


Figure A4:

Polyacrylamide gel electrophoresis of heparin chromatography fractions: Elution fractions encompassing all three absorbance peaks from figure A3 (lane number 28 is fraction A10, lane number 1 is fraction C12). Fractions pooled for next round of separation are shown in bold.

However, it surprisingly shows much higher ATP-dependent exonuclease activity (1.72 μM nucleotides/ minute/ μl fraction) than either fraction B8 or B7 (approximately 0.36 μM nucleotides/min/ μl fraction), and also shows very little ATP-independent activity in comparison. Overall, fractions, B3-C5 showed the best ATP-dependent activity, with least contamination from ATP-independent exonucleases, and also show the presence of RecBCD on gel analysis with better separation, and fewer contaminating bands. These fractions were selected for pooling and further purification using a second round of anion exchange chromatography as described above.

C) Mono-Q anion exchange (HR 5/5 /1 ml) chromatography

Figures A5 and A6 shows the chromatogram and 10% SDS-PAGE analysis respectively for this round of separation. The chromatogram shows two sharp absorbance peaks very close to each other. Polyacrylamide gel analysis of the peak fractions shows the presence of RecBCD protein (figure A6), with a complete agreement between the fractions showing the highest absorbance on the chromatogram and those showing the highest amount of RecBCD on electrophoresis. These fractions (C4-C9) correspond to elution with 0.35 M to 0.4 M salt, and total volume of 5 ml. The amount of RecBCD progressively decreases in the fractions going from C5-C9, with traces of RecBCD visible in a couple of later fractions as well (figure A6).

The ATP-dependent exonuclease activity also correlates very well with the absorbance peak, and the RecBCD peak as determined from gel analysis. A total of 12 ml of peak fractions encompassing the fractions eluted with 0.35-0.4 M salt (C4-C9), and some

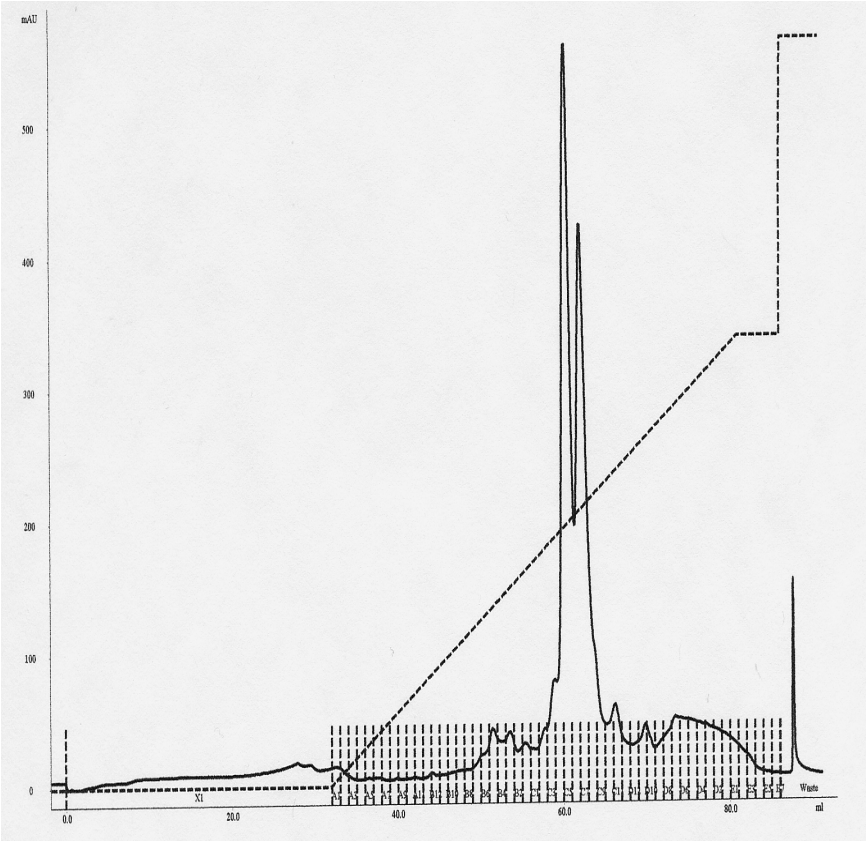


Figure A5

Chromatogram of second anion exchange (Mono-Q) chromatography: Plot of absorbance (⁻) at 280 nm (mAU) and corresponding salt concentration (···) vs fraction number. Fractions numbered A4-E5 represent elution fractions.

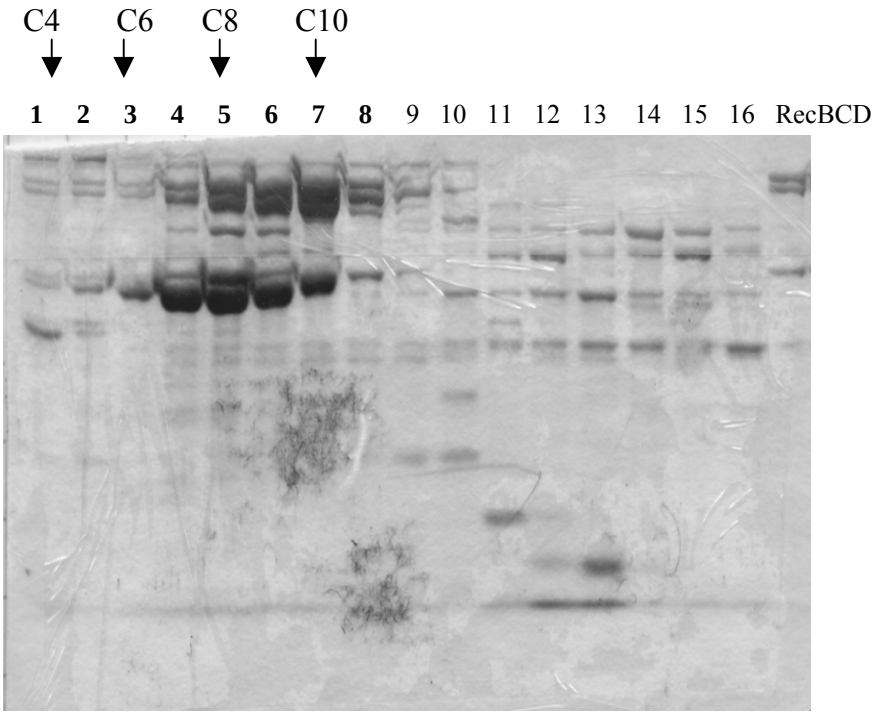


Figure A6

Polyacrylamide gel electrophoresis of Mono-Q chromatography fractions: Fractions 1 through 16 correspond to the absorbance peak (C11-B6) on the chromatogram. The fractions in bold correspond to peak fractions C4-C11 on the chromatogram, and are the ones pooled for hydroxyapatite purification.

additional fractions eluted with 0.42-0.47 M salt (C10-D11), all show high ATP-dependent exonuclease activity, while showing very little exonuclease without ATP. The fractions showing even better separation of RecBCD and fewer contaminating bands were pooled for further purification on a hydroxyapatite chromatography column.

D) Hydroxyapatite chromatography

Separation and purification of RecBCD using a gravity flow hydroxyapatite chromatography column resulted in the elution of a total of forty-three six ml fractions. Polyacrylamide gel electrophoresis analysis of odd numbered fractions localized RecBCD to 3 elution fractions appearing very early in the gradient, corresponding to elution with approximately 0.1-0.12 M salt (figure A7). These fractions show RecBCD bands, and on analysis for exonuclease activity, show ATP-dependent exonuclease, and no ATP-independent exonuclease. A total of 18 ml pure protein was pooled for dialysis against the RecBCD storage buffer (50% glycerol). Concentration of the pure dialyzed protein was estimated using absorbance at 280 nm. Approximately 2 ml of 0.5 μM pure RecBCD was obtained for use in the photocrosslinking reactions (marked lane in figure A7).

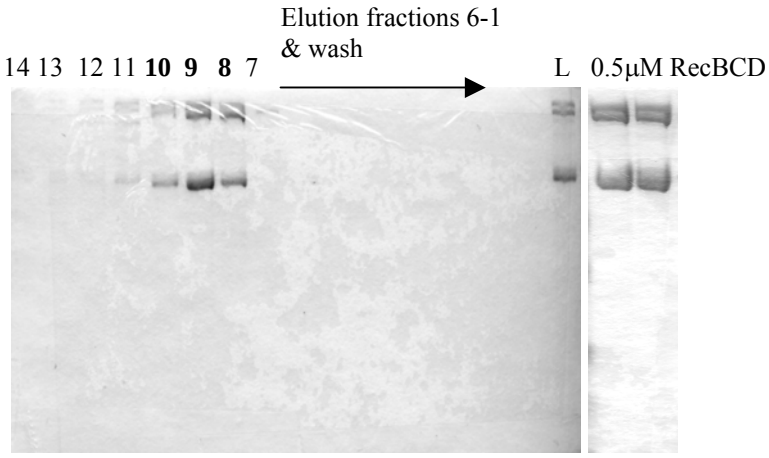


Figure A7:

Polyacrylamide gel electrophoresis of hydroxyapatite fractions: The lanes are numbered according to the fraction numbers. L refers to the sample loaded on the column (before purification). Fractions 8 through 10 show bands for RecBCD and also show ATP-dependent exonuclease activity. They were pooled and dialyzed against RecBCD storage buffer. Approximately 2 ml pure RecBCD (0.5 μM) was obtained. The lanes marked RecBCD correspond to 10.46 μl (1.72 μg) of this pure protein.

BIBLIOGRAPHY

1. Kowalczykowski, S. C. (2000). Initiation of genetic recombination and recombination-dependent replication. *Trends Biochem. Sci.* 25, 156-165.

2. Kuzminov, A. (1999). Recombinational repair of DNA damage in Escherichia coli and bacteriophage lambda. *Microbiol. Mol. Biol. Rev.* 63, 751-813.

3. McEntee, K., Weinstock, G. M. & Lehman, I. R. (1981). Binding of the recA protein of *Escherichia Coli* to single and double stranded DNA. *J.Biol.Chem* 256, 8835-8844.

4. Kowalczykowski, S. C. (1987). Mechanistic aspects of the DNA strand exchange activity of *E.coli* recA protein. *Trends. Biochem. Sci* 12, 141-145.

5. Kowalczykowski, S. C., Dixon, D. A., Eggleston, A. K., Lauder, S. D. & Rehrauer, W. M. (1994). Biochemistry of homologous recombination in *Escherichia coli*. *Microbiol. Rev.* 58, 401-465.

6. Cox, M. M. (1998). A broadening view of recombinational DNA repair in bacteria. *Genes to Cells* 3, 65-78.

7. Kuzminov, A. (1995). Collapse and repair of replication forks in Escherichia coli. *Mol. Microbiol.* 16, 373-384.

8. Cox, M. M., Goodman, M. F., Kreuzer, K. N., Sherratt, D. J., Sandler, S. J. & Marians, K. J. (2000). The importance of repairing stalled replication forks. *Nature* 404, 37-41.

9. Cox, M. M. (2001). Recombinational DNA repair of damaged replication forks in *E.coli*. *Annu. Rev. genet.* 35, 53-82.

10.Holliday, R. (1964). A mechanism for gene conversion in fungi. *Genet. Res.* 5, 282-304.

11.Meselson, M. S. & Radding, C. M. (1975). A general model for genetic recombination. *Proc. Natl. Acad. Sci.* 72, 358-361.

12.Resnik, M. A. (1976). The repair of double-strand breaks in DNA:A model involving recombination. *J.Theor.Biol* 59, 97-106.

13.Szostak, J. W., Orr-Weaver, T., Rothstein, R. J. & Stahl, F. W. (1983). The double strand break repair model for recombination. *Cell* 33, 25-35.

14.Lindahl, T. (1993). Instability and decay of the primary structure of DNA. *Nature* 362. 709-714.

15.Keyer, K., Gort, A. S. & Imlay, J. A. (1995). Superoxide and the production of oxidative DNA damage. *J. Bacteriol* 177, 6782-6790.

16.Lehmann, A. R. (1972). Postreplication repair of DNA in ultraviolet-irradited mammalian cells. *J. Mol. Biol.* 66, 319-337.

17.Rupp, W. D. & Howard-Flanders, P. (1968). Discontinuites in the DNA synthesized in an excision-defective strain of *Escherichia coli* following ultraviolet irradiation. *J. Mol. Biol.* 31, 291-304.

18.Rupp, W. D. & Howard-Flanders, P. (1971). Exchanges between DNA strands in ultraviolet-irradiated *Escherichia coli*. *J. Mol. Biol.* 61, 25-44.

19.Garrett, R. H. & Grisham, C. M. *Biochemistry*, saunders College Publishing Harcourt Brace College Publishers.

20.Dunderdale, H. J., Benson, F. E., Parsons, C. A., Sharples, Llyod, R. G. & West, S. C. (1991). Formation and resolution of recombination intermediates by *E.coli* recA and ruvC proteins. *Nature (London)* 354, 506-510.

21.Smith, G. R. (1988). Homologous recombination in procaryotes. *Microbiol. Rev.* 52, 1-28.

22.Smith, G. R. (1990). RecBCD Enzyme. In *Nucleic Acids and Molecular Biology* (Eckstein, F. & Lilley, D. M. J., eds.), Vol. 4, pp. 78-98. Springer-Verlag, Berlin, Heidelberg.

23.Roman, L. J. & Kowalczykowski, S. C. (1989). Characterization of the helicase activity of the *Escherichia coli* RecBCD enzyme using a novel helicase assay. *Biochemistry* 28, 2863-2873.

24.Roman, L. J. & Kowalczykowski, S. C. (1989). Characterization of the adenosinetriphosphatase activity of the *Escherichia coli* RecBCD enzyme: relationship of ATP hydrolysis to the unwinding of duplex DNA. *Biochemistry* 28, 2873-2881.

25.Goldmark, P. J. & Linn, S. (1972). Purification and properties of the *recBC* DNase of *Escherichia coli* K-12. *J. Biol. Chem.* 247, 1849-1860.

26.Wright, M., Buttin, G. & Hurwitz, J. (1971). The isolation and characterization from *Escherichia coli* of an adenosine triphosphate-dependent deoxyribonuclease directed by *rec B, C* genes. *J. Biol. Chem.* 246, 6543-6555.

27.Goldmark, P. J. & Linn, S. (1970). An endonuclease activity from *E.coli* absent in certain rec$^-$ strains. *Proc. Natl. Acad. Sci. USA* 67, 434-441.

28.McMilin, K. D., Stahl, M. M. & Stahl, F. W. (1974). Rec-Mediated Recombinational Hot Spot Activity in bacteriophage Lambda. I. Hot Spot Activity Associated with Spi⁻ Deletions and *bio* Substitutions. *Genetics* 77, 409-423.

29.Dixon, D. A. & Kowalczykowski, S. C. (1993). The recombination hotspot c is a regulatory sequence that acts by attenuating the nuclease activity of the E. coli RecBCD enzyme. *Cell* 73, 87-96.

30.Taylor, A. & Smith, G. R. (1980). Unwinding and rewinding of DNA by the RecBC enzyme. *Cell* 22, 447-457.

31.Ponticelli, A. S., Schultz, D. W., Taylor, A. F. & Smith, G. R. (1985). Chi-dependent DNA strand cleavage by RecBC enzyme. *Cell* 41, 145-151.

32.McEntee, K., Weinstock, G. M. & Lehman, I. R. (1979). Initiation of general recombination catalyzed in vitro by the recA protein of *Escherichia coli*. *Proc. Natl. Acad. Sci* 76, 2615-2619.

33.McEntee, K. G., Weinstock, G. M. & Lehman, I. R. (1981). DNA and nucleoside binding properties of RecA protein from *Escherichia coli*. *Prog. Nucleic. Acids. Res. Mol. Biol* 26, 265-279.

34.Little, J. W., Edmiston, S. H., Pacelli, L. Z. & Mount, D. W. (1980). Cleavage of the *Escherichia coli* lexA protein by the recA protease. *Proc. Natl. Acad. Sci* 77, 3225-3229.

35.Cox, M. M. & Lehman, I. R. (1992). RecA protein promoted DNA strand exchange. *J. Biol. Chem.* 257, 8523-8532.

36.Anderson, D. G. & Kowalczykowski, S. C. (1997). The translocating RecBCD enzyme stimulates recombination by directing RecA protein onto ssDNA in a Chi-dependent manner. *Cell* 90, 77-86.

37.Arnold, D. A., Bianco, P. R. & Kowalczykowski, S. C. (1998). The Reduced Levels of Recognition by the RecBC1004D Enzyme Reflect Its Recombination Defect in Vivo. *J. Biol. Chem.* 273, 16476-16486.

38.Connolly, B., Parsons, C. A., Benson, F. A., Dunderdale, H. J., Sharples, G. J., Lloyd, R. G. & West, S. C. (1991). Resolution of Holliday junctions in vitro requires the Escherichia coli ruvC gene product. *Proc. Natl. Acad. Sci. USA* 88, 6063-6067.

39.Taylor, A. F. & Smith, G. R. (1985). Substrate Specificity of the DNA Unwinding Activity of the RecBC Enzyme of *Escherichia coli. J. Mol. Biol.* 185, 431-443.

40.Ganesan, S. & Smith, G. R. (1993). Strand-specific binding to duplex DNA ends by the subunits of the Escherichia coli RecBCD enzyme. *J. Mol. Biol.* 229, 67-78.

41.Karu, A. E., Sakaki, Y., Echols, H. & Linn, S. (1975). The protein specified by bacteriophage. *J. Biol. Chem.* 250, 7377-7387.

42.Murphy, K. C. (1991). Gam protein inhibits the helicase and c-stimulated recombination activities of Escherichia coli RecBCD enzyme. *J. Bacteriol.* 173, 5808-5821.

43.Oliver, D. B. & Goldberg, E. B. (1977). Protection of parental T4 DNA from a restriction exonuclease by the product of gene 2. *J. Mol. Biol.* 116, 877-881.

44.Biek, D. P. & Cohen, S. N. (1986). *J. Bacteriol.* 167, 594.

45.Clark, A. J. & Margulies, A. D. (1965). Isolation and characterization of recombination deficient mutants of *E.coli* K-12. *Proc. Natl. Acad. Sci* 53, 451-459.

46.Emmerson, P. T. (1968). Recombination mutants of *Escherichia coli* K-12 that map between thyA and argA. *Genetics* 60, 19-30.

47.Lieberman, R. P. & Oishi, M. (1974). The recBC deoxyribonuclease of Escherichia coli: isolation and characterization of the subunit proteins and reconstitution of the enzyme. P*roc. Natl. Acad. Sci. USA* 71, 4816-4820.

48.Amundsen, S. K., Taylor, A. F., Chaudhury, A. M. & Smith, G. R. (1986). *recD*: the gene for an essential third subunit of exonuclease V. *Proc. Natl. Acad. Sci. USA* 83, 5558-5562.

49.Finch, P. W., Wilson, R. E., Brown, K., Hickson, I. D., Tomkinson, A. E. & Emmerson, P. T. (1986). Complete nucleotide sequence of the *Escherichia coli* recC gene and of the thyA-recC intergenic region. *Nucleic. Acids. Res.* 14, 4437-4451.

50.Finch, P. W., Storey, A., Brown, K., Hickson, I. D. & Emmerson, P. T. (1986). Complete nucleotide sequence of *recD,* the structural gene for the a subunit of Exonuclease V of *Escherichia coli*. *Nucleic. Acids. Res.*. 14, 8583-8594.

51.Finch, P. W., Storey, A., Chapman, K. E., Brown, K., Hickson, I. D. & Emmerson, P. T. (1986). Complete nucleotide sequence of the Escherichia coli recB gene. *Nucleic. Acids. Res.* 14, 8573-8582.

52.Amundsen, S. K., Neiman, A. M., Thibodeaux, S. M. & Smith, G. R. (1990). Genetic dissection of the biochemical activities of RecBCD enzyme. *Genetics* 126, 25-40.

53.Eggleston, A. K. & Kowalczykowski, S. C. (1993). The mutant recBCD enzyme , recB^{2109}CD has helicase activity but does not but does not promote efficient joint molecule formation *in vitro*. *J. Mol. Biol.* 231, 621-633.

54.Eggleston, A. K. & Kowalczykowski, S. C. (1993). Biochemical characterization of a mutant recBCD enzyme, the recB^{2109}CD enzyme, which lacks c-specific, but not non-specific, nuclease activity. *J. Mol. Biol.* 231, 605-620.

55.Holbeck, S. L. & Smith, G. R. (1992). Chi enhances heteroduplex DNA levels during recombination. *Genetics* 132, 879-891.

56.Schultz, D. W., Taylor, A. F. & Smith, G. R. (1983). *Escherichia coli* RecBC Pseudorevertants Lacking Chi Recombinational Hotspot Activity. *J. Bacteriol.* 155, 664-680.

57.Chaudhury, A. M. & Smith, G. R. (1984). A new class of Escherichia coli recBC mutants: implications for the role of RecBC enzyme in homologous recombination. *Proc. Natl. Acad. Sci. USA* 81, 7850-7854.

58.Hsieh, S. & Julin, D. A. (1992). Alteration by site-directed mutagenesis of the conserved lysine residue in the consensus ATP-binding sequence of the RecB protein of *Escherichia coli. Nucleic. Acids. Res.* 20, 5647-5653.

59.Korangy, F. & Julin, D. A. (1992). Alteration by site-directed mutagenesis of the conserved lysine residue in the ATP-binding consensus sequence of the RecD subunit of the *Escherichia coli* RecBCD enzyme. *J. Biol. Chem.* 267, 1727-1732.

60.Anderson, D. G., Churchill, J. J. & Kowalczykowski, S. C. (1999). A single mutation, RecB(D1080A,) eliminates RecA protein loading but not Chi recognition by RecBCD enzyme. *J. Biol. Chem.* 274, 27139-44.

61.Julin, D. A. & Lehman, I. R. (1987). Photoaffinity labeling of the recBCD enzyme of *Escherichia coli* with 8-azidoadenosine 5'- triphosphate. *J. Biol. Chem.* 262, 9044-9051.

62.Boehmer, P. E. & Emmerson, P. T. (1992). The RecB subunit of the Escherichia coli RecBCD enzyme couples ATP hydrolysis to DNA unwinding. *J. Biol. Chem.* 267, 4981-4987.

63.Korangy, F. & Julin, D. A. (1992). Enzymatic effects of a lysine-to-glutamine mutation in the ATP-binding consensus sequence in the RecD subunit of the RecBCD enzyme from *Escherichia coli. J. Biol. Chem.* 267, 1733-1740.

64.Korangy, F. & Julin, D. A. (1992). A Mutation in the Consensus ATP-Binding Sequence of the RecD Subunit Reduces the Processivity of the RecBCD Enzyme from *Escherichia coli*. *J. Biol. Chem.* 267, 3088-3095.

65.Chen, H.-W., Ruan, B., Yu, M., Wang, J. & Julin, D. A. (1997). The RecD subunit of the RecBCD enzyme from *Escherichia coli* is a single-stranded DNA-dependent ATPase. *J. Biol. Chem.* 272, 10072-10079.

66.Korangy, F. & Julin, D. A. (1993). Kinetics and processivity of ATP hydrolysis and DNA unwinding by the RecBC enzyme from Escherichia coli. *Biochemistry* 32, 4873-4880.

67.Korangy, F. & Julin, D. A. (1994). Efficiency of ATP Hydrolysis and DNA unwinding by the RecBC enzyme from *Escherichia coli. Biochemistry* 33, 9552-9560.

68.Taylor, A. F. & Smith, G. R. (2003). RecBCD enzyme is a DNA helicase with fast and slow motors of opposite polarity. *Nature* 423, 889-893.

69.Dillingham, M. S., Spies, M. & Kowalczykowski, S. C. (2003). RecBCD enzyme is a bipolar DNA helicase. *Nature* 423, 893-897.

70.Bianco, P. R., Brewer, L. R., Corzett, M., Balhorn, R., Yeh, Y., Kowalczykowski, S. C. & Baskin, R. J. (2001). Processive translocation and DNA unwinding by individual RecBCD enzyme molecules. *Nature* 409, 374-378.

71.MacKay, V. & Linn, S. (1974). The mechanism of degradation of duplex deoxyribonucleic acid by the *recBC* enzyme of *Escherichia coli* K-12. *J. Biol. Chem.* 249, 4286-4294.

72.Eichler, D. C. & Lehman, I. R. (1977). On the role of ATP in phosphodiester bond hydrolysis catalyzed by the recBC deoxyribonuclease of *Escherichia coli*. *J. Biol. Chem.* 252, 499-503.

73.Dixon, D. A. & Kowalczykowski, S. C. (1991). Homologous pairing in vitro stimulated by the recombination hotspot, Chi. *Cell* 66, 361-371.

74.Yu, M., Souaya, J. & Julin, D. A. (1998). The 30-kDa C-terminal domain of the RecB protein is critical for the nuclease activity, but not the helicase activity, of the RecBCD enzyme from *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 95, 981-986.

75.Yu, M., Souaya, J. & Julin, D. A. (1998). Identification of the nuclease active site in the multifunctional RecBCD enzyme by creation of a chimeric enzyme. *J. Mol. Biol.* 283, 797-808.

76.Wang, J., Chen, R. & Julin, D. A. (2000). A single nuclease active site of the *Escherichia coli* recBCD enzyme catalyses single-stranded DNA degradation in both directions. *J. Biol. Chem.* 275, 507-513.

77.MacKay, V. & Linn, S. (1976). Selective Inhibition of the DNase Activity of the *recBC* Enzyme by the DNA Binding Protein from *Escherichia coli*. *J. Biol. Chem.* 251, 3716-3719.

78.Williams, J. G. K., Shibata, T. & Radding, C. M. (1981). *Escherichia. coli* recA protein protects single stranded DNA or gapped duplex DNA from degradation by recBC DNAse. *J. Biol. Chem.* 256, 7573-7582.

79.Smith, G. R., Comb, M., Schultz, D. W., Daniels, D. L. & Blattner, F. R. (1981). Nucleotide sequence of the Chi recombinational hotspot χ^{+}D in bacteriophage l. *J. Virology* 37, 336-342.

80.Smith, G. R., Kunes, S. M., Schultz, D. W., Taylor, A. & Triman, K. L. (1981). Structure of chi Hotspots of Generalized Recombination. *Cell* 24, 429-436.

81.Smith, G. R. & Stahl, F. W. (1985). Homologous Recombination Promoted by Chi Sites and RecBC Enzyme of *Escherichia coli. BioEssays* 2, 244-249.

82.Henderson, D. & Weil, J. (1975). Recombination deficient deletions in bacteriophage l and their interaction with the Chi mutations. *Genetics* 79, 143-174.

83.Stahl, F. W., Stahl, M. M., Malone, R. E. & Crasemann, J. M. (1980). Directionality and nonreciprocality of Chi stimulated recombination in phage lambda. *Genetics* 94, 235-248.

84.Taylor, A. F., Schultz, D. W., Ponticelli, A. S. & Smith, G. R. (1985). RecBC enzyme nicking at Chi sites during DNA unwinding: location and orientation- dependence of the cutting. *Cell* 41, 153-163.

85.Taylor, A. F. & Smith, G. R. (1992). RecBCD enzyme is altered upon cutting DNA at a Chi recombination hotspot. *Proc. Natl. Acad. Sci. USA* 89, 5226-5230.

86.Spies, M., Bianco, P. R., Dillingham, M. S., Handa, N., Baskin, R. J. & Kowalczykowski, S. C. (2003). A molecular throttle: the recombination hotspot chi controls DNA translocation by the RecBCD helicase. *Cell* 114, 647-54.

87.Dixon, D. A., Churchill, J. J. & Kowalczykowski, S. C. (1994). Reversible inactivation of the Escherichia coli RecBCD enzyme by the recombination hotspot c *in vitro*: evidence for functional inactivation or loss of the RecD subunit. *Proc. Natl. Acad. Sci. USA* 91, 2980-2984.

88.Anderson, D. G., Churchill, J. J. & Kowalczykowski, S. C. (1997). Chi-activated RecBCD enzyme possesses 5'-3' nucleolytic activity, but RecBC enzyme does not: evidence suggesting that the alteration induced by Chi is not simply ejection of the RecD subunit. *Genes to Cells* 2, 117-128.

89.Anderson, D. G. & Kowalczykowski, S. C. (1997). The recombination hot spot c is a regulatory element that switches the polarity of DNA degradation by the RecBCD enzyme. *Genes & Devel.* 11, 571-581.

90.Taylor, A. F. & Smith, G. R. (1999). Regulation of homologous recombination: Chi inactivates RecBCD enzyme by disassembly of the three subunits. *Genes & Devel.* 13, 890-900.

91.Arnold, D. A., Handa, N., Kobayashi, I. & Kowalczykowski, S. C. (2000). A novel, 11 nucleotide variant of chi, chi*: one of a class of sequences defining the Escherichia coli recombination hotspot chi. *J. Mol. Biol.* 300, 469-479.

92.Hickson, I. D., Atkinson, K. E., Hutton, L., Tomkinson, A. E. & Emmerson, P. T. (1984). Molecular amplification and purification of the E. coli recC gene product. *Nucleic. Acids. Res.* 12, 3807-19.

93.Cheng, K. C. & Smith, G. R. (1987). Cutting of chi-like sequences by the RecBCD enzyme of Escherichia coli. *J. Mol. Biol.* 194, 747-750.

94.Karoui, M. E., Ehrlich, D. & Gruss, A. (1998). Identification of the Lactococcal exonuclease/recombinase and its modulation by the putative Chi sequence. *Proc. Natl. Acad. Sci.* 95, 626-631.

95.Karoui, M. E., Schaeffer, M., biaudet, V., Bolotin, A., Sorokin, A. & Gruss, A. (2000). Orientation specificity of the *Lactococcus. lactis* Chi site. *Genes to Cells* 5, 453-461.

96.Chedin, F., Ehrlich, S. D. & Kowalczykowski, S. C. (2000). The Bacillus subtilis AddAB helicase/nuclease is regulated by its cognate Chi sequence in vitro. *J. Mol. Biol.* 298, 7-20.

97.Bianco, P. R. & Kowalczykowski, S. C. (1997). The recombination hotspot Chi is recognized by the translocating RecBCD enzyme as the single strand of DNA containing the sequence 5'-GCTGGTGG-3'. *Proc. Natl. Acad. Sci. USA* 94, 6706-6711.

98.Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

99.Maniatis, T., Fritsh, E. F. & Sambrook, J. (1989). Molecular cloning. A laboratory manual. Second edition.

100.Fasman, G. D. (1975). Optical properties of nucleic acids, absorbance and CD spectra. *Handbook of Biochemistry and Molecular Biology* 1, 589.

101.Anderson, D. G. & Kowalczykowski, S. C. (1998). SSB protein controls RecBCD enzyme nuclease activity during unwinding: a new role for looped intermediates. *J. Mol. Biol.* 282, 275-285.

102.Dixon, D. A. & Kowalczykowski, S. C. (1995). Role of the Escherichia coli recombination hotspot, c, in RecABCD-dependent homologous pairing. *J. Biol. Chem.* 270, 16360-16370.

103.Chamberlin, M. & Julin, D. A. (1996). Interactions of the RecBCD enzyme from *Escherichia coli* and its subunits with DNA, elucidated from the kinetics of ATP and DNA hydrolysis with oligothymidine substrates. *Biochemistry* 35, 15949-15961.

104.Handa, N., Ohashi, S., Kusano, K. & Kobayashi, I. (1997). χ*, a χ-related 11-mer sequence partially active in an E. coli recC* strain. *Genes to Cells* 2, 525-536.

105.Norris, C., Meisenheimer, P., and Koch T. (1996). Mechanistic studies of 5'-iodouracil chromophore relevant to its use in nucleoprotein photo-cross linking. *J. Am. Chem. soc.* 118, 5796-5803.

106.Steen, H. & Jensen, O. N. (2002). Analysis of protein-nucleic acid interactions by photochemical cross-linking and mass spectrometry. *Mass spectrometry Reviews* 21, 163-182.

107.Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. 2nd edit. 3 vols, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

108.Driscoll, M. D., Sathya, G., Muyan, M., Klinge, C. M., Hilf, R., and Bambara, R. A. (1998). Sequence requirements for estrogen receptor binding to estrogen response elements. *J. Biol. Chem.* 273, 29321-29330.

109.Holt, S. E., Schuller, G., and Wilson, V. G. (1994). DNA binding specificity of the bovine papillomavirus E1 protein is determined by sequences contained within an 18-base-pair inverted repeat element at the origin of replication. *J. Virology.* 2, 1094-1102.

110.Lu, A. L., Lack, W. E., and Modrich, P. (1983). DNA determinants important in sequence recognition by *Eco*RI endonuclease. *J. Biol. Chem.* 256, 13200-13206.

111.Phillips, R. J., Hickleton, D. C., Boehmer, P. E. & Emmerson, P. T. (1997). The RecB protein of Escherichia coli translocates along single-stranded DNA in the 3' to 5' direction: a proposed ratchet mechanism. *Mol. Gen. Genet.* 254, 319-329.