Holliday junction processing enzymes in eukaryotes.

By

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ABSTRACT

Homologous genetic recombination is best understood in the model prokaryote *Escherichia coli* where the enzymatic processes have been characterised using purified proteins. Recombination involves reciprocal exchange of strands between two homologous DNA strands. The resultant Holliday junction is either branch migrated and/or resolved. Proteins, RuvA and RuvB can branch migrate the junction and RuvC can resolve the junction.

In contrast limited advancement has been made with eukaryotic systems. A study was undertaken to elucidate some of the processes that occur in eukaryotic recombination. Activities capable of processing Holliday junction-containing substrates were screened for. In addition activities capable of processing Holliday junction precursor substrates, were also investigated. Initial searches involved assaying fractionated eukaryotic cell extracts for processing activity. A later approach was to PCR potential eukaryotic homologues of prokaryotic proteins. Both approaches were successful. The latter approach resulted in the identification of the homologue of CCE1 in *S. cerevisiae* being identified in *S. pombe*. The open reading frame of YDC2_SCHPO (spCCE1) was cloned, purified, assayed biochemically and found to be a Holliday junction specific resolvase. Initial characterisation of the protein was carried out.

YDC2 being a homologue of CCE1 meant that it was likely to be a mitochondrial Holliday junction resolvase activity that could be masking a nuclear Holliday junction resolving activity. To search for another resolvase activity, a possible nuclear activity, a *S. cerevisiae* yeast strain that was a CCE1 knock out was used to resume the search for such possible activities. An initial fractionation of the knockout strain of *S. cerevisiae* revealed the presence of another Holliday junction resolving activity. This activity was partially purified and characterised.
Similar searches in mammalian cells led to the identification of an end joining activity that in experiments seemed to show a homology dependency and an annealing activity.
1. INTRODUCTION

1.1 Homologous recombination
1.2 Homologous recombination in *Escherichia coli*
1.3 Initiation of homologous recombination by RecBCD
1.4 Other pathways for initiation of homologous recombination
1.5 The *ruv* locus
1.6 Branch migration of the Holliday junction is promoted by RuvA and RuvB
1.7 RecG
1.8 RuvC
1.9 RuvABC as a complex
1.10 RuvAB and RuvC at the replication fork
1.11 RusA
1.12 T4 endonuclease VII
1.13 T7 endonuclease I
1.14 Archaea
1.15 Holliday junction-specific endonucleases in eukaryotes
   1.15.1 CCE1
   1.15.2 Other *S. cerevisiae* resolvase activities
   1.15.3 Endonuclease X1
   1.15.4 Endonuclease X2
   1.15.5 Endonuclease X3
1.15.6 Endonuclease X4
1.15.7 Mammalian resolvase activities

1.16 Structure and function
1.16.1 The structure of the Holliday junction
1.16.2 Resolvase junction binding
1.16.3 Resolvases function as a dimer
1.16.4 Holliday junction resolution
1.16.5 Proposed models for the association of a RuvABC Holliday junction complex
1.16.6 RuvA functions as a tetramer and an octamer?

1.17 Eukaryotic homologous recombination
1.17.1 Genetic Models
1.17.2 The Holliday model
1.17.3 The Meselson-Radding model
1.17.4 The Resnick model and the Double-Strand Break Repair model
1.17.5 Single-strand annealing model

1.18 The importance of recombination

1.19 Meiosis

1.20 Double-Strand Break Repair (DSBR)
1.20.1 Non-homologous end joining
1.20.2 The two pathways operate
1.20.3 Double-Strand Break Repair by homologous recombination
1.20.4 DSBR by homologous recombination: single-strand annealing
1.20.5 DSBR by homologous recombination: strand invasion

1.21 The RAD genes
1.21.1 RAD 51
1.21.2 DMC1
1.21.3 RAD52
1.21.4 RAD53
1.21.5 RAD54
1.21.6 RAD55 and RAD57
1.21.7 RAD59
1.21.8 RAD50/MRE11/XRS2

1.22 Recombination and carcinogenesis
2. METHODS

2.1 Strains and plasmids

2.2 Enzymes and reagents

2.3 Buffers and solutions
  2.3.1 DNA buffers
  2.3.2 Enzyme buffers:
  2.3.3 Protein buffers:
  2.3.4 Media:
  2.3.5 Buffers for alpha-structures:

2.4 Quantitation of DNA and protein
  2.4.1 DNA
  2.4.2 Protein

2.5 Molecular mass standards

2.6 Electrophoresis
  2.6.1 Agarose gel electrophoresis
  2.6.2 Denaturing PAGE
  2.6.3 PAGE
  2.6.4 SDS-PAGE
  2.6.5 Autoradiography and phosphorimager analysis

2.7 DNA manipulations
  2.7.1 Electroelution of DNA fragments from polyacrylamide gels
  2.7.2 Phenol chloroform extractions and ethanol precipitations
  2.7.3 Preparation of genomic DNA from S. pombe
  2.7.4 PCR using genomic DNA
  2.7.5 Purification of PCR products from agarose

2.8 Preparation of DNA substrates
  2.8.1 Preparation of synthetic Holliday junction
  2.8.2 Preparation of Chi-DNA.
  2.8.3 Preparation of gapped DNA.
  2.8.4 Extraction and Purification of pDEA-7ZF(+) from JM109
  2.8.5 Preparation of linear pDEA-7ZF(+) for annealing
3.2 Four-stranded recombination reactions promoted by RecA in vitro 101

3.2.1 Construction of gDNA 101

3.2.2 Recombination intermediates made by RecA in vitro 101

3.3 Chi-structures 106

3.4 Summary 109

4. SCHIZOSACCHAROMYCES POMBE 110

4.1 Holliday junction processing activity in fractionated S. pombe cell free extracts 110

4.2 Holliday junction resolution activity in fractionated cell-free extract from S. pombe 113

4.3 Homology search in S. pombe database 116

4.4 Cloning of a novel Holliday junction resolvase from S. pombe 116

4.5 Over-expression and purification of recombinant YDC2 119

4.6 Cloned activity is biochemically indistinguishable from the activity originally identified in cell extracts 122

4.7 Holliday junction-specific DNA binding 128

4.8 pH Optimum 128

4.9 Efficiency of junction cleavage 131

4.10 Metal ion and Metal ion concentration 131

4.11 Temperature and salt concentration optimum 136

4.12 E. coli RuvB/YDC2 branch migration 136

4.13 Discussion 139

5. S. CEREVISIAE 144

5.1 Assay for Holliday junction-processing activities in S. cerevisiae 144

5.2 Strain characterisation 145
7.3 Activities in fractionated HeLa cell extracts

Bibliography

Appendix

Publication back sleeve
LIST OF FIGURES

CHAPTER 1
Figure 1.1. The Holliday Model. 20
Figure 1.16.2. Schematic showing some conformations adopted by synthetic Holliday junctions upon protein binding. 40
Figure 1.16.4. Schematic of a Holliday junction showing the crossover and continuous strand. 44
Figure 1.16.6 RuvA as the Stator for the RuvB motor. 47
Figure 1.17.4 The double strand break repair model. 52
Figure 1.21.8. Schematic outline of protein functions in DSBR by homologous recombination. 64

CHAPTER 2
Table 2.8: Sequences of oligonucleotides used to make synthetic substrates. 78
Table 2.11: Chromatography columns used. 89

CHAPTER 3
Figure 3.1. Synthetic Holliday junction constructed by annealing four synthetic oligonucleotides. 98
Figure 3.2.1. gDNA preparation. 103
Figure 3.2.2. Formation and processing of RecA-made alpha structures. 104
Figure 3.3. Chi DNA formation. 107

Table 3.0. Possible sources of Holliday junction-processing activities. 97
Table 3.4. Holliday junction-processing activities identified. 109

CHAPTER 4
Figure 4.1. Processing of RecA-made alpha-structures by fractionated cell-free extracts from S. pombe. 111
Figure 4.2. Endonuclease activities of *S. pombe* are specific for a synthetic four-way Holliday junction and Holliday junction-containing Chi DNA. 114

Figure 4.3. Sequence comparison of *S. cerevisiae* CCE1 and *S. pombe* YDC2. 117

Figure 4.4. Cloning of the YCD2_SCHPO open reading frame from *S. pombe*. 118

Figure 4.5. Purification of YDC2. 120

Figure 4.6.1. YDC2 and *S. pombe* fraction 3 cut each strand of junction X12 at identical sites. 123

Figure 4.6.2. Shematic presentation of the central sequences of junction X12 cleaved by YDC2 125

Figure 4.6.3. Repair by DNA ligase of nicks produced by YDC2 and *S. pombe* cellular activity. 126

Figure 4.7. YDC2 binds specifically to the four-way junction X12. 129

Figure 4.8. YDC2 cleaves Holliday junctions more efficiently at alkaline pH. 130

Figure 4.9. The effect of increasing protein concentration on junction resolution. 132

Figure 4.10. YDC2 requires the presence of a specific divalent cation for resolution. 134

Figure 4.11. The effect of temperature and salt concentration on YDC2 Holliday junction resolution. 137

Figure 4.13. YDC2 resolution of junction X0. 141

**CHAPTER 5**

Figure 5.2. PCR analysis of *S. cerevisiae cce1Δ* strain SK20. 146

Figure 5.3.1. Scheme for partial purification of a resolvase activity from haploid and diploid cells of *S. cerevisiae cce1Δ* strain SK19, SK20, and AK47. 150

Figure 5.3.2. Elution profile of fractionated extracts from SK20 cells at early, mid and late log phase of growth. 153

Figure 5.3.3. Growth curve of SK20 cells 154
Figure 5.3.4. Comparison of activity from diploid AK47 cells grown in supplemented and unsupplemented media.  

Figure 5.4.1. Identification and initial fractionation of a resolvase activity from diploid cells of *S. cerevisiae cce1Δ* strain AK47.  

Figure 5.4.2. The activity from diploid *S. cerevisiae cce1Δ* cells shows specificity and selectivity for resolving four-way junctions.  

Figure 5.5.1. *S. cerevisiae cce1Δ* activity, CCE1 and YDC2 cut each strand of junction X12 at sites symmetric to the point of crossover.  

Figure 5.5.2. *S. cerevisiae cce1Δ* activity, CCE1 and YDC2 cut each strand of junction X12 at sites symmetric to the point of crossover.  

Figure 5.6. T4 DNA ligase repairs nicked DNA.  

Figure 5.7. Activity from diploid *S. cerevisiae cce1Δ* cells cuts and binds specifically to the four-way junction X12.  

Figure 5.8. Determination of the molecular weight of the Holliday junction specific endonuclease.  

Figure 5.9. RuvB-mediated branch migration.  

Table 5.2. Strain Genotype.  
Table 5.3. Chromatography column performance.  

### CHAPTER 6  

Figure 6.1 Rat testis cell-free extract fractionated on Resource S.  

Figure 6.2 HeLa cell extract fractionated on phosphocellulose.  

Figure 6.3. Dimer products form without the addition of ATP.  

Figure 6.4.1. Fraction 12 incubated with labelled linear DNA.  

Figure 6.4.2. Chi DNA reactions.  

Figure 6.5.1 Rat testis cell-free extract fractionated by gel filtration on Superdex 200.  

Figure 6.6.1. Pairing reactions.  

Figure 6.6.2. gDNA dependency of products.  

Figure 6.7. Further fractionation of partially purified HeLa cell extract.  

Figure 6.8. The presence of Rad51 in fractions from HeLa cells.
CHAPTER 7

Table 7.2. Comparison of known Holliday junction resolvases with recently identified activities.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATPγS</td>
<td>adenosine 5’-(γ-thio) triphosphate</td>
</tr>
<tr>
<td>BCDP</td>
<td>5-bromo-4-chloro-3-indolyl phosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CCE1</td>
<td>cruciform cutting enzyme 1</td>
</tr>
<tr>
<td>CIP</td>
<td>calf intestinal alkaline phosphotase</td>
</tr>
<tr>
<td>Da</td>
<td>dalton</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double-stranded DNA</td>
</tr>
<tr>
<td>gDNA</td>
<td>gapped circular duplex DNA</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>GLB</td>
<td>gel-loading buffer</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid (disodium salt)</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorter</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>IPTG</td>
<td>β-D-isopropyl-thiogalactopyranoside</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>mtDNA</td>
<td>mitochondrial DNA</td>
</tr>
<tr>
<td>NBT</td>
<td>nitro blue tetrazolium</td>
</tr>
<tr>
<td>NER</td>
<td>nucleotide excision repair</td>
</tr>
<tr>
<td>NHEJ</td>
<td>non-homologous end-joining</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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</table>
PMSF phenylmethylsulfonyl fluoride
PNK polynucleotide kinase
RNA ribonucleic acid
SDS sodium dodecyl sulphate
SSB single stranded DNA binding protein
ssDNA single-stranded DNA
TAE tris/acetate/EDTA
TBS tris buffered saline
TBE tris/borate/EDTA
TBST tris buffered saline tween
TE tris/EDTA
TEMED N,N,N',N'-(tetramethylethylenediamine)
TLCK N-tosyl-L-lysine chloromethyl ketone
TNE tris/sodium chloride/EDTA
TPCK N-tosyl-L-phenylalanine chloromethyl ketone
Tris tris (hydroxymethyl) aminoethane
Tween 20 polyoxyethylenesorbitan monolaurate
UV ultraviolet
v/v volume:volume ratio
w/v weight:volume ratio
X-gal 5-bromo-4-chloro-3-indolyl-β-galactopyranoside
1. INTRODUCTION

The aim of the project was to provide evidence for the enzymatic processing of Holliday junctions in eukaryotes, the central intermediate in homologous recombination.

1.1 Homologous recombination

Homologous recombination is important for a number of fundamental processes during a cell’s lifetime. These include double-strand break repair of damaged DNA, repair of post-replication gaps, proper segregation of chromosomes during meiosis and mitosis, maintenance of the genetic information and sequence copy number, as well as creation of new alleles and generation of genetic diversity. Homologous recombination is also thought to have a strong interdependence with DNA replication (Kogoma, 1996; Kogoma et al., 1996) and the rescue of stalled replication forks (Seigneur et al., 1998).

Understanding the molecular mechanisms of homologous recombination will help to explain some fundamental features of DNA maintenance, but could also lead to the successful targeting of gene sequences in higher eukaryotes and perhaps be of use in designing efficient gene replacement strategies. Understanding what controls gene integration and, conversely, what inhibits gene integration, would help in addressing broader issues concerning cancer. Genetic evidence now suggests that cancers can arise as a result of a defect in repair processes involving homologous recombination (Matsuda M and Yasutomi M, 1999).

The prerequisite for homologous recombination to occur, as the name implies, is that the parental DNA molecules share extensive sequence homology. This is different from site-specific recombination, which occurs between defined short sequences. These specific target sequences are recognised by the proteins that mediate the recombination process and the two recombining sites are brought
together through protein-protein interactions. This form of recombination needs to be tightly regulated, as the outcome can be very disruptive producing deletions and inversions, leading to gene inactivation and chromosomal rearrangement. In prokaryotes site-specific recombination allows the integration and excision of a bacteriophage genome into and out of the host bacterial chromosome. The rearrangement of the immunoglobulin genes through V(D)J recombination is a form of site-specific recombination in higher eukaryotes. During illegitimate recombination, another type of recombination, no sequence homology or specific sites are required. It occurs when certain genetic elements move from one chromosome location to another. Strand slippage is also a form of illegitimate recombination that can occur during replication. Often there is a small loss or gain of DNA sequence and the process is usually very deleterious. The classification of these reactions into homologous, site specific and illegitimate is for understanding purposes, as it is often the case that more than one reaction mechanism is involved.

Homologous recombination is a process whereby DNA molecules sharing extensive regions of homology interact to form daughter DNA molecules that are different from the parental DNA as a result of rearrangement and/or exchange of genetic information. The Holliday model (Holliday, 1964), formulated to explain gene conversion events in fungi, proposed that the molecular basis for this process is the physical exchange of DNA strands between the participating DNA molecules, leading to the formation of an intermediate where two DNA duplexes are joined through a crossover (Figure 1.1). This intermediate became known as the Holliday junction. The principle features of this model are central to other models proposed to explain homologous recombination, which will be discussed later in this chapter. The Holliday junction can branch migrate to extend the heteroduplex DNA, which forms the basis for transfer of information between the two parental genes.
Figure 1.1 The Holliday model. (A) Two parental homologous DNA molecules are aligned. (B) Strand exchange of DNA leads to the formation of a Holliday junction. (C) The Holliday junction can branch migrate to extend the region of heteroduplex DNA. (D) The Holliday junction can be cleaved in two possible directions A – B and C – D. Resolution of the junction in the direction A - B or C – D can lead to the production of splice (E) or (F) patch products.
cleavage A-B

splice products

cleavage C-D

patch products
Resolution of the junction by the introduction of matching nicks in the DNA phosphodiester backbone in one of two possible orientations can subsequently separate the two duplex DNA molecules and produce “splice” or “patch” recombinant products (with or without exchange of flanking markers, respectively).

1.2 Homologous recombination in *Escherichia coli*

Homologous recombination has been studied extensively in *E. coli* (reviewed in (Kowalczykowski et al., 1994; Shinagawa and Iwasaki, 1996; West, 1992; West, 1997). Many of the reactions involved have been characterised using purified proteins. The process can be divided into three stages: pre-synapsis, synapsis and post-synapsis. During synapsis homologous DNA molecules become aligned together, exchange strands and become joined together by a Holliday junction. Finally in post-synapsis the Holliday junction is cleaved to release the two DNA molecules.

1.3 Initiation of homologous recombination by RecBCD

During conjugation when the bacterial chromosome (or part of it) is transferred from one bacterium to another as a linear molecule, and in DNA double-strand break repair, duplex DNA ends are processed to allow RecA protein to search for homology. Because RecA requires the presence of a single-stranded region to form an active nucleoprotein filament on DNA, the production of single-stranded DNA is the first stage of pre-synapsis. Initiation of homologous recombination begins with the formation of DNA with a single-stranded region and a 3’ tail. The primary initiator function during conjugation is carried out by the heterotrimeric enzyme RecBCD, an ATP-dependent dsDNA and ssDNA nuclease, and a DNA helicase (Kowalczykowski and Eggleston, 1994; Roman and Kowalczykowski, 1989). RecBCD enters at the end of a linear duplex and begins to unwind DNA. The unwinding activity of RecBCD is accompanied by a simultaneous nuclease activity (Taylor and Smith, 1985). Nuclease activity continues until RecBCD reaches a correctly orientated Chi site (5’gtggtgg3’), where the enzyme pauses. This pause increases the probability of the enzyme cleaving in the vicinity of the Chi site, resulting in nicks 4-6 nucleotides 3’ of
the Chi site. The interactions with the Chi site also results in the attenuation of the 3' - 5' nuclease activity of RecBCD, and upregulation of its 5' - 3' nuclease activity. The overall result is the formation of a single-stranded 3' tail containing the Chi site (Anderson and Kowalczykowski, 1997). RecBCD can then facilitate the loading of RecA onto the Chi-containing single-stranded DNA (Anderson et al., 1997).

1.4 Other pathways for initiation of homologous recombination

The major recombination pathway in *E. coli* wild type cells depends on RecBCD. In *recBC* mutants initiation of recombination depends on other genes. This has led to the idea of multiple pathways of homologous recombination, such as the RecE pathway, seen in *recBC sbcA* mutants (Barbour et al., 1970), and the RecF pathway seen in *recBC sbcBC* mutants (Horii and Clark, 1973).

In the absence of RecBCD (or in addition to RecBCD), recombinogenic single-stranded DNA (ssDNA) could be formed by duplex DNA unwinding by helicases, by strand-specific exonucleases or by combination of these activities. The RecE (exonuclease VIII) and the RecJ protein are both single-strand specific exonucleases, which act 5'-3' and are known to play a role in recombination. These nucleases may function in combination with DNA helicases such as RecQ (Umezu and Nakayama, 1993) to generate single-stranded 3' termini. DNA helicase II, the *uvrD* gene product or DNA helicase IV, the *helD* gene product, may also act in concert with RecJ, RecN, and RecE.

RecF binds ssDNA and dsDNA in the presence of ATP (Madiraju and Clark, 1991; Madiraju and Clark, 1992) and has been shown to form a complex *in vitro* with RecO and RecR (Hegde et al., 1996). RecF, RecO and RecR are also believed to be involved in repair of DNA damage in a mechanism other than one involving recombination and may assist in replisome assembly (Discussed in Kogoma, 1997).

The production of a region of single-stranded DNA is essential for the binding of RecA (Radding, 1991). RecA catalyses pairing of homologous DNA
duplexes and strand exchange leading to the formation of a Holliday junction. RecA is a structural protein and a catalyst of 37.8 kD. In the presence of ATP RecA polymerises on ssDNA to form a right-handed presynaptic helical nucleoprotein filament within which pairing occurs (reviewed in (West, 1992)). The DNA can be partially single-stranded with a ssDNA region at the end of a linear duplex or a gap in the middle of a linear or circular duplex DNA molecule. RecA initiates filament formation at the region of ssDNA but nucleoprotein filament formation can invade the entire duplex DNA molecule. Accessory proteins may also facilitate filament assembly. SSB is believed to remove secondary structure in ssDNA. The DNA within the filament increases in pitch from 10 bp per turn to 18.6 bp per turn, as RecA imposes its own helicity upon the DNA. One helical turn of this filament consists of 6.2 RecA monomers. The RecA nucleoprotein filament forms a deep open groove where a second naked duplex DNA can be bound to a secondary DNA binding site and randomly searched for homology. When homology is established strand exchange is initiated and proceeds with a defined polarity (5' to 3' relative to the ssDNA strand within the nucleoprotein filament that stays with the filament). The strand exchange process can also be described as switching of base-pairing partners between two DNA molecules bound within the two binding sites of the RecA nucleoprotein filament (West, 1992).

In order for DNA strands to be exchanged sequence homology between the two strands must be established. This homology search does not require homologous ends in any of the chains involved. The initial alignment of homology is poorly understood. During the strand exchange reaction catalysed by RecA, complementary base pairing provides the specificity of recognition between the two DNA molecules.

Homologous alignment leads to strand exchange and the production of a Holliday junction. The Holliday junction can be processed by the RuvAB and RuvC proteins encoded by the ruv locus.
1.5 The *ruv* locus

The *ruv* locus located at minute 41 on the *E. coli* genetic map contains two operons. The *ruvA* and *ruvB* operon is controlled by the LexA repressor and is part of the SOS stress response regulon triggered by DNA damage. The second operon consisting of *ruvC* and *orf-26* is not under SOS control (Sharples and Lloyd, 1991; Takahagi et al., 1991). Mutations in all three *ruv* genes result in increased sensitivity to UV light, ionising radiation and mitomycin C, phenotypes indicating defects in DNA repair. The involvement of the *ruv* genes in recombination was demonstrated by the fact that *ruv* mutants are severely defective in recombination in a *recBC*-sbcA, *recBC*-sbcBC or *recG* background (Lloyd, 1991; Lloyd et al., 1984; Lloyd et al., 1987). RuvA is a 22 kDa DNA-binding protein that seems to target RuvB to the DNA (Parsons and West, 1993). RuvB is a 37 kDa DNA-dependent ATPase that in the presence of ATP, Mg$^{2+}$ (<10mM) and RuvA catalyses branch migration.

1.6 Branch migration of the Holliday junction is promoted by RuvA and RuvB

RuvA and RuvB promote branch migration of the Holliday junction along the two duplexes leading to the formation of heteroduplex DNA by the progressive exchange of base-pairs (Parsons et al., 1992; Shiba et al., 1991; Tsaneva et al., 1992). The heteroduplex DNA provides opportunity for transfer of genetic information from one strand to the other.

RuvA shows a high affinity for binding to Holliday junctions but also binds both ssDNA and duplex DNA (Iwasaki et al., 1992; Parsons et al., 1992). RuvA acts as a specificity factor that targets RuvB to the junction (Parsons and West, 1993) where RuvB forms hexameric rings (Stasiak et al., 1994). Binding of RuvA to DNA is structure-specific and sequence independent. In addition to binding Holliday junctions, RuvA can bind Y structures (Hiom et al., 1996). The interaction of RuvA with DNA is not dependent on ATP and is most stable in the absence of divalent cations or low Mg$^{2+}$ concentration.
RuvB is a DNA-dependent ATPase and in the presence of ATP, Mg\(^{2+}\) and RuvA, catalyses branch migration. High concentrations of RuvB alone can promote branch migration of recombination intermediates made by RecA. This reaction requires high concentrations of Mg\(^{2+}\) (>15mM) which facilitates the binding of RuvB to DNA (Muller et al., 1993; Tsaneva et al., 1993). In 2 mM MgCl\(_2\) RuvB has a weak DNA-independent ATPase activity, but in 15 mM MgCl\(_2\) the ATPase activity of RuvB can be stimulated with form I DNA (Iwasaki et al., 1992; Mitchell and West, 1994; Muller et al., 1993; Tsaneva et al., 1992).

In the presence of ATP and Mg\(^{2+}\) RuvA and RuvB form a tripartite protein complex (Hiom and West, 1995; Parsons et al., 1995) where RuvA binds to the crossover and is sandwiched between two hexameric rings of RuvB. The binding of RuvA leads to the Holliday junction within the complex adopting a square planar conformation. The hexameric RuvB rings assemble on two diametrically opposed arms of the open square junction (Parsons et al., 1995).

### 1.7 RecG

The 76 kDa protein RecG can also promote branch migration of the Holliday junction in vitro. RecG does this in an ATP-dependent manner in the presence of Mg\(^{2+}\) ions (Lloyd and Sharples, 1993). The activities of RecG seem to partially overlap with the Ruv proteins as ruv mutants are not recombination deficient whereas double ruv recG mutants are severely deficient in conjugal recombination (Lloyd, 1991; Lloyd and Buckman, 1991; Mandal et al., 1993). RecG is a structure-specific helicase that can act on both R-loops and D-loops and is implicated to function in processes other than recombination (Fukuoh et al., 1997; McGlynn et al., 1997; Vincent et al., 1996). RecG does not fit into either of the RecBCD, RecE, or RecF pathways of recombination. Probably the pathways are dependent on the DNA substrate that initiates recombination and the genetic background in which the recombination takes place.
1.8 RuvC

Finally the Holliday junction can be resolved by the 19 kDa protein RuvC (Dunderdale et al., 1991; Iwasaki et al., 1991; Sharples and Lloyd, 1991; Takahagi et al., 1991). Firstly RuvC binds the junction as a dimer (Shah et al., 1997). The DNA structure is modified by distortion of the DNA backbone (Bennett and West, 1995) and then cleaved by the introduction of two symmetrically related nicks in a pair of strands of like polarity, in the presence of either Mg$^{2+}$ or Mn$^{2+}$ (Bennett et al., 1993; Shah et al., 1997). Cleavage of the continuous (non-crossover) strands show sequence specificity for the sequence 5'WTT↓S-3' where W is A or T and S is G or C (Bennett and West, 1995). This specificity is relaxed by the presence of Mn$^{2+}$ ions. Cleavage produces 5' phosphate and the 3' OH termini that can be re-joined by ligation (Bennett et al., 1993). In vitro studies have shown that RuvC will cleave 3 and 4 stranded recombination intermediates at the junction crossover, but it fails to act on Y-junctions, G/A mismatches heterologous loops or 2-stranded branched junctions. Binding to three-stranded recombination intermediates is less stable than binding to four-stranded intermediates. In an on-going RecA recombination reaction RuvC will cleave a four-stranded intermediate but cannot cut a three-stranded intermediate, suggesting that RecA blocks the accessibility of RuvC to three-stranded junctions (Benson and West, 1994).

RuvC resolves Holliday junctions during genetic recombination and post-replication repair of DNA damage. After cleavage, ligation rejoins the 5' phosphate and the 3' hydroxyl (Bennett et al., 1993; Shah et al., 1997). Cleavage of the Holliday junction and ligation lead to splice and patch products, depending on the orientation of cleavage (Figure 1.1).

RuvC has been expressed in plant cells. RuvC was targeted to the nucleus of the plant cell by fusion to a plant viral nuclear localisation signal and shown to stimulate both genomic and extrachromosomal homologous recombination (Shalev et al., 1999).
To summarise, homologous recombination in *E. coli* is initiated at regions of ssDNA such as gaps or 3’ single-stranded tails formed at double-strand breaks by helicase and/or nuclease activities. The active RecA nucleoprotein filament nucleated at the ssDNA searches for homology and promotes the pairing and exchange of strands between homologous DNA molecules forming a Holliday junction. Extension of the heteroduplex region by branch migration of the Holliday junction can be catalysed by RuvAB or RecG. RuvC can then resolve the Holliday junction. The fact that different proteins can perform similar activities indicates that several pathways operate within a single cell.

1.9 RuvABC as a complex

Genetic analysis of the *ruv* locus has suggested that RuvABC co-operate in processing the Holliday junction (Mahdi et al., 1996; Mandal et al., 1993; Sharples et al., 1990). Further *in vitro* evidence for coupled activity has come from experiments that demonstrate that RuvB and RuvC can co-operate on junctions and promote branch migration in the absence of RuvA. Using junctions that contained a RuvC consensus sequence located in different positions relative to the crossover, cleavage was shown to be stimulated in the presence of RuvB (van Gool et al., 1998). The reduced cleavage of the junction when the consensus sequence is located away from the point of strand exchange suggests that, *in vivo*, the resolvasome would be inefficient if the Holliday junction did not have a correct sequence located at the point of crossover. The formation of a functional RuvABC complex or “resolvasome” on the Holliday junction is supported by experiments using monoclonal antibodies raised to the three Ruv proteins. Recombination reactions *in vitro* showed that the resolution of recombination intermediates by RuvC was inhibited by antibodies against RuvA and RuvB, and all three proteins co-immunoprecipitated with synthetic Holliday junctions (Davies and West, 1998; Eggleston et al., 1997). Recent evidence has demonstrated that RuvC cleavage is more efficient in reactions that contain RuvAB (Zerbib et al., 1998).
1.10 RuvAB and RuvC at the replication fork

Recent work has shown that strains that are rep, recBC ts mutants, accumulate linear DNA due to the presence of stalled replication forks. These strains are not viable at the restrictive temperature, however the lethality can be rescued by inactivation of the ruvAB operon. Similar experiments using dnaB temperature sensitive mutants showed that the accumulation of double-strand breaks was suppressed by inactivation of both RuvAB and RuvC. These results strongly suggest that RuvABC could act on stalled replication forks (Seigneur et al., 1998).

The RuvABC proteins appear to be highly conserved and widely represented in eubacteria. Homologues of the RuvA and RuvB proteins have been identified in the genomes of all completely sequenced prokaryotic species, and most contain an identifiable RuvC homologue. Other prokaryotic enzymes that cut Holliday junctions have also been identified and characterised; such as RusA, bacteriophage T4 endonuclease VII and T7 endonuclease I (reviewed in (White et al., 1997)).

1.11 RusA

RusA is a 14 kDa protein that resolves four-way junction (Sharples et al., 1994). The protein is encoded for by the rusA gene of the defective lambdoid prophage DLP12 and is constitutively repressed in E. coli (Mahdi et al., 1996).

1.12 T4 endonuclease VII

T4 endo VII was identified from T4 phage mutants that accumulated multi-branched DNA (Kemper and Janz, 1976). The 157 amino acid protein has a molecular weight of 18 kDa and cleaves a variety of substrates including four-way junctions with a preference for the continuous strand (Bhattacharyya et al.,
1.13 T7 endonuclease I

Phage T7 endo I is encoded by gene 3. Mutants that lack T7 endo I accumulate branched DNA intermediates (Tsujimoto and Ogawa, 1978) similar to the inactivation of T4 endo VII. The gene has been cloned and the protein product has been purified. The 149 amino acid endonuclease has a molecular weight of 17 kDa (de Massy et al., 1987). Similar to T4 endo VII, T7 endo I is also promiscuous about its substrate and will cleave both three and four-way junctions (de Massy et al., 1987; Dickie et al., 1987).

A common feature of all of these enzymes is that they bind to the junction as dimers and manipulate the structure of the junction, as discussed later in this chapter (reviewed in (White et al., 1997)).

Holliday junction resolvases have been categorised according to their structure and sequence selectivity. Group one, which includes RuvC, RusA, and CCE1, are structure and sequence selective. Group two, which includes T4 endo VII and T7 endo I, are less selective about structure and sequence.

1.14 Archaea

A Holliday junction resolvase functionally similar to RuvC was recently identified in the Archaea species *Pyrococcus furiosus*. The resolvase has a predicted molecular weight of 13,766 Da and shows sequence identity of ~30% to open reading frames from other archea species. The activity was shown to exist as a dimer in solution and cleaved a four-way junction with a strong
preference for the non-crossover strand. Cleavage of a three-way junction was seen but at greatly reduced efficiency (Komori et al., 1999).

1.15 Holliday junction-specific endonucleases in eukaryotes

Our understanding of the molecular mechanisms of homologous recombination in eukaryotes is still limited compared with that of prokaryotes. Despite the significant progress in understanding the proteins that promote homologous pairing and strand exchange in eukaryotes, which will be discussed later in this chapter, (reviewed in (Baumann and West, 1998)), little is known about the proteins that participate in the later stages of homologous recombination, including the processing of Holliday junctions.

Holliday junction resolvase activities have been found in a number of eukaryotic organisms (reviewed in (White et al., 1997)). However, most of these activities have not been identified at the molecular level and no resolvase has been unequivocally shown to function in the nucleus.

Yeast cells exhibit particularly high frequencies of homologous recombination and are expected to have well expressed recombination enzymes. There are several reports of Holliday junction-specific endonucleases in *Saccharomyces cerevisiae*.

1.15.1 CCE1

One of the first eukaryotic resolvases characterised was the mitochondria-specific protein, cruciform cutting enzyme 1 (CCE1) from *S. cerevisiae* (Kleff et al., 1992). This resolvase is sequence selective in cleavage showing a preference for 5’-CT↓, where ↓ indicates the point of cleavage, and a preference for the continuous strand of the junction. (Kupfer and Kemper, 1996; White and
Lilley, 1996). The enzyme does not require homology in the arms of the Holliday junction and will cleave a fixed junction providing the correct sequence is present. CCE1 functions as a dimer (White and Lilley, 1996). The resolvase has a predicted molecular mass of 41 kDa which is near to the molecular mass of 40 kDa determined by FPLC Superose 12 gel filtration, and also near to the 38 kDa as determined by SDS PAGE (Kupfer and Kemper, 1996). Resolvase activity has been tested on supercoiled and relaxed cruciforms, both of which are cleaved by CCE1. These substrates are from phage supercoiled replicative form-I DNA and contain inverted repeats cloned into unique restriction sites. The palindrome sequences form a stable cruciform structure.

*S. cerevisiae* cce1 mutants were identified as temperature sensitive strains lacking DNA cruciform cutting endonuclease activity and the *CCE1* gene was mapped to the left arm of chromosome XI (Kleff et al., 1992). *cce1Δ* mutant cells show no defect in chromosomal meiotic or mitotic recombination, but have a petite phenotype (Kleff et al., 1992), which is characteristic of non-functional mitochondria (Ezekiel and Zassenhaus, 1993; Zweifel and Fangman, 1991). CCE1 is a DNA junction-resolving enzyme involved in maintaining the stability of the mitochondrial genome in *S. cerevisiae*. The accumulation of branched mitochondrial DNA (mtDNA) structures, as a result of resolvase deficiency, can account for an increased loss of mtDNA in vegetative *cce1Δ* cells, and the altered pattern of mtDNA segregation from zygotes (Lockshon et al., 1995).

### 1.15.2 Other *S. cerevisiae* resolvase activities

Prior to the identification of CCE1 (Kleff et al., 1992), several groups reported resolvase activities in *S. cerevisiae* (Evans and Kolodner, 1987; Jensch et al., 1989; Parsons et al., 1989; Parsons and West, 1988; Symington and Kolodner, 1985; West and Korner, 1985; West et al., 1987).

### 1.15.3 Endonuclease XI

Endo XI was identified in crude cell free extracts and was partially purified from mechlorethamine-treated cells by column chromatography. The activity
was characterised using negatively supercoiled plasmids that contained inverted repeats extruded into cruciforms. Its native molecular weight was 200,000 Da, estimated by gel filtration (West and Komer, 1985; West et al., 1987). Mapping the sites of cleavage showed that when the arms of the cruciform were homologous, cleavages were symmetric. The activity could also cleave junctions asymmetrically if the arms contained heterologous sequences. The enzyme would cleave the junction at 5' A↓C↓C↓G 3' symmetrically where ↓ indicates a cleavage site. The activity was also shown to cleave 5' C↓T↓A↓C↓G↓C↓CG 3' (Parsons et al., 1989; Parsons and West, 1988). These cleavage sites are quite different to other prokaryotic and eukaryotic resolvases characterised.

1.15.4 Endonuclease X2

The partial purification and characterisation of a second Holliday junction resolvase was also reported (Evans and Kolodner, 1987; Symington and Kolodner, 1985). After the identification of CCE1, this activity was verified as CCE1 (Kleff et al., 1992).

1.15.5 Endonuclease X3

An activity was identified in *S. cerevisiae* that had similar characteristics to the bacteriophage resolvase T4 endo VII (Jensch et al., 1989). The enzyme had very similar cleavage specificity with regards to both sequence and structure. The activity eluted from gel filtration chromatography with the same molecular weight as T4 endo VII and interacted with antibodies raised against T4 endo VII.

1.15.6 Endonuclease X4

The *S. cerevisiae* nuclease Endo X4 is CCE1 (Kleff et al., 1992).
The fact that some of the activities reported (Evans and Kolodner, 1987; Symington and Kolodner, 1985) are now known to be the resolution activity of CCE1, argues that the use of a strain where the CCE1 mitochondrial resolvase gene has been deleted, would have a considerable advantage in identifying a potential nuclear resolvase, compared to groups that published reports of resolvase activity in CCE1+ cells. Residual cruciform-cutting activity was previously observed in a cce1Δ strain (Kleff et al., 1992), but was never pursued.

1.15.7 Mammalian resolvase activities

Mammalian resolvase activities have been reported by several groups and characterised to varying degrees (Couture and Chow, 1992; Elborough and West, 1990; Hyde et al., 1994; Jeyaseelan and Shanmugam, 1988; Waldman and Liskay, 1988). Some of these activities are poorly characterised and were never shown to cleave the junction symmetrically at or close to the point of crossover in strands of like polarity (Couture and Chow, 1992; Jeyaseelan and Shanmugam, 1988; Waldman and Liskay, 1988).

The mammalian activities partially purified from calf thymus and Chinese hamster ovary cells were characterised in more detail. The cleavage sites were mapped using a synthetic Holliday junction which contained a homologous core of 12 bp. The mammalian enzyme was shown to cleave this junction on strands of like polarity at sites that were symmetric. Symmetric cleavage did not require homology as cleavage was also seen on a junction with a fixed crossover point. Cleavage was specific for the junction and the enzyme would not cleave a G/A mismatch, a heterologous loop or a Y junction (Elborough and West, 1990; Hyde et al., 1994).

The molecular identity of the Holliday junction resolvases described above remains unknown. As there are no recognisable eukaryotic homologues of RuvC, or of any of the other known Holliday-junction resolvases, including that from Archaea, in the available eukaryotic sequence databases (Bult et al., 1996;
Goffeau et al., 1996; Klenk et al., 1997; Smith et al., 1997), a biochemical approach is still needed to identify these enzymes.

*S. cerevisiae* is an excellent model organism to look for a nuclear Holliday junction resolvase since it has efficient meiotic and mitotic homologous recombination. For example, the predominant repair pathway for double-strand breaks in *S. cerevisiae* is by homologous recombination, whereas mammalian cells predominantly repair double-strand breaks by non-homologous end-joining (Kanaar et al., 1998). Also, exogenous homologous sequences integrate into chromosomal DNA predominantly via homologous recombination in *S. cerevisiae* (Orr-Weaver et al., 1981). Although homologues of known resolvases, including CCE1 have not been found in the completed *S. cerevisiae* genome sequence, there is strong evidence for Holliday junction formation in *S. cerevisiae* and hence for the requirement of a nuclear resolvase. Homologues of the *E. coli* strand exchange protein RecA that can lead to Holliday junction formation have been found in yeast and mammalian cells (Shinohara et al., 1993), as discussed later in this chapter. Holliday junction intermediates in *S. cerevisiae* were first detected in 2 μm plasmid DNA from meiotic cells, both on Southern blots and by electron microscopy (Bell and Byers, 1979). The existence of Holliday junctions resulting from interhomologue exchange between chromosomes in *S. cerevisiae* was demonstrated physically using two-dimensional gel electrophoresis, both in meiotic (Collins and Newlon, 1994; Schwacha and Kleckner, 1995; Schwacha and Kleckner, 1994) and mitotic cells (Zou and Rothstein, 1997).

### 1.16 Structure and function

The Holliday junction is well established as a central intermediate formed during homologous recombination, site-specific recombination and double-strand break repair. These structures have been seen by electron microscopy of recombng DNA extracts from virally infected *E. coli* (Benbow et al., 1975; Doniger et al., 1973; Valenzuela and Inman, 1975) but not in *recA* strains (Benbow et al., 1975). Once formed, the Holliday junction can branch migrate producing heteroduplex DNA, and must be resolved to release the recombinant
products. Early studies of the kinetics of branch migration suggested that spontaneous branch migration of double-stranded DNA would be fast enough to allow for the production of heteroduplex DNA (Thompson et al., 1976). However, more recent studies suggest branch migration needs to be catalysed as a single mismatch, or deletion will impede spontaneous branch migration (Johnson and Symington, 1993; Muller et al., 1992; Panyutin and Hsieh, 1993; Panyutin and Hsieh, 1994). In addition the DNA sequence at the junction will determine which isomeric form of a folded junction is formed (Duckett et al., 1988). Therefore, spontaneous branch migration is unlikely because the junction may have to switch isomeric forms at each step.

These results imply that the Holliday junction is processed by proteins that need to interact specifically with this structure. To do this the proteins need to recognise the structure of the junction.

1.16.1 The structure of the Holliday junction

Small synthetic four-way junctions annealed from four oligonucleotides are widely used as model Holliday junctions. The structure adopted by these synthetic Holliday junctions in solution has been studied extensively using a variety of biochemical and biophysical methods. Full base-pairing of the four arms is maintained right to the crossover in these junctions, showing no evidence for single-stranded DNA at the point of strand exchange (Reviewed in (Lilley and Clegg, 1993)

The Holliday junction can adopt different structural conformations depending on the presence of metal ions. In the absence of added cations the junction has a square planar conformation with four-fold rotational symmetry. The angles between the arms of the junction are equal, and at the centre of the junction, which is open, the bases are accessible. The four arms are fully extended and unstacked. This conformation is energetically most favourable in the absence of cations.
In the presence of divalent metal ions the junction folds to form a stacked "X" structure formed by pairwise coaxial stacking of helical arms (Lilley and Clegg, 1993; Lilley and Clegg, 1993; Seeman and Kallenbach, 1994). The structure has a two-fold symmetry. The two exchanging strands pass between the two helical stacks at the point of strand exchange and the two continuous strands have continuous helical axis. The four strands, which contain different sequences, are non-equivalent and stack to minimise steric and electrostatic interactions. The pairwise stacking of the arms of the junction is accompanied by rotation of the arms. The sequence of the junction affects the stacking preference of the arms and therefore will determine the rotation of the arms into an arrangement, that is the most thermodynamically favourable combination of stacked arms. This is the likely structure in vivo.

The evidence for the structure of the four-way junction has come from a number of approaches including gel electrophoresis, fluorescence resonance energy transfer, and probing experiments.

Gel electrophoresis techniques have been used to compare the electrophoretic mobility of junctions. Six isomeric junctions were formed by cleavage with different restriction endonucleases to produce arms of different lengths. The mobility of the junctions in the presence of magnesium suggested that the angle subtended between the long arms of the junction were acute, obtuse and 180°. These results suggest that the junction adopts a stacked conformation. A different mobility pattern was seen in the absence of metal ions. Only two different mobility species, compared to three previously, were observed for the same restriction digested junctions. These results suggested a square planar confirmation for the junction (Duckett et al., 1988).

Fluorescence resonance energy transfer experiments measure the acceptor emission of two different fluorescent dyes conjugated to the 5' end of two arms of the junction. In the absence of magnesium the normalised acceptor emissions were approximately the same for the arms of the junctions when labelled in
different combinations for comparison. In the presence of magnesium two pairs of arms had increased acceptor emissions compared to other combinations of arms. These sets of results are consistent with the junction adopting a square planar structure in the absence of magnesium ions and a stacked structure in the presence of magnesium ions (Clegg et al., 1992; Murchie et al., 1989).

Chemical probing experiments using hydroxyl radicals also lead to similar conclusions about the structure of the junction in the presence of metal ions. The above mentioned experimental data also indicated that the stacked strands were antiparallel with base pairs at the point of strand exchange presenting a major and minor groove on each side of the junction (Duckett et al., 1988; Murchie et al., 1989). Different types of groove presented at two sides of the junction could effect protein binding. Cleavage sites produced by three Holliday junction resolvases have been mapped to the minor-groove side of the junction (Lilley and Clegg, 1993).

Cations present in solutions can localise to specific sites of the junction and can form a general sea of ions. The presence of sodium, magnesium or calcium cause the junction to form a stacked structure but only magnesium and calcium protect the junction from hydroxyl attack (Duckett et al., 1990). The presence of metal ions could function to overcome the charge densities at the site of strand exchange (von Kitzing et al., 1990).

Junctions can stack in a parallel or antiparallel conformation. Free energy calculations have shown that thermodynamically the junction will adopt an antiparallel conformation. The antiparallel conformation can be changed by protein interactions to the parallel conformation (Lu et al., 1990). However, free energy is not related to kinetic activation energy, which may be quite large to rotate a DNA helix.

The studies on junctions have been conducted using protein free junctions. Proteins may mould the junction into a particular structure for catalysis. Junctions constructed as mentioned will vary depending on sequence. Junctions
formed during homologous recombination will be able to branch migrate to structures that are energetically more favourable.

1.16.2 Resolvase junction binding

The binding of different resolvases to synthetic four-way junctions has been studied extensively. Gel electrophoretic mobility assays often show the presence of two junction protein complexes. The heavier complex is usually competed away by competitor DNA suggesting that the more retarded complex is not junction specific (White et al., 1997).

The binding of a resolvase to a junction is independent of cations in all cases examined. Most junction binding proteins, such as T4 endo VII, T7 endo I, RuvC, CCE1, and RuvA, change the structure of the junction, as discussed below.

Protein binding leads to the junction taking a specific novel conformation. These confirmations have been determined by extending the gel electrophoretic mobility assay using junctions with different length arms to study junction-bound complexes (Duckett et al., 1988). As with the free junction, the mobility of the junction complex depends on the angle between the two long arms of the junction. Using this assay, protein-induced changes in the structure can be revealed by comparing the patterns obtained with the free junction to that of the protein-junction complexes. The results obtained have demonstrated that resolvase binding can unfold the junction and impose different open conformations, such as square planar or two-fold symmetric (see figure 1.16.2).
Figure 1.16.2 Schematic showing some conformations adopted by synthetic Holliday junctions upon protein binding. (A) Unbound junction in the presence of magnesium ions has stacked folded structure. (B) Junction adopts square planer conformation upon binding of CCE1 or RuvA. (C) Junction structure imposed by binding of RuvC. (Adapted from (White et al., 1997)).
In a complex with RuvC the global structure of the junction was altered from stacked into an open conformation with a two-fold symmetry (Bennett and West, 1995) (Figure 1.16.2.). CCE1 holds the junction in an open-square planar conformation (White and Lilley, 1997). T4 endo VII (Giraud-Panis and Lilley, 1996; Pohler et al., 1996), RusA (Chan et al., 1998; Giraud-Panis and Lilley, 1998) and T7 endo I (Duckett et al., 1995) have also been shown to distort the junction on binding. RuvA also holds the junction in an open square planar conformation (Parsons et al., 1995). The structure of the junction in the complex was determined by the bound protein and was independent of the presence or absence of added metal ions (reviewed in (White et al., 1997)).

The particular structure of the junction adopted upon binding of the resolvase may serve to facilitate close contact between the catalytic domain of the resolvase and the particular groove of the DNA helix.

1.16.3 Resolvases function as dimers

The crystal structure of RuvC has been determined at 2.5 Å resolution. Four acidic residues were proposed to form a catalytic centre located at the bottom of a putative DNA-binding cleft (Ariyoshi et al., 1994). Support for the catalytic centre was obtained by constructing RuvC mutants that retained binding ability but not resolution activity. The crystal structure was derived from a dimeric molecule of RuvC.

Most Holliday junction resolvases form dimers in solution and bind the junction as dimers. T4 endo VII (Pohler et al., 1996), T7 endo I (Parkinson and Lilley, 1997), CCE1 (White and Lilley, 1996) and RuvC (Shah et al., 1997) have all been shown to function as dimers (Shah et al., 1997). Mutants that bind the junction but do not cleave the junction, have been used to show that a heterodimeric resolvase will nick one strand of the Holliday junction (Giraud-Panis and Lilley, 1997; Parkinson and Lilley, 1997). However, concerted cleavage of a Holliday junction by a dimeric resolvase would be the most
probable mechanism to insure that cleavage releases both duplex molecules in a Holliday junction.

Mutants that are defective in resolution of Holliday junctions have shown that binding and resolution are functionally separable (Duckett et al., 1995; Pohler et al., 1996; Sharples and Lloyd, 1993). The resolvase must therefore employ different mechanisms for junction recognition and resolution.

1.16.4 Holliday junction resolution

Endonuclease activities that cleave junctions owe their specificity to structural recognition of the junctions. Cleavage is at sites symmetric to the point of strand exchange and resolution of the junction is via nicks introduced into the continuous strands or crossover strands of the junction (Figure 1.16.4). Cleavage by RuvC and CCE1 has been shown to be on the continuous strands of the junction (Bennett and West, 1995; White and Lilley, 1996). All Holliday junction resolvases for which the gene sequence is known cleave the junction at or near the point of crossover 3’ of a thymine residue and show sequence specificity of cleavage. Although a resolvase will bind a Holliday junction irrespective of sequence, the resolvase will not cleave the Holliday junction if the correct cleavable sequence is not at or near the point of strand exchange. RuvC has been shown to function with RuvA and RuvB. A complex of the three proteins could overcome the problem of RuvC binding a Holliday junction that did not have a cleavable sequence at the point of strand exchange, by RuvAB branch migrating a cleavable sequence to the complex.
Figure 1.16.4 Schematic of a Holliday junction showing the crossover and continuous strand. The Holliday junction is not drawn to scale.
1.16.5 Proposed models for the association of a RuvABC Holliday junction complex

Experimental evidence has shown that RuvA, RuvB and RuvC can function in the presence of RecA and that RuvAB changes the orientation of Holliday junction resolution by RuvC under these conditions. These findings led to a proposal that RuvA, RuvB and RuvC could associate on a Holliday junction (Eggleston et al., 1997). A model was proposed for a mechanism that could accommodate these results. RuvA binds to the Holliday junction after the dissociation of the RecA protein filaments and holds the junction in a square planar conformation while RuvB is loaded onto the junction by interactions with RuvA.

A tripartite structure forms where the open square planar Holliday junction is sandwiched between the concave face of two RuvA tetramers, with two RuvB hexameric rings flanked on opposite arms of the junction. The dissociation of a RuvA tetramer and replacement with RuvC would form a RuvABC branch migration/resolution complex.

1.16.6 RuvA functions as a tetramer and an octamer?

A RuvABC complex has been shown to function in vitro (Eggleston et al., 1997; van Gool et al., 1999; van Gool et al., 1998; Zerbib et al., 1998) and the genetic evidence suggests that the three proteins function together in vivo (Mahdi et al., 1996; Mandal et al., 1993; Sharples et al., 1990). The binding of RuvC has been shown to unfold the Holliday junction into a two-fold symmetric, “open X” structure (Bennett and West, 1995). However, this structure is different from the open square planar structure of the junction complexed with RuvA (Parsons et al., 1992), which is maintained on the addition of RuvB (Yu et al., 1997). The open square planar structure enables DNA strands to be pumped through the RuvB octameric rings without inhibition by coaxial stacking of the DNA arms. The simultaneous binding of
RuvA and RuvC to the junction requires that one or both would have to compromise their preferred way of interaction with the junction.

The crystal structure of *E. coli* RuvA alone and bound to a Holliday junction reveals a tetramer of RuvA that holds the junction in an open square planar conformation (Hargreaves et al., 1998; Rafferty et al., 1996). The RuvA subunits are related by four-fold symmetry in the tetramer, which has a negatively charged convex face and a positively charged concave face (Rafferty et al., 1996). The detailed structure of the concave surface suggested how a Holliday junction could be docked onto the surface. The structure of the *E. coli* RuvA-junction complex solved to 6Å proposed that RuvA bound to the junction as a tetramer (Hargreaves et al., 1998). One tetramer bound to one side of the junction would allow RuvC to bind and function with RuvB as a RuvABC complex.

The crystal structure of *Mycobacterium leprae* RuvA bound to a junction was solved at 3 Å and also revealed an open square planar junction and a similar structure of RuvA (Roe et al., 1998). However, in the crystal structure of the *M. leprae* RuvA-junction complex, the Holliday junction was sandwiched between two RuvA tetramers in an octameric complex (Roe et al., 1998). An octameric structure could stabilise the complex during branch migration whereas a tetrameric structure might be unstable due to the torque generated by the counterrotating RuvB motor (Figure 1.16.6).

Electron microscopy and biochemical evidence led to the proposal of a model of the Holliday junction bound by an oligomer (tetramer or octamer) of RuvA (Hiom and West, 1995; Parsons et al., 1995). Later experimental evidence from electron microscopy and mass analysis of scanning transmission electron microscopy images of *E. coli* RuvAB-junction complexes proposed two tetramers of RuvA (Yu et al., 1997). *E. coli* RuvA has been shown to exist as a tetramer in solution by gel filtration but binding studies have shown the formation of both tetrameric and octamer complexes with junction DNA (Parsons et al., 1992; Whitby et al., 1996).
Figure 1.16.6. RuvA as the Stator for the RuvB motor. (A) Schematic diagram of the proposed RuvAB branch migration complex based on (Parsons et al., 1995). In the “unstable” form with only one RuvA tetramer bound, the torque generated by the counter-rotating RuvB motors would cause the RuvA tetramer to rotate around its own axis, disrupting interactions with DNA. In the “stable” form with the RuvA octamer described here, the twisting of one RuvA tetramer is opposed by the counter motion of the other tetramer, stabilising the complex for branch migration. In a putative RuvABC resolution complex, interactions between a RuvC dimer and RuvB motors might provide sufficient stability for limited branch migration even when only one RuvA tetramer is bound. (B) Space-filling picture of the *M. leprae* RuvA octamer shell. The C-terminal domains (in red) which are essential for the Ruv B interactions, are arrayed on either side of the mouths of the tunnels.
UNSTABLE Motor(s)

Stator

UNSTABLE

Rotor

STABLE Motor(s)

Stator

STABLE

Rotor

a

b
*M. leprae* RuvA has been shown to form an octamer in solution and neutron scattering contrast variation and modelling suggest octamer binding to a Holliday junction whereby two tetramers of RuvA sandwich the junction (Chamberlain et al., 1998).

There is evidence in support of both models. Both models could show a functional form of the protein junction complex. RuvABC will function as a complex, which would agree with the RuvA tetramer-junction complex where RuvC could be accommodated in the complex. This is also in agreement with genetic evidence that proposes all three Ruv proteins function together. RuvA and RuvB are part of the SOS repair response to DNA damage. Induction of RuvA and RuvB could co-ordinate enhanced RuvC cleavage of a Holliday junction, as the RuvC cleavage of a Holliday junction is inefficient unless a correct cleavage sequence is at or near the point of crossover of the junction. RuvC could bind to a junction already bound by a tetramer of RuvA. However, junction cleavage was shown to be inhibited by high concentrations of RuvA (Whitby et al., 1996), a situation that would be encountered by the induction of RuvA.

The octamer of RuvA with RuvB on a junction could be a functionally specialised enzymatic form for efficient branch migration through regions of damaged DNA or heterology.

RuvAB is very efficient at branch migration and can branch migrate a Holliday junction through a nucleosome. Although not a situation encountered by RuvAB, this does suggest that eukaryotic analogues would be able to cope with chromatin (Grigoriev and Hsieh, 1998). The open square planar structure taken by CCE1 would be appropriate to cooperate with a RuvAB-like analogue as there would be no inhibition from helical stacking of the junction. CCE1 would not encounter a nucleosome problem, a nuclear resolvase probably would.
1.17 Eukaryotic homologous recombination

1.17.1 Genetic Models

Analysis of recombination is more amenable in lower eukaryotes and fungal genetic studies led to various models being proposed to explain crossing over (intergenic recombination), intragenic gene conversion events and general mechanisms of homologous recombination (reviewed in (Paques and Haber, 1999)).

1.17.2 The Holliday model

The Holliday model suggests a molecular basis to explain a gene conversion event and its association to crossing over during meiosis (Holliday, 1964). Various models were put forward at that time. However, this remarkable model was proposed long before proteins that process the Holliday junction were known. According to this model, homologous DNA molecules align and symmetric nicks introduced in both molecules allow the exchange of DNA strands from one molecule to another leading to production of heteroduplex DNA (Figure 1.1). Branch migration extends the region of heteroduplex DNA leading to gene conversion or post-meiotic segregation. The Holliday junction formed can be resolved in either of two orientations to produce either patch or splice products. The flanking DNA regions of the patch recombinant product remain the same as the parental DNA molecules (no crossing over). Conversely, the flanking markers are different to the parental markers when resolution of the Holliday junction produces splice products (gene conversion accompanied by crossing over). This model explains the altered linkage observed during fungal meiosis and conceptualises gene conversion and crossing over as outcomes of one molecular event.

1.17.3 The Meselson-Radding model

The Meselson-Radding model (Meselson and Radding, 1975) aimed to explain gene conversion bias on one strand, and was later modified (Radding, 1982) to
account for genetic evidence suggesting that the strand receiving the invading strand was where the initiating single-strand nicking had occurred. A single-strand DNA break is invaded by a strand from the homologous chromatid to form a D-loop. The D-loop is nicked and the non-invasive 3’ end acts as a primer for DNA synthesis. Branch migration and nicking can lead to Holliday junction formation after isomerization.

1.17.4 The Resnick model and the Double-Strand Break Repair model
Evidence for double strand breaks as possible initiators of recombination was growing and gave rise to several models proposed to explain the experimental findings (Resnick, 1976; Szostak et al., 1983). In the double-strand break repair model (DSBR) (Szostak et al., 1983) the site of the DSB is enlarged by exonucleolytic activity to produce a double-strand gap. In this model gene conversion results from two mechanisms: repair of the double strand gap using information from a homologous DNA molecule, and the formation of heteroduplex DNA adjacent to the gap. This model was later modified to accommodate experimental evidence suggesting that instead of a double-strand gap, DSBs were processed to give long (>1 kb) 3’ single strand tails (Figure 1.17.4) (Sun et al., 1991). It was proposed that one of these tails invades a homologous duplex partner. The D-loop created is enlarged by DNA repair synthesis enabling the pairing with the 3’ end of the second strand leading to the formation of a second Holliday junction. Cleavage of the junctions in the same orientation produces patch products, cleavage in the opposite orientation results in splice products.
Figure 1.17.4. The Double-Strand Break Repair model. (A) A DSB is initiated in one of the DNA molecules. (B) 5' – 3' exonucleolytic activity produces extensive 3' single-strand overhangs. (C) One 3' end invades the intact DNA molecule creating a D-loop. (D) DNA repair synthesis primed from the other 3' single-strand overhang enlarges the D-loop. (E) Repair synthesis also occurs from the second 3' end and two Holliday junctions are formed. Resolution of the Holliday junction can lead to (F) splice products or (G) patch products.
1.17.5 Single-strand annealing model

The single-strand annealing model was proposed following the observation that recombination between repetitive sequences in mammalian cells resulted in the loss of intervening sequences. The model proposes annealing of single-stranded tails rather than strand invasion. DSB are processed by 5'-3' exonuclease activity. Complementary single-strands anneal, and non-paired overhangs are removed by single-strand endonuclease or exonuclease activity. Heteroduplex molecules can be repaired by DNA polymerase and DNA ligase activity.

Most models propose a four-strand branched structure, the Holliday junction, as a key intermediate in homologous recombination. This does not mean that other models that do not propose the Holliday junction intermediate, e.g. the synthesis dependant strand annealing model (reviewed in (Paques and Haber, 1999)), are wrong and should not be considered. Genetic studies may suggest certain explanations and a pathway, but this does not mean that this pathway is the main or only pathway. Different substrates are processed by specific proteins depending on which protein is expressed. Recombination pathways will probably be initiated depending on what particular DNA substrate is available. A possible competition for DNA substrates by different molecular pathways has been proposed and is discussed below. A single model is unlikely to explain every facet of genetic evidence.

These models provide a conceptual framework that suggests what DNA intermediates and biochemical activities might be involved and can help to design experiments to look for these intermediates and enzymes.

1.18 The importance of recombination

In eukarotic cells recombination plays a fundamental role in several important processes. The roles of homologous recombination in meiosis and in double-strand break repair (DSBR) need to be emphasised in particular.
1.19 Meiosis

Recombination between homologous chromosomes is strictly required for the proper segregation of chromosomes during meiosis. Meiosis cannot occur in the absence of homologous recombination.

During meiosis one round of DNA replication is followed by 2 rounds of division. During pachytene of prophase I the replicated condensed homologous chromosomes have come together as bivalents. At diplotene, when chromatids start to separate, chiasmata, or points of crossover can be seen at sites where DNA molecules have recombined. During anaphase the first division separates the bivalents into sister chromatid pairs. At the second division the sister chromatid pairs segregate into different daughter cells. In *S. cerevisiae* meiotic homologous recombination is initiated by double-strand breaks.

1.20 Double-Strand Break Repair (DSBR)

Double-strand breaks occur in prokaryotes and eukaryotes, and can be of multiple origin. They can be caused by both exogenous agents, including chemical DNA-damaging agents or physical agents such as ionising radiation, and endogenous agents, such as oxidative DNA damage, site specific endonucleases, or replication arrest at replication forks due to a stalled or defective DNA helicase (Michel et al., 1997). Double-strand breaks (DSB) also occur naturally during V(D)J recombination to produce variation in antigen receptor sites (Gellert, 1997).

Double-strand break repair and recombination are closely related events. One cannot be discussed without the other. In *S. cerevisiae* several recombination pathways are induced by double-strand break repair and meiotic recombination is initiated as a result of DSBR. Double-strand breaks in the genome need to be repaired to maintain the cell’s viability and preserve the organism’s genome.
Failure to repair a DSB will lead to cell death. Inaccurate repair can lead to mutations in genes, loss of genetic material, genetic rearrangements or even cancer.

Repair of double-strand breaks can be by either homologous recombination or non-homologous end joining (NHEJ).

1.20.1 Non-homologous end joining

In vertebrates the predominant pathway for DSBR is the Ku-dependant NHEJ. NHEJ repairs DSB by directly joining the DNA ends without using an undamaged template to restore the genetic information. A small deletion often results in this repair process that does not involve long regions of homology. NHEJ in yeast requires the $RAD50$, $MRE11$, $XRS2$, $YKU70$, $YKU80$, and $LIG4$ genes. MRE11, RAD50 and XRS2 act in a complex and are proposed to be linked to the DNA damage response via the cell cycle. In meiotic and mitotic cells the RAD50/MRE11/XRS2 protein complex is involved in processing DSB to form single-strand tails (Sun et al., 1991). The recombination event is dependent on short stretches of homology but does result in deletions or insertions of sequences at the joint.

The analysis of X-ray-sensitive mammalian cell lines has led to the identification of 4 complementation groups involved in NHEJ: $XRCC4$, $XRCC5$, $XRCC6$, and $XRCC7$. The products of the $XRCC5$ and $XRCC6$ genes are the Ku80 and Ku70 subunits of the DNA-dependent protein kinase, respectively (Taccioli et al., 1994). Mammalian cells lacking Ku70 and Ku80 that are crucial for NHEJ exhibit extreme sensitivity to ionising radiation.

1.20.2 The two pathways operate

Both yeast and mammalian systems have in place the Ku-dependent and the Rad52-dependent pathways for repair of DSB. The induction of the pathways in
response to DNA damage is not understood. Genetic and biochemical evidence suggests that the two pathways are complementary. 

*Rad52*−/− knockout mice show reduced homologous recombination but still exhibit resistance to ionising radiation (Rijkers et al., 1998). Other homologues of Rad52 have not been found so it seems likely that ionising radiation resistance is due to processing via a NHEJ pathway, which is the predominant pathway in these cells.

In chicken, DSBs induced by γ-radiation seem to be repaired by NHEJ in G1-early S phase of the cell cycle, and are repaired by recombination in late S-G2 phase of the cell cycle (Takata et al., 1998), both pathways having a role in chromosomal DNA maintenance in the cell cycle.

RAD54−/−/Ku70−/− double mutants were significantly more sensitive to ionising radiation than single mutants. Double mutants have greatly increased frequencies of chromosomal aberrations.

Rad52 has been shown to bind DNA ends (Van Dyck et al., 1999) suggesting that both pathways could compete for DSBs. Repair of DSB in higher eukaryotes is predominantly by the Ku-dependent NHEJ pathway. How NHEJ is chosen as a mechanism of DSBR over Rad52-dependent homologous recombination is not known. Why higher eukaryotes have adopted an error-prone repair pathway is also not known. It has been suggested that the predominance of a repair pathway evolved because of differences in genome constitution. Lower eukaryotes have a greater percentage of coding sequences in their genomes and accurate repair by a Rad52-dependent mechanism would be advantageous, whereas the genomes of higher eukaryotes have a large percentage of non-coding repetitive sequences that could be repaired quickly by a NHEJ mechanism (Hiom, 1999). Transcriptional or translational control of expression of proteins from both pathways may be how lower and higher eukaryotes direct the repair of DSB. Control may be at the protein level by interaction with other proteins leading to modification of protein structure.
1.20.3 Double-Strand Break Repair by homologous recombination

Repair by homologous recombination is considered a precise repair mechanism that requires a homologous chromosome or sister chromatid to act as a template for repair. This ensures that DNA sequences are not lost.

Repair of DSB by homologous recombination can be achieved by a mechanism involving single-strand annealing or strand invasion. Both mechanisms require RAD52. The process involves resectioning of DNA ends. The nucleolytic resectioning can extend for several kilobases. ssDNA is generated at a faster rate in rad52 mutants than in wild type cells (Sugawara and Haber, 1992). In S. cerevisiae repair of DSB is more frequently by RAD52-dependent homologous recombination involving Rad50/Mre11/Xrs2, compared to NHEJ.

1.20.4 DSBR by homologous recombination: single-strand annealing

Single-strand annealing occurs at repeated DNA sequences. Exonucleases produce single-stranded tails that can anneal to complementary sequences. After further nucleolytic trimming the two strands are ligated. In S. cerevisiae this pathway requires RAD52, RAD1 and RAD10 (Haber et al., 1993; Ivanov and Haber, 1995). No other NER genes are involved in the repair of HO-induced DSB.

1.20.5 DSBR by homologous recombination: strand invasion

The strand invasion mechanism uses an intact homologous chromosome as a template to direct repair of the DSB. Proteins involved in the repair mechanism are part of the RAD52 epistasis group. This group includes RAD50, RAD51, RAD 52, RAD53, RAD54, RAD55, RAD57, RAD59, MRE11, and XRS2. The proposed functions of these proteins has come from both genetic and biochemical analysis of the molecular mechanism of these proteins in vitro and in vivo. It should be remembered that although in vitro assays have implicated these proteins to particular functions, the substrates used in these assays might bias the experimental results, as might the fact that S. cerevisiae strains are not
isogenic. One strain may have a subtle difference in its phenotype as a result of genotype differences between strains. However a consensus picture is forming.

1.21 The *RAD* genes

The *RAD* genes were discovered due to their sensitivity to radiation (Game and Mortimer, 1974). Three epistasis groups of *RAD* genes have been identified by genetic analysis in *S. cerevisiae*: the *RAD3* group involved in nucleotide excision repair (NER); the *RAD6* group associated with error prone/post-replicational repair and the genes from the *RAD52* group required for the repair of DSBs by a recombinational mechanism. Most genes of the *RAD52* group are also involved in meiotic recombination.

1.21.1 RAD 51

Initial *in vitro* strand-transfer assays used to look for eukaryotic recombination activities proved disappointing, as they tended to highlight exonuclease activities. Research was focused instead on mutants in *S. cerevisiae* that showed phenotypes similar to *E. coli* recA mutants. One such mutant was *rad51*.

*rad51* mutants of *S. cerevisiae* were shown to be defective in the repair of DNA damage caused by methyl-methane sulphonate and ionising radiation, and were defective in spontaneous or induced mitotic recombination (Shinohara et al., 1992). Cloning and sequencing revealed that *S. cerevisiae* RAD51 (scRAD51) is a homologue of the prokaryotic protein RecA (Aboussekhra et al., 1992; Shinohara et al., 1992). Since these initial findings, several *RAD51* homologues have been identified in yeast, chicken and mammals.

Biochemical experiments using purified scRAD51 give indications about the molecular mechanism of its crucial role in both mitotic and meiotic recombination, and in the repair of DNA double-stranded breaks. scRAD51 is a DNA-dependant ATPase that coats ssDNA to form a helical filament that is structurally very similar to the RecA filament (Ogawa et al., 1993) and is
proposed to scan the genome for a homologous sequence during recombination. scRAD51 has been shown to mediate DNA pairing and initiate strand exchange in a similar way to RecA, but showing opposite polarity of strand exchange (Sung, 1994; Sung and Robberson, 1995; Sung and Stratton, 1996), although some results suggest transfer in either direction (Namsaraev and Berg, 1997). The scRAD51-ssDNA filament interacts with dsDNA to form a joint molecule. RPA (replication protein A), a heterotrimeric single-strand binding complex, helps at this stage (Sugiyama et al., 1997; Sung, 1994; Sung and Robberson, 1995) by reducing secondary structure, although incubation of ssDNA with RPA and scRAD51 simultaneously results in less strand exchange (Sung, 1997). scRAD52 seems to enable filament formation by scRAD51 in the presence of RPA and ssDNA.

The mammalian Rad51 has very similar functions to the yeast RAD51 and shows 76% identity at the amino acid level (Ogawa et al., 1993). The human testis RAD51 protein is also a structural homologue of RecA. It forms filaments on ssDNA and has been shown to promote homologous pairing and strand transfer reaction in vitro in an ATP-dependant manner. The protein binds both ssDNA and dsDNA but binding to dsDNA is stronger compared to the bacterial RecA protein (Baumann et al., 1996; Benson et al., 1994). The ATPase activity of hRAD51 has a $K_{\text{cat}}$ of 0.16 ATP/min in the presence of ssDNA (Baumann et al., 1996), which is significantly lower than that of the E. coli RecA at 30 ATP/min (Roca and Cox, 1990). Like scRAD51, the hRAD51-dependent joint molecule formation between circular ssDNA and linear duplex DNA was stimulated by RPA reducing secondary structure but RPA also competes with Rad51 for ssDNA binding (Baumann and West, 1997).

Mammalian RAD51 an essential gene in mice, (Tsuzuki et al., 1996) has been shown to interact with p53 (Buchhop et al., 1997; Sturzbecher et al., 1996) and the tumour suppressors BRCA1 (Scully et al., 1997) and BRCA2 (Sharan et al., 1997; Wong et al., 1997).
1.21.2 DMC1

DMC1 was first identified in S. cerevisiae as a meiotic RecA homologue (Bishop et al., 1992). Mouse and human DMC1 homologues were subsequently found showing 54% sequence identity to the yeast protein (Habu et al., 1996). DMC1 and RAD51 are a phylogenetically distinct subclass of genes homologous to RecA, as discussed in (Stassen et al., 1997).

In yeast DMC1 is required to convert DNA double strand break recombination intermediates into homologous joint molecules. Mutation in the gene leads to defects in reciprocal recombination, abnormal formation of synaptonemal complexes, accumulation of DSB recombination intermediates, and arrest in late meiotic prophase (Bishop et al., 1992; Li et al., 1997; Schwacha and Kleckner, 1997).

The gene product of the human DMC1 is expressed only in meiotic tissue although a shortened form exists in mouse somatic tissue (Habu et al., 1996). The mouse DMC1 gene is expressed in meiosis and is detected from leptotene to zygotene in spermatocytes. DMC1 is required for homologous synapsis of chromosomes in meiosis (Yoshida et al., 1998). hDMC1 has ATPase activity and ssDNA binding activity (Li et al., 1997).

1.21.3 RAD52

RAD52 is required in DNA repair and for all homologous recombination events (reviewed in (Game, 1993)). Mutations in the gene lead to extreme X-ray sensitivity (Resnick, 1969), inability to repair DSBs (Resnick and Martin, 1976), and decreased spontaneous and induced mitotic recombination (Prakash and Baquer, 1980). Expression of the scRAD52 is transcriptionally induced by entry of cells into meiosis (Cole et al., 1989). Rad52p binds ssDNA, mediates DNA strand annealing and increases the co-operativity of Rad51 binding to ssDNA, (Mortensen et al., 1996) stimulated by RPA (Shinohara et al., 1998; Sugiyama et al., 1998; Sung, 1997). Rad52p enhances dissociation of RPA from ssDNA and promotes RPA transfer to the displaced strand preventing reversal of strand exchange (New et al., 1998; Shinohara and Ogawa, 1998).
Rad52p interacts with the middle subunit of RPA (Shinohara et al., 1998). In addition, Rad52 increases the annealing rate of complementary ssDNA and could help Rad51 initiate joint molecule formation. Rad52p forms ring structures *in vitro* that interact with DNA (Shinohara et al., 1998). Rad52p also interacts with Rad51p (Donovan et al., 1994; Milne and Weaver, 1993; Schild, 1995; Shinohara et al., 1992). Rad52p does not catalyse the invasion of dsDNA by ssDNA.

hRad52 shows similar properties to the *S. cerevisiae* homologue. Both homologues enhance dissociation of RPA from ssDNA and promotes RPA transfer to the displaced strand preventing reversion of strand exchange (Benson et al., 1998). hRad52 binds directly to linear duplex DNA ends forming complexes that can be visualised by electron microscopy. These complexes stimulate ligation of linear duplex DNA with blunt ends and cohesive ends (Van Dyck et al., 1999). hRad52 protein also promotes single-strand annealing of complementary strands of DNA between single-stranded DNA and double-stranded DNA (Reddy et al., 1997).

### 1.21.4 RAD53

Identified as a member of the RAD52 epistasis group, RAD53 has an essential cell-cycle checkpoint function that controls cell-cycle progression in response to DNA damage (Neecke et al., 1999), rather than a direct role in the mechanism of recombinant DNA molecule formation.

### 1.21.5 RAD54

RAD54 exhibits homology to a group of proteins that may all encode DNA helicases. The exact function of Rad54 in not known but it does interact with Rad51 in humans (Golub et al., 1997) and in yeast (Clever et al., 1997) and has been shown to assist scRad51 in promoting homologous pairing between ssDNA and dsDNA (Petukhova et al., 1998). scRAD54 and hRAD54 belong to the SNF2/SW12 family of chromatin remodelling proteins (Emery et al., 1991;
Schild et al., 1992) that are DNA-dependent ATPases. scRAD54 is transcriptionally induced by entry of cells into meiosis (Cole et al., 1989).

A second homologue of scRAD54 - RDH54 has been identified in *S. cerevisiae* but its exact function is not known (Klein, 1997; Shinohara et al., 1997).

Human RAD54 contains a helicase motif, but no helicase activity has been demonstrated (Swagemakers et al., 1998).

Two *rad54* knockout cell lines have been reported, they are both highly sensitive to ionising radiation but showed varying degrees of immunoglobulin gene conversion. In *rad54*<sup>−/−</sup> cells derived from the chicken cell line DT40, targeted integration of DNA was two orders of magnitude lower, suggesting double-strand break repair and homologous recombination are linked (Bezzubova et al., 1997). However, a mouse knockout suggested the gene was not needed to generate functional immunoglobulin and T-cell receptor genes (Essers et al., 1997).

### 1.21.6 RAD55 and RAD57

In budding yeast RAD55 and RAD57 are also a RecA homologues, showing homology to RAD51 (Kans and Mortimer, 1991; Lovett, 1994). Both contain a potential nucleotide-binding domain similar to that in RAD51 (Heyer, 1994). RAD55 and RAD57, which form a heterodimer, do not appear to participate directly in strand exchange but may assist this process as they are reported to have a similar role to the bacterial RecO and RecR proteins (Sung, 1997). RecO and RecR help RecA assemble onto ssDNA in the presence of SSB (Shan et al., 1997; Umezu and Kolodner, 1994). Both RAD55 and RAD57 contain Walker-type ATPase motifs and mutations in the RAD55 Walker motif affect recombination but mutations in the RAD57 Walker motif do not (Johnson and Symington, 1995). Over-expression of RAD51 can compensate for the absence of RAD55 or RAD57 (Hays et al., 1995).
1.21.7 RAD59

RAD59 is a homologue of RAD52 in *S. cerevisiae* required for RAD51-independent recombination (Bai and Symington, 1996).

1.21.8 RAD50/MRE11/XRS2

Nine genes responsible for the production of double-strand breaks have been identified in *S. cerevisiae* by null mutants. Among these is *Spo11*, responsible for the meiotic DNA cleavage (Keeney et al., 1997). Three of these, *MRE11*, *RAD50* and *XRS2*, have identical phenotypes. A common feature of RAD50 and MRE11 is their nuclease activity.

RAD50 is an ATP-dependent binding protein which contains a purine nucleotide-binding domain (Raymond and Kleckner, 1993). The DNA binding is ATP-dependent. MRE11 is a 3'-5' exonuclease that facilitates the production of double strand breaks together with Rad50 and XRS2 (Pauli and Gellert, 1998). hMre11 also cleaves DNA hairpins, a function also catalysed by RAG1 and RAG2 (Gellert, 1997) and SbcC/D (Connelly et al., 1998).

The biochemical properties of these proteins may vary *in vivo* when all recombination proteins under their respective control mechanisms are present.

MRE11, RAD50, and XRS2 have multiple functions *in vivo*. They are involved in both pathways of DSBR, via homologous recombination and NHEJ. During meiosis they are involved in the DSB formation and resecting of the double-strand ends. Their other functions include telomere maintenance, removal of Spo11 in meiosis and checkpoint regulation (reviewed in (Haber, 1998)). Rad32, the *S. pombe* homologue of Mre11, is also required for DNA repair, meiotic recombination, NHEJ and telomere length maintenance (Wilson et al., 1999).

The probable functions of these genes in recombination are summarised in figure 1.21.8.
Figure 1.21.8. Schematic outline of protein functions in DSBR by homologous recombination. DSBs can be processed by the meiotic proteins Rad50p, Mre11p, and Xrs2p. The production of a 3' single-stranded tail can be processed by Rad51p, Rad52p, Rad54p, Rad55p, Rad57p, and RP-A protein. The single-strand tail can invade homologous duplex DNA leading to joint molecule formation. The crossover results in heteroduplex DNA that can be extended by branch migration. Resolution, DNA synthesis and ligation complete the repair process.
1.22 Recombination and carcinogenesis

Rad51p has been implicated to associate with the tumour suppressors BRCA1 (Scully et al., 1997) and BRCA2 (Sharan et al., 1997; Wong et al., 1997) suggesting defective homologous recombination could lead to tumour formation. Recently, mutations at functional regions of the Rad54p as a result of aberrant splicing were shown in primary cancers (Matsuda M and Yasutomi M, 1999).

A human homologue of RecQ, a prokaryotic DNA helicase, has been found and shown to be responsible for the symptoms of Bloom’s Syndrome, a genetic disorder accompanied by chromosome instability and radiation sensitivity (Ellis et al., 1995).

Nijmegen breakage syndrome has been shown to be due to a mutation in the NBS1 gene that is the human homologue of Xrs2 and is part of a Mre11/Rad50 complex. Patients show an increased incidence of cancer (Carney et al., 1998; Matsuura et al., 1998; Varon et al., 1998).

From the above brief description of the genes involved in homologous recombination it becomes clear that the eukaryotic homologues could have similar enzymatic activities to the prokaryotic proteins. RAD50 is a homologue of SbcC, one of the two polypeptides in the E. coli ATP-dependent ds-DNA exonuclease SbcCD (Connelly et al., 1997), and Mre11 is a homologue of SbcD (Sharples and Leach, 1995). In addition, RecA structural homologues have also been found in Archaea and are likely to be functional homologues (Kawarabayasi et al., 1998; Sandler et al., 1996; Woods and Dyall-Smith, 1997). Dominant recombination defective RAD51 mutations have been found in residues conserved in numerous bacterial RecA proteins (Chanet et al., 1996). Several proteins show similarities structurally and/or functionally to the prokaryotic protein RecA and they show us that a complex picture is beginning to emerge where the proteins may have evolved from a single gene.
Although homologues of prokaryotic recombination proteins have been identified, the eukaryotic system does differ greatly from the prokaryotic system in its complexity. There appears to be a growing number of proteins that have multiple functions \textit{in vivo} and readily form large complexes. The RAD genes which have been identified are all involved in stages of recombination that precede the formation of heteroduplex DNA and Holliday junction formation. The diversification of the RAD genes could be extended to genes functioning in the late stages of recombination. These genes are yet to be identified.

In summary, the eukaryotic activities produce recombinogenic single-stranded DNA tails, Rad51 forms nucleoprotein filaments on single-stranded DNA that are structural and functional homologues of the RecA filament. It appears that the RecA pathway has been conserved in evolution. The Holliday junction is a crucial intermediate in this pathway.

1.23 Description of the thesis

The late stages of recombination, branch migration and resolution of the Holliday junction, are well reported in prokaryotes, this is not the case for recombination in eukaryotes. Identifying eukaryotic proteins that catalyse the late stages of recombination is imperative for the understanding of recombination. With this in mind screening for these activities was initiated.

A Holliday junction resolvase was identified in cells of \textit{S. pombe}. A putative \textit{S. pombe} homologue of the \textit{S. cerevisiae} mitochondrial CCE1, identified in the sequence database, was cloned and purified. The purified enzyme had very similar biochemical properties to the cellular activity, which was most likely the mitochondrial enzyme. To identify a possible nuclear resolvase a \textit{S. cerevisiae} strain deleted for the mitochondrial resolvase activity was used to continue to look for resolvase activities. A second resolvase activity was identified and characterised. Screening of mammalian extracts also identified the presence of recombination proteins in fractionated extracts.
2. METHODS

Standard molecular biology protocols were followed for all DNA manipulations (Sambrook et al., 1989).

2.1 Strains and plasmids

Eukaryotic cells

HeLa cells were kindly cultured and supplied washed by Dr. Mina Edwards.

Yeast

\textit{Saccharomyces cerevisiae}

SK19 (\textit{MAT\alpha}, \textit{leu2}, \textit{trp1}, \textit{ura3}, \textit{his3}, \textit{ade2}, \textit{nuc1::LEU2}, \textit{cce1\Delta::LEU2}) (Kleff et al., 1992)

SK20 (\textit{MATa}, \textit{leu2}, \textit{trp1}, \textit{ura3}, \textit{nuc1::LEU2}, \textit{cce1\Delta::LEU2}) (Kleff et al., 1992)

AK47 (\textit{MAT\alpha\ MATa}, \textit{leu2/leu2}, \textit{trp1/trp1}, \textit{ura3/ura3}, \textit{his3/HIS3}, \textit{ade2/ADE2}, \textit{nuc1::LEU2/nuc1::LEU2}, \textit{cce1\Delta::LEU2/cce1\Delta::LEU2})

\textit{S. cerevisiae} yeast strains SK19 and SK20 were a kind gift from Dr. R Sternglanz (SUNY at Stony Brook USA) and Prof. B. Kemper (Institute for Genetics, University of Cologne, Germany). Strain BJ2168 was a gift from Prof. P. Piper.

\textit{Schizosaccharomyces pombe}

Wild type 972h

Plasmids

pDEA-7Zf(+) (3.0 kb) was constructed by replacing the \textit{Sca I-Bsa I} fragment of pGEM-7Zf(+) (Promega) with the \textit{Sca I-Bsa I} fragment of pBR322 (Shah et al., 1994). pDEA2 (3.543 kb) contains a 643 nucleotide fragment of pACYC184 (nt 2057-2700) ligated to the 2.9 kb \textit{Sph I – Nsi I} fragment of pGEM-7Zf(+) (Adams et al., 1994). The supercoiled plasmids pDEA2 and pDEA-7Zf(+) were
a kind gift from Dr. D. E. Adams. pET21a(+) was purchased from Novagen and used according to their recommended protocols.

2.2 Enzymes and reagents

\textit{E. coli} RuvA and RuvB were purified by Dr. K. Hiom according to published protocols (Tsaneva et al., 1992). \textit{E. coli} RuvC was a gift from Dr. F. Osman purified according to published protocol (Dunderdale et al., 1994). RecA was purified as described in section 2.13.2. YDC2 was purified as described in section 2.13.1, essentially as published (Oram et al., 1998). \textit{S. cerevisiae} CCE1 protein was a gift from Dr. M. White (CRC Nucleic Acid Structure Group, University of Dundee). Anti-hRAD51 serum and hRAD51 were a gift from Dr. S.C. West (Imperial Cancer Research Fund, South Mimms). Enzymes were diluted to working concentrations in enzyme dilution buffer.

Vent®DNA polymerase, double-stranded virion φX174DNA and restriction enzymes were obtained from New England BioLabs. T4 polynucleotide kinase, terminal deoxynucleotidyl transferase (calf thymus), T4 DNA ligase, the Klenow fragment of DNA polymerase, φX174 form I DNA digested with \textit{Hinf I}, 1 kb DNA ladder, lambda DNA \textit{Hind III} digest markers and proteinase K were obtained from Promega. All enzymes were used according to their supplier’s instructions.

Reagents were obtained from BDH, Fisons, or Sigma, unless otherwise stated, and were of analytical grade. Ammonium persulphate molecular biology certified, bromophenol blue, Coomassie Brilliant Blue R-250, protein standards, molecular biology certified SDS, xylene cyanol, Bradford reagent, and Macro-Prep®DEAE, CM and High Q chromatography media were obtained from Bio-Rad. Acrylamide:bisacrylamide 40% w/v [19:1] and 30% [29:1] solutions were from NBL Gene Sciences Limited. IPTG and X-gal were from Melford Laboratories Limited. BSA was from Boehringer Mannheim. Phenol obtained from Sigma was saturated with 10 mM Tris-HCl (pH 8.0), 1 mM EDTA.

68
Bacto®Tryptone, Bacto®Peptone, yeast extract, yeast nitrogen base without amino acids and ammonium sulphate, and yeast nitrogen base without amino acids were from Difco. Luria agar and Terrific Broth were from Gibco BRL.

Phosphocellulose was from Whatman, and Sepharose CL2B-300 was from Sigma. All other resins used, unless otherwise mentioned, were from Pharmacia.

Microcon concentrators were purchased from Amicon. Schleicher & Schuell Bio-Trap-Membranes BT1 and BT2 were purchased from Western Laboratory Services Limited. Qiagen, Qiaex II, and Qiaquick DNA purification kits were purchased from Qiagen.

Unlabeled synthetic oligonucleotides were obtained from Genosys. \( [\gamma^{32}\text{P}]\text{ATP} \) and \( [\alpha^{32}\text{P}]\text{CTP} \) was from New England Nuclear, and dideoxy\( [\alpha^{32}\text{P}]\text{ATP} \) was from Pharmacia Amersham Biotech. X-OMAT XAR-5 and BioMax MS-1 film was purchased from Kodak.

### 2.3 Buffers and solutions

#### 2.3.1 DNA buffers

**Bacteriophage precipitation solution**: 20% (w/v) polyethylene glycol 8000, 3.75 M ammonium acetate. Made by adding equal volumes of 40% (w/v) polyethylene glycol 8000 and 7.5 M ammonium acetate.

**Binding buffer**: 50 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), 1 mM DTT, 100 µg/ml BSA, 5% (v/v) glycerol, 2.5 µg/ml \( \phi X174 \) form I DNA digested with \( \text{Hind} I \).

**Denaturing loading buffer**: 80% (v/v) de-ionised formamide, 2 mM EDTA, 0.2% (w/v) bromophenol blue, 0.2% (w/v) xylene cyanol in TBE.

**Sample-loading buffer 6X**: 0.25 % (w/v) Bromophenol blue, 0.25 % (w/v) xylene cyanol FF, 30 % glycerol (v/v).

**P1 buffer**: 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 µg/ml RNaseA.

**P2 buffer**: 200 mM NaOH, 1% (w/v) SDS.
P3 buffer: 5 M Potassium acetate, 11.5% (v/v) glacial acetic acid.

SSC 20X: 3 M NaCl, 0.3 M sodium citrate, adjusted to pH 7.0 with NaOH

TAE: 40 mM Tris base, 1 mM EDTA, 0.11% (v/v) glacial acetic acid.

TBE: 89 mM Tris, 89 mM boric acid, 2 mM EDTA.

TE: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0).

2.3.2 Enzyme buffers:

Branch migration buffer: 20 mM Tris-HCl (pH 8.0), 10 mM MgCl$_2$, 1 mM ATP, 1 mM DTT, 100 μg/ml BSA, 10 mM phosphocreatine, 5 U/ml phosphocreatine kinase.

Calcium branch migration buffer: 50 mM Tris-HCl (pH 8.0), 15 mM CaCl$_2$, 1 mM DTT, 50 μg/ml bovine serum albumin, (1 mM ATP), (0.25 mM ATP$_y$S).

Cutting buffer 1: 50 mM Tris-HCl (pH 8.0), 15 mM MgCl$_2$, 1 mM DTT, 100 μg/ml BSA. Unless specified, cutting buffer 1 was used.

Cutting buffer 2: 50 mM Tris-HCl (pH 8.0), 10 mM MgCl$_2$, 50 mM KCl, 1 mM DTT, 100 μg/ml BSA.

Enzyme dilution buffer: 20 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), 0.5 mM DTT, 100 μg/ml BSA, 150 mM NaCl, 20% (v/v) glycerol.

RecA strand exchange buffer: 20 mM Tris-HCl (pH 7.5), 15 mM MgCl$_2$, 2 mM DTT, 2 mM ATP, 100 μg/ml BSA.

Stop buffer 5X: 100 mM Tris-HCl (pH 7.5), 200 mM EDTA (pH 8.0), 2.5% (w/v) SDS, 10 mg/ml proteinase K.

2.3.3 Protein buffers:

Coomassie stain: 10% (v/v) glacial acetic acid, 10% (v/v) propan-2-ol, 0.1% (w/v) Coomassie brilliant blue.

Destain: 10% (v/v) glacial acetic acid 10% (v/v) propan-2-ol.

Hypotonic lysis buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 5 mM DTT.

P buffer: 20 mM Potassium phosphate (pH 6.8), 10% (v/v) glycerol, 0.1 mM EDTA (pH 8.0), 1 mM DTT.

R buffer: 20 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 1 mM DTT, 1 mM EDTA.
**SDS-PAGE Reservoir buffer:** 25 mM Tris base (pH 8.8), 190 mM glycine, 0.1% (w/v) SDS.

**SDS loading buffer 2X:** 125 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 2 mM DTT.

**SDS buffer A 2X:** 750 mM Tris-HCl (pH 8.8), 0.2% (w/v) SDS.

**SDS buffer B 2X:** 250 mM Tris-HCl (pH 6.8), 0.2% (w/v) SDS.

**Sucrose/glycerol buffer:** 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 2 mM DTT, 25% (w/v) sucrose, 50% (v/v) glycerol.

**TBS:** 10 mM Tris-HCl (pH 8.0), 150 mM NaCl.

**TBST:** 0.05% (v/v) Tween® 20 in TBS.

**TED:** 20 mM Tris-HCl (pH 8.0), 1 mM EDTA 1 mM DTT.

**Western blot transfer buffer:** 39 mM glycine, 48 mM Tris base, 0.037% (w/v) SDS (electrophoretic grade), 20% (v/v) methanol.

### 2.3.4 Media:

**LB media:** 1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl (2% (w/v) Bacto-agar for plates).

**Terrific Broth:** 4.7% (w/v) Terrific Broth

**M9 plates:** M9 salts (2X)/500 ml: 6 g Na₂HPO₄, 3 g KH₂PO₄, 0.5 g NaCl, 1 g NH₄Cl, Dissolved in H₂O and adjusted to pH 7.4 using NaOH. Agar/H₂O (2X)/500 ml: 15 g/500 ml. Autoclaved separately. In a 60-65°C water bath 500 ml 2X salts and 2X agar are mixed followed by the addition of: 2 ml 1 M MgSO₄, 10 ml 20% (w/v) glucose, 0.2 ml 0.5 M CaCl₂, 1 ml 0.1 % (w/v) vitamin B1.

**Yeast minimal media:** 0.67% (w/v) Bacto-yeast nitrogen base (without amino acids), 2% (w/v) glucose (2% (w/v) Bacto-agar for plates).

**YE media:** 0.5% (w/v) yeast extract, 3% (w/v) glucose, (2% (w/v) Bacto-agar for plate).

**YPG media:** 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose (2% (w/v) Bacto-agar for plate).
2.3.5 Buffers for alpha-structures:

**Sepharose column buffer:** 20 mM Tris-HCl (pH 7.5), 1 mM DTT, 5 mM MgCl₂, 100 µg/ml BSA.

**Neutral density gradient sucrose solutions:** 5% (w/v) or 20% (w/v) sucrose (ultra DNase, RNase free), in 10 mM Tris-HCl (pH 7.5), 1 M NaCl, 10 mM EDTA.

**TNE:** 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA.

2.4 Quantitation of DNA and protein

2.4.1 DNA

DNA concentrations were determined by measuring the absorption at 260 nm in quartz cuvettes using a Philips PU8720 spectrophotometer. Calculations were based on the following spectrophotometric conversions, 1A\textsubscript{260} unit of double stranded DNA = 50 µg/ml, and 1A\textsubscript{260} unit of single stranded DNA = 33 µg/ml. Concentrations are expressed in moles of nucleotide residues.

2.4.2 Protein

Protein was quantified using a Bio-Rad Protein Assay Kit, which is based on the Bradford protein assay (Bradford, 1976) and was used according to the manufacturers recommended protocol. Protein solutions were made to a volume of 800 µl with H\textsubscript{2}O and Bradford dye reagent concentrate added to 1 ml. Colour was developed for 15 minutes at room temperature. Absorbance was measured at 595 nm. BSA was used to produce a standard curve from which unknowns could be read.

2.5 Molecular mass standards

**1kb DNA ladder (Promega):**
10k, 8k, 6k, 5k, 4k, 3k, 2.5k, 2k, 1.5k, 1k, 750, 500, 250, 253 bp.

**Lambda DNA-\textit{Hind} III Digest (Promega):**
23130, 9416, 6557, 4361, 2322, 2027 bp
SDS-PAGE markers, low range (Bio-Rad):
Phosphorylase B (rabbit muscle) 97,400 Da, bovine serum albumin 66,200 Da, hen egg white ovalbumin 45,000 Da, bovine carbonic anhydrase 31,000 Da, soybean trypsin inhibitor 21,500 Da, hen egg white lysozyme 14,400 Da.

Gel filtration markers (Bio-Rad):
Bovine thyroglobulin (670,000 Da), bovine gamma globulin (158,000 Da), chicken ovalbumin (44,000 Da), horse myoglobin (17,000 Da) and vitamin B-12 (1,350 Da).

2.6 Electrophoresis

2.6.1 Agarose gel electrophoresis
DNA samples were de-proteinised by the addition of 0.2 volumes 5X stop buffer and incubation at 37°C for 10 minutes. Sample loading buffer, 0.2 volumes was added, and samples were separated on 1.0 - 1.5% (w/v) agarose gels in TAE buffer, run using the Bio-Rad wide mini sub cell apparatus. Electrophoresis was at 4-8 V/cm for 2-4 hours in TAE buffer with buffer recirculation at room temperature. Agarose gels contained 0.5 µg/ml ethidium bromide. DNA was visualised by transillumination under short wavelength UV and photographed using the ‘uvi tec’ imaging system. Radiolabelled bands were visualised by autoradiography.

2.6.2 Denaturing PAGE
Reaction samples containing labelled DNA were incubated at 95°C for 3 minutes in denaturing loading buffer. Gels were poured and run in the Bio-Rad Sequi Gen sequencing cell (38x30cm) or using the Cambridge Electrophoresis Limited protein analysis and DNA sequencing apparatus (20x16cm). Gels contained 8% polyacrylamide (19:1, acrylamide:bisacrylamide supplied as a 40% (v/v) solution) and 7M urea in TBE buffer. Polymerisation was initiated with 0.1% (w/v) ammonium persulphate and 0.1% (w/v) TEMED. Gels were
pre-run for 1 hour at 65 W or 25 W to reach 50°C and samples were run at 65W or 25 W for 40-60 minutes. Samples for high resolution mapping of cleavage sites were analysed on 12% sequencing gels. Gels were fixed in 10% (v/v) acetic acid 10% (v/v) methanol for 10 minutes, dried and analysed by autoradiography or phosphorimager.

2.6.3 PAGE

Assays for Holliday junction processing activities were stopped by the addition of 5X stop buffer and incubation for 10 minutes at 37°C. 6X sample-loading buffer was added and samples run on 6% polyacrylamide gels poured using the Bio-Rad Mini PROTEAN II and polymerised as above. Gels were run for 90 minutes at 6-8 V/cm. Gels were dried and visualised as described for denaturing PAGE.

2.6.4 SDS-PAGE

Proteins were analysed by SDS-PAGE using a method based on Laemmli (Laemmli, 1970). Protein samples were heated to 100°C for 3 minutes in an equal volume of 2X SDS gel-loading buffer. Gels were poured and run in both Bio-Rad Mini PROTEAN II and PROTEAN II xi apparatus. Running gels were 12 or 15% in 1X SDS buffer A prepared from a 30% stock solution (29:1 acrylamide:bisacrylamide). Stacking gels contained 6% polyacrylamide in 1X SDS buffer B prepared from a 30% stock solution (29:1 acrylamide:bisacrylamide). Polymerisation was as described in section 2.6.2. Samples were run in SDS-PAGE reservoir buffer at 5 V/cm through the 6% stacking gel and at 10 V/cm through the running gel. Bio-Rad low range molecular weight standards were used for the production of a molecular weight standard curve from which unknowns could be read. Proteins were stained with Coomassie stain for 1 hour. Background stain was removed by several changes of destain solution. Gels for photography were stored in 10% (v/v) glacial acetic acid. After photography gels were dried between 2 sheets of cellophane for storage.
2.6.5 Autoradiography and phosphorimager analysis

Gels containing radiolabelled substrate, both agarose and polyacrylamide, were dried on Whatman 3MM filter paper exposed to film and developed using an X-OGRAPH compact x2 film processor. Alternatively, gels were exposed to BAS-MP 2040 imaging plates for a maximum of 24 hours, and analysed in the Fujifilm FLA2000 using Fujifilm image gauge V3.01 software.

2.7 DNA manipulations

2.7.1 Electroelution of DNA fragments from polyacrylamide gels

The DNA bands of interest were excised from the polyacrylamide gel and the trimmed gel fragment was placed in a Bio-Trap electroelution chamber (Schleicher & Schuell). The electroelution chamber and Bio-Trap-Membranes BT1 and BT2 were used as recommended by the manufacturer. DNA was electroeluted for 1 hour at 100 V in 0.5 X TBE buffer. After electroelution the current was reversed for 1 minute and the DNA was recovered from the collection chamber by pipette. DNA samples were dialysed against either H\textsubscript{2}O or TE buffer. All steps were carried out at 4°C.

2.7.2 Phenol chloroform extractions and ethanol precipitations

Equal volumes of solvent were added to samples and mixed. The aqueous phase was separated by centrifugation and collected. Chloroform contained isoamyl alcohol at a ratio of 24:1 (v/v). Other solvents used were butan-2-ol and diethyl ether. Phenol-chloroform 1:1 (v/v) extractions were repeated until no interphase was seen between the aqueous and organic phase.

DNA was precipitated from the aqueous phase by the addition of 1/10 volume 3 M sodium acetate (pH 7.0 for dsDNA, or pH 5.2 for ssDNA) and 2.5 volumes 100% ethanol. The ethanol solution containing the DNA was kept at -20°C for at least 30 minutes. Precipitated DNA was collected by centrifugation, washed with 70% ethanol and dried at 37°C. Dried DNA was dissolved in TE buffer and stored at -20°C.
2.7.3 Preparation of genomic DNA from *S. pombe*

Wild type *S. pombe* at $5 \times 10^7$ cells per ml were pelleted at 3000g for 5 minutes at 20°C. Pellets were resuspended in H$_2$O and centrifuged again as before. Cells were resuspended in 1.2 M D-sorbitol, 50 mM citrate buffer pH 5.6, 50 mM sodium phosphate, before the addition of 5 mg/ml Sigma lysing enzyme from *Trichoderma harzianum*. The cells were incubated at 37°C for 2 hours. Aliquots were checked for spheroplast formation (spheroplasts were lysed in 0.3 M mannitol, but remained intact in 1.2 M mannitol). Approximately 80% of the cells were lysed after 2 hours. After digestion spheroplasts were centrifuged at 1000 g for 5 minutes and resuspended in TE pH 7.5 followed by the addition of SDS to 1% (w/v). Cells were incubated at 65°C for 3 minutes to lyse. 5M potassium acetate was added to a final concentration 1.5 M and the solution was incubated for 1 hour on ice. Debris was pelleted at 3000 g for 15 minutes at 4°C. 0.3 volumes ice-cold isopropanol was added to the supernatant, which was incubated on ice for 5 minutes. Nucleic acids were pelleted by centrifugation at 17000 g for 15 minutes at 4°C. The partially dried pellet was resuspended in 5X TE pH 7.5 and RNase was added to a final concentration of 0.02 mg/ml. The reaction mixture was incubated for 1 hour at 37°C. After digestion protein was removed by the addition of SDS to 0.5% (w/v) and proteinase K to a final concentration of 0.05 mg/ml, and incubation for 30 minutes at 50°C. The solution was extracted with phenol-chloroform. DNA was precipitated by the addition of 0.1 volumes 5 M NaCl and 2.5 volumes 100% ethanol. The DNA solution was gently rocked and DNA was spooled from the solution using a glass rod. DNA was washed with 70% ethanol dried and dissolved in H$_2$O. A sample was loaded onto a 0.8% agarose gel which showed that the DNA was in the order of approximately 23000 bp in size. The resuspended DNA was aliquoted into 10 µl aliquots and stored at -20°C.

2.7.4 PCR using genomic DNA

PCR reactions were set up using Vent® DNA polymerase in a total volume of 50 µl (2 U Vent polymerase, 5 µl 10X ThermoPol buffer, 1 µl dNTP mix (10 mM of each dNTP), primer 1, 50 pmol, primer 2, 50 pmol, genomic DNA $>10^4$
copies of target sequence, 50 µl mineral oil). 30 rounds of PCR were programmed into a Hybaid thermocycler. Step one, 5 minutes at 95°C for one cycle. Step two, 30 cycles of 1 minute at 95°C, 1 minute at 47°C and 2 minutes at 74°C. Step three, the last round of PCR, was 1 minute at 95°C, 1 minute at 47°C and 5 minutes at 74°C for one cycle. Amplification was checked by running 5 µl of the PCR reaction next to molecular weight markers on a 1% agarose gel.

2.7.5 Purification of PCR products from agarose

The PCR products were concentrated using Amicon microconcentrators and electrophoresed on 1% agarose gels. The product was readily visible by UV transillumination of the ethidium bromide-stained gel. The correct size band was excised from the gel and purified using Qiaex II gel extraction kit according to the manufacturer’s recommendations.

2.8 Preparation of DNA substrates

2.8.1 Preparation of synthetic Holliday junction

The sequences of oligonucleotides used are given in Table 2.8. The synthesised oligonucleotides were gel-purified using the Bio-Rad PROTEAN II apparatus with 1.5 mm spacers. 8% polyacrylamide gel was poured containing 7M urea in TBE. Oligonucleotides were heated to 95°C for 3 minutes and an equal volume of denaturing loading buffer was added. The gel was run at 300V for 4 hours at 4°C. Oligonucleotides were visualised by UV shadowing on a fluorescent plate using incident illumination with a long wavelength UV light source, excised from the gel, and electroeluted from the gel slice. If needed, the oligonucleotides were concentrated by precipitation with ethanol.

The oligonucleotides were dialysed against TE buffer and concentrations were determined by measuring the absorbance at 260 nm. Oligonucleotides used for sequencing were dialysed against H$_2$O.

The synthetic substrates were assembled from various combinations of oligonucleotides, as shown in table 2.8.
Table 2.8: Sequences of oligonucleotides used to make synthetic substrates. All sequences are shown 5' to 3'. Homologous regions are shown in bold.

<table>
<thead>
<tr>
<th>Four-way junction X12 (“mobile”): oligonucleotides 1, 2, 3 and 4</th>
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<tbody>
<tr>
<td><strong>1 (61-mer)</strong></td>
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<td></td>
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<tr>
<td><strong>2 (62-mer)</strong></td>
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<td></td>
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<tr>
<td><strong>3 (63-mer)</strong></td>
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<td></td>
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<tr>
<td><strong>4 (62-mer)</strong></td>
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<tr>
<th>Linear duplex L12: oligonucleotides 2 and 5</th>
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<tr>
<td><strong>5 (62-mer)</strong></td>
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<tr>
<th>Four-way junction XO (“static”): oligonucleotides 1, 6, 7 and 8</th>
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<tr>
<td><strong>6 (62-mer)</strong></td>
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<td></td>
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<tr>
<td><strong>7 (63-mer)</strong></td>
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<tr>
<td><strong>8 (62-mer)</strong></td>
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<th>Three-way junction Y0: oligonucleotides 1, 8 and 9</th>
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<tr>
<td><strong>9 (62-mer)</strong></td>
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<th>Linear duplex L0* (with 6 mismatched bases): oligonucleotides 2 and 10</th>
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<tr>
<td><strong>10 (62-mer)</strong></td>
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For each construct, 1 µg of one oligonucleotide was labelled at either the 5’ end with [γ-32P] ATP (3000 Ci/mmol, from New England Nuclear) using PNK, or the 3’ end with dideoxy [α-32P] ATP (3000 Ci/mmol, from Amersham) and terminal transferase. The labelling reaction was in a total volume 10 µl 1X Pharmacia’s One-phor-all buffer that included 1 µg oligonucleotide and 10 U PNK. After 30 minutes at 37°C the labelling reaction was stopped by the addition of 1 µl 0.5 M EDTA and incubation at 65°C for 10 minutes. Unincorporated label was removed using a G25 microspin column (Pharmacia). Annealing mixtures contained 4 µg of each of the unlabelled oligonucleotides and 1 µg of the labelled oligonucleotide in 1X SSC buffer. The annealing mixture was heated at 95°C for 3 minutes and then transferred to a water-bath at 68°C for 10 minutes. The waterbath was then switched off to allow slow cooling. SDS was added to the annealing mixture to 1.5% SDS, 6X gel loading buffer was added and the junction was purified by electrophoresis through 8% polyacrylamide for 2 hours at 300 V in 1x TBE. The junction band was excised from the gel, electroeluted and dialysed against TE buffer. Concentrations were determined by calculation of specific radioactivity using the DE81 paper binding method (Sambrook et al., 1989).

2.8.2 Preparation of Chi-DNA.

DNA Chi structures were prepared from plasmid intermediates formed in vivo by XerC-mediated site-specific recombination (McCulloch et al., 1994). E. coli strain RM40 and plasmid pSD115 (as described in (McCulloch et al., 1994)) were kindly supplied by Prof. D. Sherratt (University of Oxford). RM40/pSD115 cells were grown at 37°C in LB medium containing 100 µg/ml ampicillin and 50 µg/ml diaminopimelic acid to an A600 of 0.5. For induction of XerC expression isopropyl β-D-thiogalactoside (IPTG) was added to a concentration of 1 mM and the cells were harvested after one hour. Plasmid DNA was isolated using a Qiagen column, digested by Sty I and Sca I and fractionated by electrophoresis on 1% agarose gels. The Chi DNA band was excised, electroeluted and labelled at the Sty I ends using the Klenow enzyme.
and [α-\(^{32}\)P] dCTP (3000 Ci/mmol). The labelled Chi DNA was gel-purified again as above.

### 2.8.3 Preparation of gapped DNA.

The annealing reaction contained 180 µg linear pDEA-7Zf(+) mixed with (excess) 250 µg circular pDEA-7Zf(+) ssDNA in 3 ml 50% (v/v) formamide, 10 mM EDTA (pH 8.0). The annealing mixture was dialysed at room temperature for 2 hours against 200 ml 95% (v/v) formamide solution, for 2 hours against 200 ml 50% (v/v) formamide solution, for 2 hours against 200 ml TNE buffer and overnight against 200 ml TE. The annealing reaction was then analysed by 1.5% agarose gel electrophoresis.

The dialysed annealing reaction was applied to two 1.5% preparative agarose gels in 0.2 volumes 6X gel loading buffer. The preparative gels were run at 5-6 V/cm for 3-4 hours. The gel was stained for 25 minutes in ethidium bromide in TAE and viewed under long-wave UV. The gDNA was excised and gel slices were put in dialysis sacs containing a minimum volume of TAE and electroeluted overnight at 50 V. The following day current was briefly reversed and a small section of the gel stained to confirm complete electroelution.

The DNA solution was collected and was extracted once with 1 volume phenol, once with 1 volume chloroform, once with 1 volume diethyl ether, 4 times with 1 volume butan-1-ol and twice with 1 volume diethyl ether. The DNA solution was left to evaporate any remaining ether, and DNA was precipitated with ethanol.

The DNA precipitate was collected at 39000 g for 1 hour at 4°C. The pellet was dissolved in 500 µl TE and dialysed against 2 litres of TE at 4°C.

### 2.8.4 Extraction and Purification of pDEA-7Zf(+) from JM109

JM109 (recA1, supE44, endA1, hsdR17, gyrA96, relA1, thiΔ(lac-proAB)) harbouring plasmid pDEA-7Zf(+) was streaked onto LB agar plates containing
100 µg/ml ampicillin. One colony was inoculated into LB media containing ampicillin (100 µg/ml) and grown for 7 hours. The inoculum was then divided into 4 x 5 litre flasks containing 500 ml Terrific Broth containing ampicillin (100 µg/ml) and left to grow overnight with aeration at 37°C. Cells were collected at 5000 g for 30 minutes at 4°C.

The bacterial pellet was resuspended in 50 ml P1 buffer. 50 ml of P2 buffer was added to the suspension. And after 5 minutes at room temperature 50 ml of chilled P3 buffer added. The solution was incubated on ice for 30 minutes after which the solution was centrifuged at 10000 g for 45 minutes at 4 °C. The supernatant was removed and centrifuged for 30 minutes as before. The plasmid DNA was purified using a Qiagen-tip 2500. The final DNA pellet was dissolved in 800 µl TE.

2.8.5 Preparation of linear pDEA-7Zf(+) for annealing

200 µg pDEA-7Z was digested with Bsa I in NEB buffer 4. Linearisation was checked on a 0.8 % agarose gel. After complete digestion with Bsa I, the linearised plasmid was digested with Pst I. Digestion of the plasmid with both enzymes produced two fragments one 2727 bp and one 274 bp. The digest was extracted with phenol-chloroform, back extracted with TE, extracted with chloroform and ethanol precipitated. The DNA was dissolved in TE and applied to a 5 - 20 % sucrose density gradient and centrifuged at 26000 rpm for 16 hours at 4°C in the Beckman SW28 rotor. The gradients were pumped out from the bottom of the 40 ml polyallomer centrifuge tubes and 0.5 ml fractions were collected. 10 µl samples were assayed on a 0.8 % agarose gel. Fractions containing the linear plasmid DNA, were pooled and ethanol precipitated. The final pellet was resuspended in 300 µl TE.

2.8.6 Preparation of single-stranded DNA

Bacterial strain JM109/pDEA-7Zf(+) was streaked out on M9 minimal plates containing 100 µg/ml Ampicillin and 0.1% (w/v) glucose. A single colony was inoculated into 2 ml LB containing 0.1% (w/v) glucose and ampicillin (100
1 ml of this overnight culture was used to inoculate 50 ml LB media containing glucose and ampicillin. The culture was grown at 37°C. After 3.5 hours 20 ml of culture was inoculated with 2.6 ml of helper bacteriophage M13K07 (10^{10} pfu/ml) and incubation continued for a further 1.5 hours. 4 ml of the infected culture was then used to inoculate 4 x 400 ml of LB containing 0.1% (w/v) glucose and ampicillin (100 μg/ml). The cultures were grown overnight at 37°C.

The overnight cultures were then centrifuged twice at 5000 g at 4°C for 30 minutes and 20,000 U DNase I (Sigma) and 1 ml RNase A (10 mg/ml) were added to the supernatant (1600 ml). The supernatant was incubated at 37°C for 30 minutes after which 400 ml bacteriophage precipitation solution was added. The solution was kept on ice for 1 hour. The bacteriophage was pelleted by centrifugation at 11000 g for 15 minutes at 4°C. The pellet was resuspended in 20 ml TE and the ssDNA was purified by phenol-chloroform extraction. 7.5 M ammonium acetate (0.5 volumes) was added to the ssDNA solution and ssDNA was ethanol precipitated. The ssDNA pellet was dissolved in TE.

**2.8.7 3’ End-labelling of linear pDEA-7Zf(+)***

pDEA-7Z(+1) or pDEA2 were digested with Pst I and labelled in One-phor-all buffer (Promega) using [α^{32}P] ddATP and terminal deoxynucleotidyl transferase. Incubation was at 37°C for 90 minutes and the reaction was stopped by the addition of EDTA and SDS to 40 mM and 1% (w/v) final concentrations, respectively. Unincorporated label was removed by using S-400 micro-spin columns (Pharmacia). The reaction mix was extracted with phenol-chloroform and back extracted with 25 μl TE. The aqueous phases were pooled and applied to a second microspin column. The column eluent was made 50% (v/v) with 100% ethanol. The DNA stock was stored at 4°C.
2.8.8 Initial quantitation of labelled linear DNA and RecA required for production of alpha-structures

The method used in the production of alpha-structures is essentially as described by Müller and West (Muller and West, 1994).

A bulk RecA reaction was set up in RecA buffer, containing 560 ng gDNA, 11 μg RecA 10 mM phosphocreatine, and 5 U/μl phosphocreatine kinase. The reaction mixture was divided into 4 x 20 μl aliquots and incubated for 5 minutes at 37°C. Increasing concentrations of labelled linear plasmid DNA was added to each 20 μl aliquot. After 10 minutes at 37°C the reaction was stopped with 5X stop buffer. After 5 minutes at 37°C 6X GLB was added and samples were run on a 1.2% agarose gel. The volume of labelled linear plasmid DNA that gave the best yield of alpha-structures was used in subsequent experiments. A similar pilot experiment was performed keeping gDNA and linear DNA constant while varying the concentration of RecA to establish the optimum concentration of RecA. These experiments established the optimum conditions for the large-scale preparation of deproteinised alpha-structures.

2.8.9 Large scale purification of deproteinised α-structures

Alpha-structures were formed in RecA strand exchange buffer containing gDNA and RecA at concentrations determined as described previously. The reaction mixture was incubated for 5 minutes at 37°C after which labelled linear pDEA-7Zf(+) was added. Following incubation at 37°C for 8 minutes exactly the reaction was stopped by the addition of SDS and proteinase K to a concentration of 0.5% (w/v) and 2 mg/ml respectively, and was further incubated at 37°C for 15 minutes. The reaction mixture was applied to a Sepharose CL-2B column pre-equilibrated in 2 column volumes of Sepharose column buffer. The column was washed with Sepharose column buffer and 1 drop fractions were collected. The radioactivity of the fractions was monitored and the peak of fractions containing labelled substrate were pooled. The alpha-structures were stored at 4°C and used within 2-3 days.
2.9 Yeast culture

*S. pombe* cells were grown in YE media and *S. cerevisiae* were grown in YPG. Cells were streaked on YE or YPG agar plates from frozen cultures. Single colonies were used to inoculate 10 ml overnight cultures, incubated at 30°C with aeration. The overnight culture was then used to inoculate 1 litre of media in 2 litre flasks with 100μl. At timed intervals samples were removed and the cells counted using a haemocytometer. Cell culture was continued until late log phase of growth to construct a complete growth curve. Cell cultures could then be harvested at the required growth phase by monitoring the cell density.

2.10 Crude cell extracts from eukaryotes

2.10.1 Lysis of *S. pombe* and *S. cerevisiae*

*S. pombe* and *S. cerevisiae* BJ2168 strain (described in (Sorger and Pelham, 1988)) were both lysed using the same protocol. The cells were cooled on ice and harvested at 3000 g for 10 minutes washed with sterile water, and centrifuged again. Pellets were stored at -70°C if not used immediately.

The following extraction buffer was prepared: 25 mM Tris-HCl (pH 7.5), 15 mM MgCl2 100 mM NaCl, 2 mM NaF, 80 mM β-glycero-phosphate, 20 mM EGTA, 1 mM DTT, 0.1% TritonX-100. Immediately before use p-nitrophenyl phosphate and Na3VO4 were added to a final concentration of 15 and 1 mM, respectively. N-tosyl-L-phenylalanine chloromethyl ketone and, N-tosyl-L-lysine chloromethyl ketone (both at 50 μg/ml), aprotinin and bestatin (both at 2 μg/ml), leupeptin (0.5μg/ml), pepstatin (1 μg/ml) and 1 mM phenylmethylsulphonyl fluoride were added to the extraction buffer just before use and were present in all column and dialysis buffers.

Cells were broken in a pre-cooled Biospec bead beater using 0.5 mm glass beads, at a 1:1 ratio of beads to cell paste and 10 bursts of 10 seconds with 50 seconds cooling in between. This was repeated after 20 minutes cooling on ice. During the whole process the apparatus was kept on ice. Fresh protease
inhibitors were added and the extract was centrifuged at 10,000 g at 4°C for 20 minutes. This was followed by 60 minutes centrifugation at 4°C at 200,000 g. Fractions from all subsequent chromatography steps were concentrated 10-50 fold using microcon concentrators and assayed.

2.10.2 Lysis of cce1Δ S. cerevisiae strains and fractionation of the cell extract

The initial lysis of S. cerevisiae strain SK20 was the same as the lysis protocol described in the previous section with some modifications. cce1Δ strains were grown to a density of 1–3x10⁷ cells/ml in 8x1 litre cultures in YPG media at 30°C. Cells were harvested by centrifugation at 3,000 g for 5 minutes at 4°C, washed with H₂O, pelleted as before and stored at -70°C until needed. The cell paste was resuspended in extraction buffer (50 mM Tris-HCl (pH 7.5), 15 mM MgCl₂, 100 mM NaCl, 2 mM NaF, 80 mM β-glycero-phosphate, 20 mM EGTA, 1 mM DTT, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol) containing the following protease inhibitors: aprotinin (2 µg/ml), bestatin (40 µg/ml), leupeptin (0.5 µg/ml), pepstatin (1 µg/ml) and phenylmethylsulphonyl fluoride (1 mM). Cells were broken by vortexing in glass tubes with 0.5 mm glass beads, using 1 minute bursts and cooling for 1 minute on ice between bursts. The extract was centrifuged at 30,000 g for 60 minutes at 4°C to remove cell debris. All subsequent purification steps were at 4°C. The clear supernatant (fraction I) was applied to Macro-Prep DEAE (Bio-Rad) ion exchange column equilibrated in buffer A (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 10% glycerol) containing 0.1 M NaCl. The column was washed with buffer A and protein fractions were eluted using a 0.1-1.0 M NaCl gradient. Fractions collected were transferred to ice for storage. Aliquots were concentrated 5 to 10-fold using Amicon Mirocon centrifugal filter devices with a 10,000 Dalton molecular weight cut-off and assayed for Holliday junction cleavage. The active fractions were pooled (fraction II), dialysed against buffer A, applied to a pre-packed HiTrap heparin-Sepharose column (Amersham-Pharmacia Biotech) and eluted with 0.1–1.0 M NaCl gradient in buffer A. Fractions were assayed as described above. Active fractions from heparin-Sepharose were pooled (fraction
IQ), concentrated to 50 μl using Microcon concentrators and fractionated by FPLC gel filtration on Superose 12 HR column equilibrated in buffer A containing either 0.1 M or 1.0 M NaCl. The column was run at 1 ml/min and 0.5 ml fractions were collected and assayed. Active fractions were pooled (fraction IV), dialysed against buffer A and applied to a Resource S ion-exchange column (Amersham-Pharmacia Biotech). Proteins eluted from Resource S using a 0.1-1.0 M NaCl gradient were assayed, active fractions were pooled (fraction V) and used to characterise the Holliday junction resolvase activity.

2.10.3 Preparation of a crude extract from rat

Two rat testes (approximate weight 3.8 g) were slit with a scalpel, the contents squeezed into 5 ml TED and NaCl added to a final concentration of 0.1 M. The rat testes were homogenised by 25 strokes in a Dounce homogeniser. Cell disruption was verified by sample examination under a microscope. The homogenate was then stirred on ice for 45 minutes. Cell debris was removed by centrifugation at 12,000 rpm for 10 minutes at 4°C. Further clarification was achieved by centrifugation at 45,000 rpm for 1 hour 20 minutes in a Beckman 75 Ti rotor. The clear supernatant was made 10 % with respect to glycerol using TED/0.1M NaCl/80 % glycerol. The supernatant was then applied to a chromatography column.

2.10.4 Preparation of a crude extract from HeLa cells

HeLa cell-free extracts were prepared essentially as described in (Wood et al., 1988). HeLa cells (10⁹) were grown washed and pelleted to obtain an approximate packed cell volume (PCV). The cells were resuspended in 4X PCV of hypotonic lysis buffer and left to swell for 20 minutes on ice. The cells were then broken and homogenised by 40 strokes in a glass size A homogeniser. Protease inhibitors were added just prior to homogenising. For each 1 ml of PCV; 10 μl 1M PMSF, 10 μl 1mg/ml leupeptin, 10 μl 1mg/ml pepstatin, 50 μl 2mg/ml aprotinin, 5 μl 20 mg/ml TPCK, 2.5 μl 20 mg/ml TLCK, and 10 μl 2 mg/ml Bestatin were added. Following the addition of 4 PCV of sucrose glycerol buffer and 1 PCV saturated ammonium sulphate solution neutralised to pH 7.0, the solution was stirred for 30 minutes on ice before centrifugation in
the Beckman 70 Ti rotor for 3 hours at 42,000 rpm. Ammonium sulphate (0.33 g/ml), was added to the carefully removed supernatant that was stirring on ice. After 1 hour when the ammonium sulphate was dissolved, 10 µl of 1 M NaOH/g of ammonium sulphate was added. The precipitate was pelleted at 8000 g for 20 minutes at 4°C and fast frozen for storage at -70°C. The pellet was thawed on ice and resuspended in R buffer. Dialysis was against R buffer at 4°C for 4 hours. After centrifugation to remove any insoluble material in the Beckman microfuge R at full speed for 10 minutes at 4°C the supernatant was loaded onto a 1 ml phosphocellulose column.

2.11 Protein fractionation

All protein fractionation steps were carried out at 4°C. All protein fractions and column buffers were stored on ice during fractionation. After centrifugation of crude cell-free extracts to remove cell debris and insoluble material, supernatants were passed through 0.45 micron filters before loading onto ion-exchange columns. Production of a cell-free extract, application to a column, elution from the column, and assaying of fractions was completed in 1 day. Subsequent dialysis, column chromatography stages, and assaying of fractions were completed in 1 day. The fast processing of cells was essential to minimise loss of enzymatic activity.

2.11.1 Gel filtration

Gel filtration resins were equilibrated in TED/10% glycerol buffer containing either 0.1 M or 1.0 M NaCl. Protein samples were filtered through 0.45 micron filters and concentrated to the appropriate application volume of the particular column. Protein elution was monitored by the UV absorbance at 280 nm and fractions were tested for activity. Proteins of known molecular mass were run as standards under identical conditions. The void volume of the column was determined using Blue Dextran 2000. Different gel filtration columns were used to confirm calculated molecular mass.
2.11.2 Ion-exchange chromatography (all ion-exchange columns)

Columns were equilibrated in ice-chilled TED/0.1 M NaCl/10% glycerol buffer. All column buffers and elution buffers were filtered through 0.45 micron filters and degassed. The cleared supernatant was applied to an ion-exchange column. Protein application was monitored by UV absorbance at 280 nm. The column was washed until a stable baseline of UV absorbance was achieved. A 0.1 - 1.0 M NaCl gradient in TED/10% glycerol was applied to the column to elute bound protein. Gradient volume was 10 column volumes. The heparin-Sepharose chromatography stage used during the purification scheme of resolvase activity from AK47 cells involved washing the column with TED/0.5 M NaCl/10% glycerol buffer before elution using a 0.5 - 1.0 M NaCl gradient in TED/10% glycerol. Column fractions were stored on ice pending assaying or flash frozen in liquid N2 and stored at -70°C. Columns used are presented in table 2.11.
**Table 2.11: Chromatography columns used**

<table>
<thead>
<tr>
<th>Column</th>
<th>Pilot scale</th>
<th>Large scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio-Rad MacroPrep</td>
<td>0.5 ml and 1 ml Pharmacia K 9/15 column</td>
<td>25 ml Pharmacia XK 16/20 column</td>
</tr>
<tr>
<td>DEAE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pharmacia Hi Trap</td>
<td>1 ml pre-packed</td>
<td>5 ml pre-packed</td>
</tr>
<tr>
<td>Heparin-Sepharose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whatman P11 phosphocellulose</td>
<td>1 ml or 3 ml Pharmacia K 9/15 column</td>
<td></td>
</tr>
<tr>
<td>Pharmacia HiTrap Octyl-Sepharose</td>
<td>1 ml pre-packed</td>
<td></td>
</tr>
<tr>
<td>Pharmacia Mono S HR 5/5</td>
<td>1 ml pre-packed</td>
<td></td>
</tr>
<tr>
<td>Pharmacia Mono Q HR 5/5</td>
<td>1 ml pre-packed</td>
<td></td>
</tr>
<tr>
<td>Resource S</td>
<td>1 ml pre-packed</td>
<td></td>
</tr>
<tr>
<td>Resource Q</td>
<td>1 ml pre-packed</td>
<td></td>
</tr>
<tr>
<td>Source 15S</td>
<td></td>
<td>5 ml Pharmacia XK 16/20 column</td>
</tr>
<tr>
<td>Bio-Rad MacroPrep CM</td>
<td>1 ml Pharmacia K 9/15 column</td>
<td></td>
</tr>
<tr>
<td>Bio-Rad MacroPrep High Q</td>
<td>1 ml Pharmacia K 9/15 column</td>
<td></td>
</tr>
<tr>
<td>Superose12 HR 10/30</td>
<td>24 ml Pre-packed</td>
<td></td>
</tr>
<tr>
<td>Superdex 75</td>
<td>100 ml Pharmacia XK 16/70 column</td>
<td></td>
</tr>
<tr>
<td>Superdex 200</td>
<td>100 ml Pharmacia XK 16/70 column</td>
<td></td>
</tr>
</tbody>
</table>
2.12 Biochemical in vitro assays

2.12.1 Assay for resolvase activity

Cleavage reactions contained up to 2 μl of the fractions and 1 - 5 ng of \(^{32}\)P-labelled junction (typically around \(10^4\) cpm) in 10 μl cutting buffer 1. Assays for \(S.\ pombe\) fractions were in cutting buffer 2. A 500-fold molar excess of \(Hinf I\) cut \(\phi X174\) form I DNA was used as competitor DNA. The ratio reflects DNA concentrations in nucleotide residues. The reactions were incubated for 30-60 minutes at 30°C. Control reactions with 5 μg/ml RuvC were incubated at 37°C and those with 10 μg/ml YDC2 and 2μl CCE1 (diluted 1:100 in enzyme dilution buffer) at 30°C. Reactions were stopped by the addition of stop buffer and further incubated at 30°C for 15 minutes. Products were analysed by electrophoresis on 6% neutral polyacrylamide gels or, 8 or 12% denaturing polyacrylamide gels. The gels were dried and visualised by autoradiography or phosphorimager.

2.12.2 Ligation assays

For the ligation experiments, a resolvase assay was performed as above. One half of the reaction mixture was stopped and processed as described. T4 DNA ligase (10 units) and ATP (1 mM final concentration) were added to the remaining sample. The reactions were further incubated for 30 min at 37°C, then stopped and processed as before.

2.12.3 Assays for cutting of Chi structures

Assays for cutting of Chi structures were as described for the synthetic substrates using around \(10^4\) cpm of \(^{32}\)P-labelled DNA per assay. Reactions (20 μl) were stopped and deproteinised by adding 5 μl of stop buffer 5X followed by incubation for 15 min at 37°C. Products were analysed by 1.2% agarose gel electrophoresis and autoradiography.
2.12.4 DNA binding assays

Binding reactions contained about 5 ng of labelled synthetic junction and 2 µl of protein sample in a total volume of 10 µl binding buffer and a 500-fold molar excess of *Hinf* I-cut φX174 form I DNA used as competitor. The ratio reflects DNA concentrations in nucleotide residues. Control reactions contained either no added protein or 5 µg/ml RuvC or 10 µg/ml YDC2. Reactions were incubated on ice for 20 minutes. Samples were loaded directly onto 6% neutral polyacrylamide gels in TBE in sample gel-loading buffer. Electrophoresis was at 10 V/cm at 4°C with continuous buffer re-circulation. Bands on dried gels were visualised by autoradiography or phosphorimager.

2.12.5 Stimulation of RuvB-mediated branch migration

RuvB (600 nM) was pre-incubated with 5 ng of ³²P-labelled synthetic Holliday junction in calcium branch migration buffer at 34°C for 5 minutes. YDC2, 10 µg/ml, or 2 µl fraction V resolvase activity from AK47 cells, was added and incubation was continued for a further 30 minutes. RuvAB was used as a control. Reactions were stopped with 5X stop buffer and incubation continued for 10 minutes. Samples were loaded on neutral 6% polyacrylamide gels in gel loading buffer. Electrophoresis was at 10 V/cm. Bands on dried gels were visualised by autoradiography or phosphorimager.

2.12.6 Assay of crude extracts for branch migration activity

Alpha-structures 0.75 ng/µl were purified by gel filtration chromatography in 20 mM Tris-HCl (pH 8.0), 1 mM DTT, 0.1 mg/ml BSA, and 5 mM MgCl₂, which is a suitable for branch migration. ATP was added to the intermediates to give a final concentration of 2 mM. The purified *Escherichia coli* proteins RuvA and RuvB were used as controls for branch migration. Both proteins were diluted from stock solutions with enzyme dilution buffer and used at a final concentration of 200 nM. Test samples and controls were incubated for 30 minutes at 37°C. The reactions were stopped with 5X stop buffer followed by incubation for 10 minutes at 37°C, 6X GLB was added to the samples before loading onto a 1.5% agarose gel. Agarose gels were run in buffer containing
ethidium bromide (0.5 µg/ml) for 180 minutes at 5 V/cm with buffer recirculation. Gels were dried and autoradiographed.

2.12.7 Assays for ligation/pairing activity

Reactions in 1X RecA buffer contained 25 ng gDNA and 30 ng labelled linear duplex DNA. Similar reactions used 30 ng of linear DNA alone, 30 ng linear DNA and 25 ng ssDNA, and 30 ng linear DNA and 25 ng supercoiled plasmid DNA. Pairing reactions containing 2 species of linear duplex DNA contained 15 ng of each species. 1-2 µl of sample to be tested was then added to the reaction mixtures and after 10 minutes at 37°C the reaction was stopped with 5X stop buffer. Control reactions were incubated at 37°C for 20 minutes with RecA and at 20°C for 20 minutes with T4 DNA ligase. After 5 minutes at 37°C 6X GLB was added to the samples and product bands were separated on a 1.2% agarose gel. Gels were dried and products were visualised by autoradiography.

2.12.8 Antibody inhibition assays

Fractions were pre-incubated with hRad51 antibodies for 15 minutes followed by the addition of substrates. Assays for pairing/ligation were carried out as above.

2.13 Protein purification

2.13.1 Cloning and expression of S. pombe YDC2

The YDC2_SCHPO open reading frame was amplified by PCR as described in 2.7.4. An Nde I restriction site was engineered at the start codon of the open reading frame sequence in frame with the start codon in the T7 expression vector pET21a(+) (from Novagen), and a Xho I restriction site was introduced at the 3’ end of the sequence, after the natural stop codon. The PCR product was cleaved with Nde I and Xho I, cloned into the Nde I and Xho I sites of pET21a(+). Ligation reactions were as recommended by Novagen. The recombinant plasmid pET21-YDC2 was used to transform E. coli strain BL21(DE3) (F-,ompT, hsdS, (rB, mB) dcm, gal, λ(DE3)).
For expression of the YDC2 protein, cells were grown in LB medium containing 100 µg/ml ampicillin at 37°C to an A600 of 0.5 and induced with IPTG (0.1 mM final concentration) for 18 hours at 30°C. The induced cells were harvested, resuspended in TED buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 mM DTT, 10% (v/v) glycerol) and lysed by treatment with lysozyme and 0.1% TritonX-100 in 0.1 M NaCl as described (Tsaneva et al., 1992). The lysate, containing only a fraction of the over-expressed protein, was subjected to column chromatography on phosphocellulose, Heparin Sepharose HiTrap, and Mono Q FPLC.

2.13.2 Purification of RecA protein

*E. coli* strain JCI2772 (described in (Uhlin and Clark, 1981)) was grown in LB containing ampicillin (100µg/ml) at 30°C overnight. 4 litres of cells were pelleted at 5000 g for 30 minutes at 4°C and resuspended in ice-chilled 50 mM Tris-HCl (pH 7.5), 10% sucrose such that a 1/1000 dilution had an OD595 of approximately 0.4. The suspension was stored at -70 °C. Cells were thawed to room temperature and made 100 mM Tris-HCl (pH 7.5) and 10% (w/v) sucrose. The suspension was cooled on ice and the cells were lysed by the addition of lysozyme to a concentration 0.8 mg/ml in 100 mM Tris-HCl (pH 7.5). The solution was stirred for 5 minutes and made 5 mM EDTA (pH 8.0), 0.5% Brij 58, 1 mM DTT. Stirring was continued for 30 minutes before centrifugation in the Beckman 45 Ti ultracentrifuge rotor for 70 minutes at 42,000 rpm.

Polymin P (pH 7.9) was added to the supernatant to 0.5% final concentration. This mixture was stirred for 30 minutes on ice and then centrifuged at 12000 g for 10 minutes at 4°C. The precipitate was resuspended in 30 ml R buffer containing 150 mM ammonium sulphate. The suspension was centrifuged as before, and the pellet was resuspended in 24 ml R buffer containing 300 mM ammonium sulphate and again stirred and centrifuged. Additional ammonium sulphate was added to the supernatant to a concentration of 0.28 g/ml. After 30 minutes the solution was centrifuged for 30 minutes at 12000 g at 4°C. The pellet was resuspended in 50 ml P buffer containing 200 mM NaCl and dialysed
overnight. After dialysis the solution was applied to a 20 ml phosphocellulose column equilibrated in P buffer containing 200 mM NaCl. The column flow-through was collected and dialysed against P buffer.

Half the dialysed solution was applied to an 11 ml ssDNA cellulose column equilibrated in P buffer to ensure the column was not overloaded. The column was washed with 2 volumes of P buffer containing 50 mM NaCl. RecA was eluted from the column with buffer containing 1 mM ATP. The peak of protein as measured on SDS PAGE was precipitated overnight with 0.28 g/ml ammonium sulphate. The precipitate was pelleted for 30 minutes at 12000 g and resuspended in R buffer containing 0.28 g/ml ammonium sulphate. The solution was centrifuged as before and resuspended in R buffer/20% (v/v) glycerol. The RecA protein was stored at -70°C. The ssDNA cellulose column was washed in P buffer containing 1M NaCl and then equilibrated in P buffer where upon the second half of the RecA solution was applied and the procedure repeated. Protein concentration was calculated assuming a 10 mg/ml solution of RecA has an OD280 = 5.17. Purity was checked by SDS PAGE.

2.14 Western blot analysis of HeLa cell fractions
Potential antigens were separated on SDS-PAGE using 12% gels. Proteins were electroblotted to Bio-Rad Trans-Blot® nitrocellulose in transfer buffer at 150 mA for 2 hours at 4°C. Non-specific binding sites were blocked by incubating the membrane in TBST, containing 5% (w/v) low fat dried milk (block solution) for 15 minutes. Anti-hRad51 antibody was diluted in the block solution, 1:1000. The membrane was incubated with the primary antibody for 1 hour at room temperature. Unbound antibody was removed by three 5 minute washes in block solution. Anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate was added to the membrane diluted 1:30,000 in block solution as recommended by Sigma. The membrane was washed 3 times in TBST as before. The membrane was finally washed with two quick washes of TBS to remove residual Tween® 20. Colour development was using Sigma Fast™ BCIP/NBT buffered substrate tablets (1 tablet/10 ml H2O). The membrane was
incubated in the substrate solution for 10 minutes. Membranes were air-dried before photography.

2.15 Homology searches and protein sequence comparisons.

Sequence data base searches were performed using the blast search engines at the National Center for Biotechnology Information at the NIH. Protein sequence comparisons were based on the ClustalW 1.7 multiple sequence alignment program (Thompson et al., 1994).
3. SEARCHING FOR HOLLIDAY JUNCTION PROCESSING ACTIVITIES IN EUKARYOTIC CELL EXTRACTS: THE APPROACH

Experimental evidence has demonstrated that homologous recombination plays an important part in many biological processes. A Holliday junction is likely to be central to the homologous recombination process. The formation of Holliday junctions has also been demonstrated experimentally. To search for Holliday junction-specific enzymes efforts were focused in two parallel directions. One approach taken was to screen cell-free extracts for biochemical activities capable of processing Holliday junction-containing substrates. The other approach involved a homology search of protein sequence databases with the protein sequences of known Holliday junction-specific enzymes.

The biochemical approach involved screening various eukaryotic cells for Holliday junction processing activities. The substrates used were synthetic Holliday junctions, alpha-structures (RecA-made recombination intermediates), and Holliday junction-containing molecules formed in vivo by XerC-mediated site-specific recombination, (Chi DNA structures). Cell extracts were made from a variety of biological sources and assayed. The choice of eukaryotic cells or tissue was decided by the advantages offered by each eukaryotic source as summarised in table 3.0.

Yeast as an organism is very amenable to genetic analysis and the genotype can also be manipulated to create strains with phenotypes that could be advantageous to protein purification. This could include protease and/or nuclease deficient strains, cell cycle mutant strains, meiotically inducible strains, RAD mutants, and Pol mutants. DNA damage can be repaired by a number of integrated pathways. Genetic evidence has revealed that removal of genes involved in a specific DNA repair pathway causes the organism to repair DNA damage using an alternative pathway. For example, mutating the RAD27 gene in S. cerevisiae leads to an increased rate of recombination following
DNA damage (Reagan et al., 1995; Symington, 1998; Vallen and Cross, 1995). This could be an approach to enrich the cells for recombination activity.

Table 3.0. Possible sources of Holliday junction processing activities.

<table>
<thead>
<tr>
<th>Source</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Complete genome sequence known, can be synchronised, amenable to genetic analysis, extensively researched genetics, homologous recombination is very frequent, DNA sequences easily manipulated to exact locations.</td>
</tr>
<tr>
<td><em>S. pombe</em></td>
<td>Cell cycle well characterised and understood, mutants available, closer to higher eukaryotes than <em>S. cerevisiae</em>, homologous recombination is very frequent.</td>
</tr>
<tr>
<td>Rat testis</td>
<td>Proliferating pre-meiotic cells and cells undergoing meiosis, large amounts of tissue obtainable.</td>
</tr>
<tr>
<td>HeLa cells</td>
<td>Highly active proliferating cells, widely studied.</td>
</tr>
</tbody>
</table>

3.1 Synthetic Holliday junction

A synthetic four-way junction containing a 12 bp region of homology was constructed by the annealing of four synthetic 61-63 bp oligonucleotides together (Figure 3.1). To produce a labelled synthetic junction, one oligonucleotide can either be labelled at the 5'-end with [γ-32P] ATP using PNK or at the 3'-end labelled with [α-32P] dideoxy ATP using terminal transferase prior to annealing. In figure 3.1 oligonucleotide number 2 is drawn labelled. The labelled substrate can be used to assay for Holliday junction branch migration or resolution.
Figure 3.1. Synthetic Holliday junction constructed by annealing four synthetic oligonucleotides. The junction is labelled on one oligonucleotide as indicated. Substrate and products can be analysed by both denaturing and native PAGE. Resolution of the junction labelled on strand 2 in a west-east direction or a north-south direction can be visualised by electrophoresis on a native polyacrylamide gel. Resolution of the junction in a west-east direction would not be seen on a denaturing gel, as strand 2 would not be cut. Resolution occurs in both orientations if the preferred sequences of the resolvase are present at or near the point of crossover of the two DNA strands that make the Holliday junction.
Strand 1

Strand 2

Strand 3

Strand 4

Resolution

Products analysed by native PAGE

Products analysed by denaturing PAGE
The central 12 bp region of homology could allow the junction to be positioned anywhere within the homologous core and therefore have different nucleotide sequences at the point of crossover. This junction is referred to as junction X12 because of the 12bp region of homology.

A similar junction X0 with oligonucleotide in common with X12 was constructed by annealing four oligonucleotides that have no homologous sequences. These will therefore form a junction with a fixed crossover point. The junction is referred to as junction X0 because the junction contains no region of homology.

The substrate and products can be analysed by both native and denaturing polyacrylamide gel electrophoresis and autoradiography as shown schematically in figure 3.1. A symmetric cut on both strands of a synthetic junction labelled on strand 2 in a west-east or a north-south direction can be detected by native PAGE. However, resolution of the junction in a west-east direction would not be revealed on a denaturing gel as the labelled strand 2 would remain uncut. A resolvase will cleave a junction in both orientations if the preferred sequence of the resolvase is present at or near the point of crossover of the Holliday junction. If a particular junction such as X0 does not contain cleavable sequences symmetric to the point of crossover on both pairs of arms, resolution could occur in one orientation. Such partial cleavage could pass undetected using denaturing PAGE if the cleaved strand was not labelled.

To map the cleavage sites of a resolvase requires constructing four junctions where only one strand is labelled (1, 2, 3, or 4) and analysing the products by high resolution denaturing PAGE. Sequencing ladders can be run in parallel to enable determination of the sequence in the junction where cleavage has taken place. This analysis also reveals if cleavage is on opposing strands and symmetric to the point of crossover. A single-strand nicking activity like Rad1/Rad10 could appear as a resolvase on a denaturing gel, as could a single strand-specific nuclease (West, 1995). Demonstrating that resolution is not the result of a non-specific nuclease nicking duplex DNA or a nuclease nicking the
junction at the point of crossover due to the single-strand character of this region is important in providing evidence of a true resolvase.

Synthetic Holliday junctions can also be used to detect branch migration. The products of branch migration can only be seen on a native gel. Use of both electrophoretic systems is needed to demonstrate different types of activities.

3.2 Four-stranded recombination reactions promoted by RecA in vitro

RecA has been shown to promote both three and four-stranded strand exchange reactions in vitro. We used the four-strand reaction developed by Muller (Muller and West, 1994) which forms a Holliday junction recombination intermediate.

3.2.1 Construction of gDNA

RecA requires a region of ssDNA to initiate pairing of homologous sequences. The single-stranded region can be provided by a species of DNA molecule referred to as gapped circular duplex DNA (gDNA). The gDNA consists of circular plasmid (pDEA7z-f(+)) that contains a 174 bp region of ssDNA (Figure 3.2.1). To produce the gDNA, plasmid DNA is digested with restriction enzymes to produce two fragments one 2827 bp and the other 174 bp. The 2827 bp fragment is annealed to a full-length single stranded form of the circular plasmid. The ssDNA is produced in vivo by utilising the origin of replication of the filamentous bacteriophage f1 contained within the plasmid.

3.2.2 Recombination intermediates made by RecA in vitro

Purified RecA protein is used to pair the gDNA to linearised homologous plasmid labelled with $\alpha$-$[^{32}\text{P}]$ddATP. RecA binds to the single-stranded region in the gDNA and nucleates the polymerisation of nucleoprotein filament that invades the dsDNA region and covers the whole plasmid. This activated RecA nucleoprotein plasmid will pair with the labelled homologous linear DNA and will promote strand exchange and formation of Holliday junction intermediates, also referred to as alpha-structures (Figure 3.2.2A).
If the RecA strand exchange reaction is allowed to proceed to completion the labelled linear strands will be completely exchanged with the gDNA to produce labelled nicked duplex DNA and gapped linear DNA, and no Holliday junction-containing intermediate. The RecA reaction is stopped to minimise the production of nicked circular DNA and maximise the production of Holliday junction-containing alpha-structures. The Holliday junction-containing DNA molecules are deproteinised and used to assay cell-free extracts for Holliday junction-processing activities.

A second plasmid (pDEA2z-f+) can also be used in the construction of recombination intermediates. The second plasmid is similar to the first plasmid except it contained a region of heterology. A RecA pairing reaction is set up between labelled linear DNA constructed from the second plasmid, (pDEA2z-f+) and gDNA constructed using the first plasmid (pDEA7z-f+). The strand exchange reaction proceeds until the region of heterology is reached and is blocked at this point. A high proportion of the gDNA would be used in forming blocked alpha-structures and no nicked circular DNA would be formed.

Deproteinised RecA-made alpha-structures can be used as a substrate to detect reactions catalysed by proteins in eukaryotic crude extracts. A sample of crude extract is incubated with the recombination intermediates. The reaction products are deproteinised, analysed by agarose gel electrophoresis and visualised by autoradiography as described in Methods. *E. coli* proteins, RuvA and RuvB, and RuvC can be used as controls for branch migration and resolution, respectively (Muller et al., 1990) (Figure 3.2.2.B).
Figure 3.2.1. gDNA preparation. Restriction enzyme-digested plasmid DNA (red) is annealed to the circular single-stranded form of the plasmid (blue) to produce gDNA (blue and red) that contains a short region of ssDNA.
Figure 3.2.2. Formation and processing of RecA-made alpha structures. (A) RecA can pair homologous gDNA and linear duplex DNA and promote strand exchange. The reaction proceeds via Holliday junction-containing intermediates referred to as RecA-made intermediates or alpha-structures. The alpha-structures are deproteinised before use in assays. The Holliday junction can branch migrate in either of two directions. Forward branch migration forms nicked circle and gapped linear DNA species. Reverse branch migration produces the starting products gDNA and linear duplex DNA. Resolution of the Holliday junction can form both nicked circle DNA and gapped linear DNA, and linear dimer products. (B) Products of branch migration of the Holliday junction by RuvAB and resolution of the Holliday junction by RuvC can be separated by gel electrophoresis and visualised by autoradiography. Control reactions contained no protein.
Lin 3001 bp RecA strand exchange

\[ \begin{array}{c}
32P \\
\text{Linear pDEA-7Z} \\
\text{Gapped DNA}
\end{array} \]

RuvAB 'reverse' branch migration

RuvAB 'forward' branch migration

RecA strand exchange

RuvC cleavage (W-E)

Nicked Circle

Alpha-structure

Linear dimer

Gapped Linear

B

\begin{array}{c|c|c}
\text{Control} & \text{RuvAB} & \text{RuvC} \\
\hline
\text{Alpha-structures} & \text{Linear dimer} & \text{Nicked circle} \\
\text{Linear\Gapped linear} & & \\
\end{array}
Incubation of a fractionated cell-free extract with gDNA and labelled linear DNA can be used as an assay for homologous pairing and strand exchange activities analogous to RecA. The reaction products would be deproteinised, analysed by agarose gel electrophoresis and visualised by autoradiography to see if recombination intermediates have been made. RecA would be used as a control.

RecA-made intermediates were used initially to screen for Holliday junction processing activities, as they are more representative of recombination intermediates formed in vivo. These intermediates have been used with success to detect RuvC in cell-free extracts (Connolly and West, 1990) and in reconstitution experiments using RecA and RuvC (Dunderdale et al., 1991). RecA-made intermediates have also been used to demonstrate RuvAB branch migration of a Holliday junction (Tsaneva et al., 1992; Tsaneva et al., 1993).

### 3.3 Chi-structures

Holliday junction-containing molecules can be prepared from plasmid intermediates formed in vivo by XerC-mediated site-specific recombination (McCulloch et al., 1994). Cells containing a substrate plasmid that can undergo XerC recombination are grown and the expression of XerC is induced which leads to the transient formation and accumulation of “figure eight” structures. These Holliday junction-containing “figure eight” plasmids can be purified and cut with restriction enzymes to produce topologically unconstrained Holliday junction-containing Chi DNA (Figure 3.3). The Holliday junction pictured can be imagined in the same conformation as the synthetic Holliday junction drawn in figure 3.1. The westerly Styl end of the junction can be turned out of the page towards the reader 180° about the west-east axis, while the westerly Scal end is revolved to the earlier position of the Styl end. Chi structures are much larger than the synthetic Holliday junction and are therefore analysed on agarose gels. Three of the possible four resolution products can be visualised following autoradiography of the dried agarose gel.
Figure 3.3. Chi DNA formation. Chi DNA is prepared from plasmid intermediates formed in vivo by XerC-mediated site-specific recombination. Plasmid containing cells are grown and upon induction of XerC expression, figure eight structures are formed. Plasmid regions shown in red are recognised and acted upon by XerC recombinase. Figure eight structures are digested with restriction enzymes to form Chi DNA. Resolution products of Chi DNA can be separated on agarose gels where three of the possible four resolution products can be visualised by autoradiography.
XerC induction

Sty I / Sca I cleavage

Resolution

W/E: *Sty I + *Sty I **2.06 kb
Sca I + Sca I (2.33 kb)

N/S: *Sty I + Sca I *1.89 kb
*Sty I + Sca I *2.50 kb
3.4 Summary

Both the biochemical and the homology search approach to identifying Holliday junction processing activities were successful as summarised in table 3.4.

Table 3.4. Holliday junction-processing activities identified.

<table>
<thead>
<tr>
<th>Source</th>
<th>Biochemical approach</th>
<th>Genetic approach</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. pombe</em></td>
<td>Resolvase activity found.</td>
<td>Homologue of <em>ccel</em> identified.</td>
</tr>
<tr>
<td></td>
<td>(Chapter 4)</td>
<td>(Chapter 4)</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Resolvase activity found.</td>
<td>No homologues identified.</td>
</tr>
<tr>
<td></td>
<td>(Chapter 5)</td>
<td></td>
</tr>
<tr>
<td>Rat testis</td>
<td>Ligation activity found.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Chapter 6)</td>
<td></td>
</tr>
<tr>
<td>HeLa cells</td>
<td>Ligation activity found.</td>
<td>No homologues identified.</td>
</tr>
<tr>
<td></td>
<td>(Chapter 6)</td>
<td></td>
</tr>
</tbody>
</table>
4. **SCHIZOSACCHAROMYCES POMBE**

Although high frequencies of homologous recombination have been observed in *S. pombe*, no Holliday junction resolution activities were reported at the start of this work.

As part of the screen for Holliday junction processing activities cell-free extracts from the fission yeast *S. pombe* were fractionated and assayed initially using RecA-made alpha structures and later using a synthetic Holliday junction and Chi DNA.

### 4.1 Holliday junction processing activity in fractionated *S. pombe* cell free extracts

Cell-free extract obtained from one litre of yeast culture was applied to a 1 ml DEAE chromatography column and eluted with a 0.1 - 1.0 M NaCl gradient. The crude extract, column flow through (unbound material), and eluted fractions were assayed for Holliday junction processing activities using alpha-structures. This assay allows for both branch migration and resolution of the Holliday junction to be detected in the presence of ATP. An ATP regeneration system was used to ensure that ATP is not depleted by ATPases present in the extract. *E. coli* proteins RuvAB and RuvC were used as controls for branch migration and resolution of the Holliday junction, respectively. As seen in Figure 4.1, no bands corresponding to branch migration products were formed in the assays. Fractionation of crude cell extracts on various other chromatography supports provided no evidence for branch migration activities in the fractions (data not shown). In contrast, fractions eluted from the DEAE chromatography column contained activity producing linear dimer products characteristic of Holliday junction resolution (Figure 4.1B and C). Two peaks of activity eluted from the column (Figure 4.1C). One peak of activity eluted during the gradient at low salt concentration (lane c) and another peak of activity eluted during the gradient at higher salt concentration (lane f).
Figure 4.1. Processing of RecA-made alpha-structures by fractionated cell-free extracts from *S. pombe*. (A) Schematic of the formation of alpha-structures by RecA and processing of Holliday junctions. (B) Processing of RecA-made alpha-structures by RuvAB, RuvC, and crude cell-free extract from one litre cell culture. (C) Fractionation of the cell-free extract by DEAE chromatography using a 0.1-1.0 M NaCl gradient. The crude extract and fractions from the DEAE column were assayed using RecA made alpha structures. Lane c peak of activity eluting during the gradient at low salt concentration. Lane f peak of activity eluting during the gradient at higher salt concentration.
Gapped DNA

= >

Linear DNA

alpha-structure

RuvAB branch migration
RuvC cleavage (N-S)

= >

Nicked Circle

Gapped Linear

= +

Linear Dimer

RuvC cleavage (W-E)

B

<table>
<thead>
<tr>
<th>Junction</th>
<th>RuvAB</th>
<th>RuvC</th>
<th>Extract</th>
</tr>
</thead>
</table>

α-structures

Nicked circle
Linear dimer
Gapped linear/linear

C

0.1 - 1.0 M NaCl gradient

α-structures
Linear dimer product
Fractions that produced the dimer products needed to be assayed again to see if the activity would resolve other Holliday junction-containing substrates, the synthetic Holliday junction and Chi DNA. The activity that eluted during the gradient at low salt concentration (Figure 4.1, lane c) was tested with synthetic Holliday junction as a substrate. Although it appeared to resolve the RecA-made intermediates this fraction would not cut the synthetic Holliday junction, nor would it cut the Chi DNA (data not shown). In control experiments using labelled linear DNA alone as a substrate, linear dimer product formation was observed (data not shown). This indicated the presence of a DNA ligation activity in the fraction. The production of the dimer product and assays for ligation will be discussed in detail in chapter 6 where a ligation activity was characterised further. The fractions that eluted later in the gradient (Figure 4.1C, lane f) cut the synthetic Holliday junction and Chi DNA as shown below.

4.2 Holliday junction resolution activity in fractionated cell-free extract from *S. pombe*

Fraction f (Figure 4.1C, lane f) from the DEAE chromatography fractionation was assayed using the synthetic Holliday junction X12 (Figure 4.2A) labelled on strand 2. One discrete cleavage site was observed by denaturing PAGE which was very close to the cut produced by RuvC used as a control. This result demonstrates cleavage of the junction close to the crossover point and suggests some sequence specificity at the resolution stage.

It has been shown previously that nucleases such as Rad1/Rad10 and S1 nuclease can nick small synthetic Holliday junctions around the crossover point due to some single-strand character of DNA in this region, to give apparent resolution products. To rule out this possibility active fractions were also tested for Holliday junction resolution using another substrate, Chi-DNA (Figure 4.2B). The cellular activity cut the Chi-DNA to give all the labelled products in ratios consistent with resolution of the junction in both orientations. The activity was, therefore, a true and novel Holliday junction resolvase. This was an important result.
Figure 4.2. Endonuclease activities of *S. pombe* are specific for a synthetic four-way Holliday junction and Holliday junction-containing Chi DNA. (A) Reactions with 5 ng of DNA were incubated in 10 µl reaction buffer with either RuvC (5 ng) or 2 µl *S. pombe* cellular activity. The substrate used was junction X12 labelled on strand 2. Incubation was for 30 minutes at 30°C (or 37°C for RuvC). Analysis was by denaturing 8% polyacrylamide gel electrophoresis followed by autoradiography. (B) Reactions contained $^{32}$P-labelled Chi DNA with either 5 ng RuvC, or 2 µl cellular activity, in 20 µl reaction buffer, as indicated. After incubation for 20 minutes at 30°C (or 37°C for RuvC) reactions were stopped by addition of SDS (to 0.5%) and Proteinase K (to 1 mg/ml). Products were analysed by 1.2% agarose gel electrophoresis and visualised by autoradiography. (C) Schematic presentation of Chi DNA structures, labelled at the *Sty* I ends. Resolution of the Holliday junction in two orientations (North-South and East-West) produces four nicked linear DNA species (three of them labelled) of the sizes indicated.
4.3 Homology search in *S. pombe* database

In budding yeast the only known resolvase is CCE1. CCE1 has been shown to be localised and to act in the mitochondria. The possibility arose that the fission yeast resolvase demonstrated earlier in figure 4.2, could also be a mitochondrial activity. While the purification of the resolvase activity was in progress, a search in the *S. pombe* sequence data base revealed an open reading frame on chromosome I which encoded a predicted 30.2 kDa protein (YDC2_SCHPO) (Accession number Q10423) showing ~30% identity to the *S. cerevisiae* CCE1. One alignment of CCE1 and its putative *S. pombe* homologue is given in figure 4.3, showing a number of short conserved regions spread throughout the sequences. At this stage the work split into two directions. One direction was to clone the YDC2_SCHPO open reading frame by PCR amplification and express the protein. A second direction was to work alongside a post-doctorate in the laboratory to develop a fractionation scheme to partially purify and characterise the cellular activity.

4.4 Cloning of a novel Holliday junction resolvase from *S. pombe*

The YCD2_SCHPO open reading frame was amplified from *S. pombe* 972h- chromosomal DNA by PCR (Figure 4.4). Primer sequences were;

5’ primer

5’-ATATATCATATGGCTACTGTGAATCAGTTTTACAGC;

3’ primer

5’-ATATCTCGAGCTATTTGCAACAACTGTATTGCAAAAGTTTC.

A *Nde* I restriction site was engineered at the start codon of the sequence in frame with the start codon in the T7 expression vector pET21a (+), and a *Xho* I restriction site was introduced at the 3’ end of the sequence, after the natural stop codon. The linkers added are underlined in the sequences above. The PCR product was cleaved with *Nde* I and *Xho* I, cloned into the T7 expression vector pET21a (+) and the recombinant plasmid pET21-YDC2 was used to transform *E. coli* strain BL21 (DE3).
Alignment program. Identical amino acids are indicated in red and conserved residues in blue.

Figure 4.3. Sequence comparison of S. cerevisiae CCE1 and S. pombe YDC2. The figure shows an alignment of the CCE1 protein (533 amino acids) with the open reading frame of YDC2 (258 amino acids) based on the ClustalW 1.7 multiple sequence alignment. The figure shows an alignment of the CCE1 protein (533 amino acids) with the open reading frame of YDC2 (258 amino acids) based on the ClustalW 1.7 multiple sequence alignment.

| 258 | ---------- | K-DMADS ALIASGIROAN -- | VEER ---- | YDC2 |
| 353 | | | | | CCE1 |
| 220 | | | | | AD2 |
| 268 | | | | | CCE1 |
| 150 | | | | | AD2 |
| 179 | | | | | CCE1 |
| 62 | | | | | AD2 |
| 90 | | | | | CCE1 |
Figure 4.4. Cloning of the YCD2_SCHPO open reading frame from *S. pombe*.
4.5 Over-expression and purification of recombinant YDC2

To express the YDC2 protein *E. coli* strain BL21 (DE3) containing plasmid pET21-YDC2 was grown in LB medium containing ampicillin and induced with IPTG. Induced cells were harvested and lysed by treatment with lysozyme and Triton X-100. The induced cells clearly produced a strong novel protein band on SDS-PAGE with a molecular weight of 30 kDa. To check the solubility of the induced protein, cell debris and any inclusion bodies were removed from induced cells by centrifugation to produce a clear supernatant. Comparison of this supernatant with total cells on SDS PAGE revealed that a large percentage of YDC2 protein was insoluble (data not shown). Growth of cells at lower temperatures, 25 and 30°C, and lower IPTG concentrations, 0.1mM did improve the amounts of soluble YDC2 in the clear supernatant fraction. The lysate, containing only a fraction of the over-expressed protein, was subjected to column chromatography (Figure 4.5A), purification was monitored by assaying for Holliday junction resolution. Figure 4.5B shows the scheme used for the purification of the cellular activity for comparison. Figure 4.5C shows that the protein expressed from the YDC2 open reading frame was purified to near homogeneity. Its molecular weight was 29.5 kDa as observed by SDS-PAGE, in reasonable agreement with the DNA sequence that encodes a predicted 30.2 kDa protein. The purified protein was used in subsequent experiments to characterise the enzyme.
Figure 4.5. Purification of YDC2. (A) Scheme used to purify YDC2 over-expressed in *E. coli* BL21 (DE3). (B) Scheme used to purify cellular acity. (C) SDS-PAGE of YDC2 (2.4 µg) on a 12% gel stained with Coomassie® Brilliant Blue R250. *M*₀ standards were ‘low range’.
4.6 Cloned activity is biochemically indistinguishable from the activity originally identified in cell extracts

Once the protein YDC2 was cloned and purified a comparison could be made between the activity of the cloned enzyme and the cellular activity. A detailed characterisation of the purified protein could also be achieved. Assays using the purified protein would also reveal the preferred cleavage sites and optimal reaction conditions of the yeast resolvase enabling a comparison with other recombination proteins.

The cleavage sites of both the purified YDC2 and the cellular activity were mapped at high resolution on all four strands of junction X12. The activity produced symmetrical nicks in strands of the same polarity close to the crossover point. Strands 2 and 4 were cut more efficiently than 1 and 3 in this experiment (Figure 4.6.1). Both activities showed a strong structure selectivity and some sequence preference. All the main cut sites mapped 3’ of a thymine residue. A preliminary consensus sequence of 5’-CXT↓-3’ could be derived from this data, with a preference of 5’-CCT↓-3’ (Figure 4.6.2).

Resolution of a Holliday junction produces a nick in the DNA backbone. The 5’ phosphate and 3’ hydroxyl need to be ligated together for a viable outcome of the resolution process. A resolvase activity must produce nicks in the DNA that can be repaired. To test if the cleavage sites can be ligated, reaction products are incubated with T4 DNA ligase. The uncleaved junction will be visualised as a single labelled oligonucleotide band by denaturing PAGE. The cleavage products will run further through the polyacrylamide gel. If the T4 DNA ligase can ligate the nicked duplex to restore the DNA backbone of the cleaved strand, the ligated products will now run on denaturing PAGE as full-length oligonucleotides. The results of this experiment show this. Both the cellular and the purified YDC2 resolvase activities produced nicks that could be efficiently repaired by T4 DNA ligase (Figure 4.6.3.).
Figure 4.6.1. YDC2 and *S. pombe* fraction 3 cut each strand of junction X12 at identical sites. Four preparations of junction X12, each labelled uniquely at the 3’ end of one strand, as indicated, were used in separate reactions containing 5 ng of DNA and ~0.5 μg YDC2 or 2 μl of fraction 3 in 20 μl reaction buffer. Control reactions contained no added protein. Samples were incubated at 30°C for 60 min and processed as described in Methods. The products were resolved by 12% PAGE and visualised by autoradiography. The bands produced are marked with asterisks. Maxam and Gilbert sequencing reactions specific for purines (A+G) or pyrimidines (T+C) are shown for each labelled junction. This figure was kindly produced by Dr. M.Oram.
Strand 1
A + G
C + T
Frac. 3
YDC2

Strand 2
A + G
C + T
Frac. 3
YDC2

Strand 3
A + G
C + T
Frac. 3
YDC2

Strand 4
A + G
C + T
Frac. 3
YDC2
Figure 4.6.2. Schematic presentation of the central sequences of junction X12 cleaved by YDC2. The positions of cleavage sites identified (Figure 4.6.1) are indicated with green arrows of varying size to illustrate the differences in cutting efficiencies. RuvC cleavage sites are also shown for comparison with blue arrows (Bennett et al., 1993). The central region of homology in junction X12 is shown in red. The position of crossover is shown arbitrarily.
Figure 4.6.3. Repair by DNA ligase of nicks produced by YDC2 and *S. pombe* cellular activity. (A) Schematic diagram of a 4-way junction, labelled on one strand (*). Resolution of the junction via a cut on the labelled strand yields a much shorter labelled product. The full-length strand is restored when the nick is repaired by DNA ligase. (B) Ligation of nicks produced by the recombinant YDC2 on strands 2 and 4 of junction X12. (C) Ligation of all four strands of X12 cut by fraction 4. Cutting assays contained 5 ng of junction X12 labelled uniquely at the 3' end of the indicated strand and 2 μl of either YDC2 or fraction 4 as described previously. After incubation for 60 min at 30°C, one half of each reaction was stopped. The remainder of the reaction was treated with 10 units of T4 DNA ligase in the presence of 1 mM ATP for a further 30 min at 37°C. The reactions were analysed by denaturing 12% polyacrylamide gel electrophoresis followed by autoradiography. This figure was kindly produced by Dr. M. Oram.
A. Resolvase → X → Ligase

B. YDC2
- + +
Ligase
- - +

C.

<table>
<thead>
<tr>
<th></th>
<th>Strand 1</th>
<th>Strand 2</th>
<th>Strand 3</th>
<th>Strand 4</th>
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<tbody>
<tr>
<td>Fraction 4</td>
<td>- + +</td>
<td>- + +</td>
<td>- + +</td>
<td>- + +</td>
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<tr>
<td>Ligase</td>
<td>- - +</td>
<td>- - +</td>
<td>- - +</td>
<td>- - +</td>
</tr>
</tbody>
</table>
4.7 Holliday junction-specific DNA binding

To test for Holliday junction binding, YDC2 was analysed by gel electrophoretic mobility shift experiments. The cloned and purified YDC2 bound specifically to junction X12 in the presence of a 500-fold excess of linear duplex DNA (Figure 4.7). The appearance and accumulation of a protein-junction complex could be observed as a prominent retarded band in binding reactions containing increasing amounts of recombinant YDC2. A second and heavier protein-DNA complex could be observed at higher protein concentrations. The major protein-junction complex showed a slower electrophoretic mobility than a RuvC-junction complex used as a control. As RuvC (19 kDa) has been shown to bind to Holliday junctions as a dimer (Shah et al., 1997), this result is consistent with YDC2 (30 kDa) also binding as a dimer. The affinity for the junction was much greater than the affinity of YDC2 for duplex DNA as the presence of a 1000-fold molar excess of linear duplex competitor DNA did not prevent binding.

4.8 pH Optimum

YDC2 protein was used in several assays to ascertain a pH for optimum cutting. Different buffers at overlapping pH were used to confirm that a preferred pH was not the result of preferred buffer. The results in figure 4.8 present evidence for both pH and buffer preference. Considering pH alone, figure 4.8 shows that YDC2 resolved the junction more efficiently at alkaline pH. Resolution was seen in both phosphate and Tris buffers that extend into an alkaline pH. This demonstrates that the improved cleavage seen at alkaline pH was a genuine pH effect and not the result of buffer type. Considering buffer type, YDC2 resolved efficiently in both Tris and phosphate buffers. YDC2 did not resolve the Holliday junction in citrate. The limited resolution seen at pH 6.0 in phosphate buffer was not seen in citrate buffer at the same pH. It could be that the enzyme does exhibit a preference for phosphate buffer to citrate buffer or that citrate buffer inhibits the enzyme.
Figure 4.7. YDC2 binds specifically to the four-way junction X12. Increasing amounts of YDC2 were incubated on ice with $^{32}$P-labelled junction X12 pre-mixed with a 500-fold excess of linear duplex DNA, as described in Methods. A control reaction contained RuvC. After 20 min, gel-loading buffer was added and samples were separated on a 6% polyacrylamide gel. The gel was dried and bands visualised by autoradiography.
Figure 4.8. YDC2 cleaves Holliday junctions more efficiently at alkaline pH.
Reactions with 5 ng of DNA were incubated in 10 μl reaction buffers of differing pH with YDC2. The substrate used was junction X12 labelled on strand 2. Reactions with YDC2 protein also contained a 500-fold molar excess of linear duplex DNA. Incubation was for 30 minutes at 30°C. Analysis was by native 6% PAGE followed by autoradiography.
4.9 Efficiency of junction cleavage

RuvC at high protein concentrations inhibits its own cutting of a Holliday junction. To find optimum concentration of YDC2 for cleavage, Holliday junction resolution assays were conducted using increasing concentrations of YDC2. Cutting increased as YDC2 concentration was increased, and eventually reached a plateau. This suggests that YDC2 does not inhibit its own ability to cleave a junction at high protein concentrations. The purified YDC2 protein resolved ~50% of the labelled synthetic Holliday junction at concentrations above 320 nM (Figure 4.9A and B). This concentration was used in other experiments.

4.10 Metal ion and Metal ion concentration

All Holliday junction-specific endonucleases cleave four-way junctions in a divalent metal dependent manner. To discover the metal ion required to support cleavage, YDC2 was incubated with labelled junction in the presence of different metal ions. YDC2 cleaved the junction in the presence of Mg$^{2+}$ and Mn$^{2+}$ but would not cut in the presence of Zn$^{2+}$ or Cu$^{2+}$ (Figure 4.10A). Reactions using increasing concentrations of metal ion Mg$^{2+}$ were used to determine the optimum concentration for cleavage. The optimum concentration of magnesium was 15 mM although cutting was observed at between 5-20 mM magnesium sulphate (Figure 4.10B). Although YDC2 would not cut in the absence of magnesium, binding to the junction was observed in the presence of EDTA (Figure 4.7).
Figure 4.9. The effect of increasing protein concentration on junction resolution. (A) Increasing concentrations of YDC2 were incubated with 5 ng of junction X12 labelled at the 5’ end. After incubation for 35 minutes at 30°C the reaction was stopped. The reactions were analysed by native 6% polyacrylamide gel electrophoresis dried on 3 MM Whatman paper and quantified using a phosphorimager as described in methods. (B) Quantified results were used to plot a graph of the effect of increased protein concentration on percentage of junction cut.
Figure 4.10. YDC2 requires the presence of a specific divalent cation for resolution. (A) Reactions with 5 ng of DNA were incubated in 10 μl reaction buffers containing differing metal ions with YDC2. (B) Reactions with 5 ng of DNA were incubated with YDC2 in 10 μl reaction buffers containing increasing amounts of magnesium chloride. The substrate used in both experiments was the junction X12 labelled on strand 2. Reactions contained a 500-fold molar excess of linear duplex DNA. Incubation was for 30 minutes at 30°C. Analysis was by native 6% PAGE followed by autoradiography.
<table>
<thead>
<tr>
<th>Junction</th>
<th>Magnesium</th>
<th>Manganese</th>
<th>Copper</th>
<th>Zinc</th>
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<table>
<thead>
<tr>
<th>Junction</th>
<th>Mg$^{2+}$ concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>10</td>
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</table>
4.11 Temperature and salt concentration optimum

YDC2 would efficiently cleave Holliday junctions at temperatures between 25°C and 34°C (Figure 4.11A). The optimum temperature for cleavage by the resolvase was ~ 30°C. Temperatures above 34°C did not improve the cutting efficiency. YDC2 would also cut at RT. These results are consistent with the growth temperature of budding yeast.

The efficiency of YDC2 cleavage remained the same at up to 200 mM NaCl concentration tested (Figure 4.11B). The presence of potassium chloride was also tested as it had been used in some resolution assays. The efficiency of YDC2 cutting was not affected by KCl at a concentration of 50 mM.

4.12 *E. coli* RuvB/YDC2 branch migration

YDC2 was shown to bind specifically to a synthetic Holliday junction. Cleavage of the Holliday junction was demonstrated only when the correct sequence was present in the junction. Presenting YDC2 with a junction containing the preferred sequence for cutting *in vitro* is probably quite a different situation from the one faced by YDC2 *in vivo*. Despite the high AT content of mtDNA, YDC2 may not always be presented with its recognition sequence. The resolvase could work in conjunction with a DNA helicase like the *E. coli* protein RuvB to branch migrate the complex to a preferred cleavage site. To investigate if YDC2 could possibly branch migrate as a complex we conducted experiments to test whether see if YDC2 could in any way work in conjunction with RuvB. RuvB-YDC2 branch migration assays were conducted under the same conditions used to demonstrate branch migration by RuvC (van Gool et al., 1998). YDC2 was used at varying concentrations and with different orders of addition of YDC2 and RuvB. Branch migration was not seen in any experiments.
Figure 4.11. The effect of temperature and salt concentration on YDC2 Holliday junction resolution. (A) Reactions with 5 ng of DNA in 10 μl were incubated with YDC2 and incubated for 30 minutes at 25, 30, 34, or 37°C, as indicated. (B) Reactions with 5 ng of DNA were incubated with YDC2 in 10 μl reaction buffers containing increasing amounts of sodium chloride, as indicated. Incubation was for 30 minutes at 30°C. The substrate used in both experiments was the junction X12 labelled on strand 2. Reactions contained a 500-fold molar excess of linear duplex DNA. Analysis was by native 6% polyacrylamide gel electrophoresis followed by autoradiography.
### A

<table>
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### B

<table>
<thead>
<tr>
<th>Junction</th>
<th>Na⁺ conc.(mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50</td>
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</tbody>
</table>

![Image of gel electrophoresis with bands for each temperature and Na⁺ concentration]
4.13 Discussion

A novel eukaryotic Holliday junction resolvase was identified in *S. pombe* cell extracts using a biochemical approach. The cellular activity was shown to be a true resolvase as it cleaved Holliday junction-containing Chi-DNA in both orientations (N-S, E-W) and cut the synthetic four-way junction X12. There was no activity on duplex DNA, mismatched regions or a three-way junction (data not shown). In parallel, the putative CCE1 homologue of *S. pombe* was cloned and expressed in *E. coli* and shown to be a functional Holliday junction resolvase in vitro. The two enzymes were biochemically indistinguishable in all our experiments: they showed the same substrate specificity, nicked identical sites in synthetic junctions and produced ends which were efficiently repaired by DNA ligase. Different resolvases can produce a very specific pattern of major and minor cleavage sites on synthetic junctions (Dunderdale et al., 1994; White and Lilley, 1996), but these sometimes individual spectrum of cleavage sites cannot be used as evidence for identity.

The results in this chapter also show that our initial assay conditions were fortuitously near to optimum. YDC2 protein efficiently cleaved the synthetic Holliday junction at alkaline pH in Tris-HCl buffer, and the magnesium concentration of 10 mM was near to the optimum 15 mM. YDC2 would bind both junctions X12 and X0 in the presence of EDTA but would not produce a band shift on PAGE electrophoresis with duplex DNA and duplex DNA containing a 6 bp region of non-homology (data not shown). We can conclude that the protein binds to the junction in a cation-independent structure specific manner. Binding of the junction as a dimer is probably the functional quaternary structure of the protein for resolution. The concentration of YDC2 required to obtain band shifts was higher than concentrations published by other groups who also reported similar results independently. However, other groups used NaCl at a concentration of 200 mM in their binding buffers and did not use competitor DNA (Whitby and Dixon, 1997; White and Lilley, 1997). Their later experiments using competitor DNA in the absence of NaCl show band shift results at YDC2 concentrations very close to ours. The use of competitor
DNA in all reactions was considered to reflect the \textit{in vivo} reaction conditions more accurately than reactions without competitor DNA.

Figure 4.7 shows that at high concentrations of YDC2 (800 nM) a second protein-DNA complex forms. This presumed complex of two dimers of YDC2 binding to the junction is probably not the functional form of the protein. As the graph figure 4.8B shows, increasing protein concentration above 800 nM did not improve cutting. This further suggests that the heavier YDC2-junction complex is not a functional form of the enzyme and that YDC2 functions as a dimer. The second YDC2 dimer could bind elsewhere on the arms of junction or not have direct access to a cleavable site.

Resolution of the junction required the presence of either magnesium or manganese and demonstrated a certain degree of sequence specificity. This is in contrast to binding that was magnesium-independent and showed no sequence specificity. Cleavage of the junction X12 was symmetric and on strands of like polarity. The cleavage site was always 3' of a thymine nucleotide at or near the point of crossover. The sequences in junction X12 present a variety of possible cleavage sites. This junction was cut with a preference for 5'-CXT-3'. Clearly, this sequence needs to be present in a specific structural context, such as distance from the crossover point and overall conformation of the junction. Cleavage of junction X0 was not seen initially using junction labelled on strand 6. However when junction X0 was labelled on strand 7, some inefficient cleavage was detected by denaturing PAGE (Figure 4.13A). The inefficient cleavage of junction X0 should be expected as the junction does not contain symmetric cleavable sites located at or near the point of crossover (Figure 4.13B).
Figure 4.13. YDC2 resolution of junction X0. (A) YDC2 was incubated with 5ng junction X12 or junction X0, as indicated. Junction X12 was labelled on strand 2, junction X0 was labelled on strand 7. Both junctions were labelled at the 5' end. After incubation for 35 minutes at 30°C reactions were stopped, analysed on 8% denaturing PAGE and visualised by phosphorimagner. (B) Schematic presentation of the central sequences of the four-way junction X0 and (C) the three-way junction.
YDC2 has been reported to cleave a 3-way junction (Whitby and Dixon, 1998) with very low efficiency. The Y junction used in our experiments, labelled on strand 8, would not reveal cleavage of the Y-junction (data not shown). This is probably due to the fact that our 3-way junction does not contain a cleavable sequence located in a specific structural context (Figure 4.13C). Construction of a different Y-junction containing YDC2’s preferred consensus sequence at the crossover would probably demonstrate that an Y-junction could be cut.

These results suggest that YDC2 behaves functionally in a way similar to the other resolvases RusA, RuvC, and CCE1, and differs from the resolvases T7 endonuclease 1, and T4 endonuclease 7 that have a more relaxed structural and junction sequence requirements. The characteristics of YDC2 show a conservation of function between YDC2 and CCE1 as well as some conservation of protein sequence. We can conclude that YDC2 is a true homologue of CCE1.
5. *S. cerevisiae*

5.1 Assay for Holliday junction-processing activities in *S. cerevisiae*

Crude cell-free extracts from *S. cerevisiae* BJ2168 were fractionated on chromatography supports (DEAE, Heparin-Sepharose, ResourceQ, ResourceS). Fractions were tested for branch migration of alpha-structures made by RecA-mediated strand exchange. Fractionated extracts did not show branch migration products under conditions where *E. coli* RuvAB control proteins formed nicked circle products (data not shown). Fractions were not tested for Holliday junction resolution as CCE1 activity could not be differentiated from other endogenous resolvases.

The only eukaryotic Holliday junction-specific endonucleases identified at the gene level so far, are the mitochondrail resolvases from yeast. We wanted to identify a resolvase activity or Holliday junction-processing activity that acted on nuclear DNA. The advantages of the yeast *S. cerevisiae* are that the repair of DSBs occurs predominantly by homologous recombination, they have an active recombination apparatus, a large yeast cell mass can be easily grown to early log stage of growth and cell densities of \(1 \times 10^8\) cells can be harvested. In addition, a strain of *S. cerevisiae* was available that was a *ccele*\(1\) null mutant. Using a strain where the mitochondrial resolvase gene had been deleted would be a considerable advantage to identifying a nuclear resolvase compared to groups that published results of resolvase activity prior to the identification of CCE1.

*S. cerevisiae* strains SK19 and 20

Both strains SK19 and SK20 were kindly provided by Dr. R. Sternglanz. Prof. B. Kemper kindly sent us SK20. These strains were constructed during the cloning of *CCE1*. The entire *CCE1* coding region with the exception of the first codon was deleted and replaced by *LEU2* in these two strains (Kleff et al., 1992). SK19 and SK20 were of opposite mating types and were crossed to produce a diploid strain AK47. All three strains were used to test for Holliday junction-processing activities.
5.2 Strain characterisation

PCR and Southern analysis

The absence of the *CCE1* gene was confirmed by PCR (Figure 5.2). Genomic DNA from SK20, the first strain tested for Holliday junction-processing activity, was used as a template for the amplification of the *CCE1* gene using the following primers:

      5’primer - ATGTCGACAGCCACAGAAAGCTAA
      and 3’primer - TTAGTCATTGTTGTAAGTGTTCTGC.

Genomic DNA from *S. cerevisiae* strain BJ2168 was also used as a template for the amplification of the *CCE1* gene as a control. The *CCE1* gene would not amplify by PCR using SK20 genomic DNA, however *CCE1* was amplified to give the predicted 1062 bp fragment using strain BJ2168 genomic DNA. Both genomic DNAs were also used to amplify the *LEU2* gene to ensure that the SK20 genomic DNA used was amenable to PCR. The primers for *LEU2* were:

      5’primer - CCCCTAAGAAGATCGTCGTTT
      and 3’primer - TCTTAACTTCTTCCGCGACA.

The *LEU2* gene was amplified using both genomic DNAs to produce the expected 1074 bp fragment (this fragment is 21bp smaller than the expected *LEU2* gene due to the design of the primers).

To verify that *CCE1* was deleted in both haploid SK19 and SK20, the strains were kindly checked by Southern blot analysis by Judit Arenas using *CCE1* and *LEU2* to probe genomic DNA. Both Southern analysis and PCR confirmed the reported genotype of the strains which were both deficient for the *CCE1* gene. Control analysis showed the presence of the *LEU2* gene.
Table 5.2: PCR analysis of *S. cerevisiae* cce1Δ strain SK20. Both *S. cerevisiae* BJ2168 and SK20 genomic DNA were tested by PCR. Lane a Promega 1kb DNA ladder. Lanes b and d BJ2168 Lanes c and e SK20. Lanes b and c primers were for CCE1. Lanes d and e primers were for LEU2.
The published mating types of the two strains differed from the provided mating type. Strain analysis demonstrated that SK19 was \( MAT_\alpha \) and SK20 was \( MATa \). This was later confirmed with both groups that donated the strains. To see if the resolvase activity could be enriched at a specific stage of the cells cycle, experiments were undertaken that used alpha factor to synchronise the cells. During these experiments SK20 underwent the characteristic "smooing" of cells, confirming the SK20 mating type as \( MATa \). FACS analysis provided evidence for a doubling of DNA content in the diploid AK47.

**Phenotype analysis**

To check the phenotypes of strains SK19, SK20 and AK47, all auxotrophic markers were tested by growing each strain separately on several minimal media plates containing a combination of supplements (adenine sulphate, uracil, \( l^- \)tryptophan and \( l^- \)histidine-HCl all at 20 mg/litre) minus one supplement. The strain phenotype agreed with the reported genotype in all cases.

**Table 5.2. Strain Genotype**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK20</td>
<td>( MATa, leu2, trp1, ura3, nuc1::LEU2, cce1\Delta::LEU2 )</td>
</tr>
<tr>
<td>SK19</td>
<td>( MAT_\alpha, leu2, trp1, ura3, his3, ade2, nuc1::LEU2, cce1\Delta::LEU2 )</td>
</tr>
<tr>
<td>AK47</td>
<td>( MATa MATa, leu2/leu2, trp1/trp1, ura3/ura3, his3/HIS3, ade2/ADE2, nuc1::LEU2/nuc1::LEU2, cce1\Delta::LEU2/cce1\Delta::LEU2 )</td>
</tr>
</tbody>
</table>

**5.3 Detection and fractionation of a Holliday junction resolvase**

Initially crude extracts prepared from \( cce1\Delta \) cells were loaded onto various chromatography supports. Fractionated extracts were tested for Holliday junction resolution using synthetic Holliday junctions. A Holliday junction activity was identified and results are shown later in this chapter. A purification
scheme needed to be devised to allow characterisation of the activity. The activity could not be detected in crude extracts.

Table 5.3. Chromatography column performance

<table>
<thead>
<tr>
<th>Column</th>
<th>Performance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macro-Prep DEAE</td>
<td>Activity bound, activity present in flow-through.</td>
</tr>
<tr>
<td>Heparin-Sepharose</td>
<td>Activity bound, no activity in flow-through.</td>
</tr>
<tr>
<td>Resource S</td>
<td>Activity bound, strong nuclease when used early in scheme, good separation when used later in the scheme.</td>
</tr>
<tr>
<td>Source 15 S</td>
<td>Activity bound, used for large scale, equivalent to Resource S.</td>
</tr>
<tr>
<td>Superose 12</td>
<td>Good resolution, reproducible, limited by sample volume.</td>
</tr>
<tr>
<td>Mono Q FPLC</td>
<td>Activity bound, sharp protein peaks eluted.</td>
</tr>
<tr>
<td>Macro-Prep CM</td>
<td>Activity bound, activity present in flow through. DEAE column retained more activity or CM column retained more inhibitors of the activity.</td>
</tr>
<tr>
<td>Resource Q</td>
<td>Activity bound, Resource S gave sharper protein peaks.</td>
</tr>
<tr>
<td>Macro-Prep High Q</td>
<td>Activity bound, activity present in flow-through DEAE column retained more activity or High Q retained more inhibitors of the activity.</td>
</tr>
</tbody>
</table>

Macro-Prep DEAE was a good column for initial capture of the activity. There was frequently some activity present in the flow-through that could be bound to another column, but the majority of the activity was bound to the DEAE column. Making an approximate comparison between total protein in the crude extract and the column binding capacity suggested that the column was not overloaded and using a larger DEAE column resulted in diluting the activity at this stage, that was detrimental to its longevity. Stronger anion-exchange resins like Macro-Prep High Q did not significantly capture more activity.
Various combinations of columns were also tested. Although heparin-Sepharose would bind all the activity without activity flowing through the column, the purification scheme could be extended further if DEAE was used as the first chromatography step. Use of a Resource S column early in the fractionation scheme revealed the presence of a strong nuclease activity. However use of the Resource S column in the late stages of purification resulted in good separation of the activity from other proteins and the activity eluted from the column as a tight peak. For small-scale work (up to 8 litres) purification scheme A was used, for large-scale work, scheme B was used (Figure 5.3.1). In scheme A fractions that eluted from Resource S were small enough to be concentrated to the maximum sample loading volume of the gel filtration column, 240 µl. This was not the case in scheme B as the fractions were too large to concentrate at this stage.

**Ammonium sulphate precipitation**

Ammonium sulphate precipitated the activity at between 20 and 40% saturation. Although reproducible on a small scale (1-2 litres of cell culture), the ammonium sulphate precipitation was less reproducible with larger cell cultures. Another problem encountered in large-scale work was the tendency of the activity to precipitate on dialysis at high protein concentrations. Most likely the resolvase co-precipitated with other proteins as some activity in the precipitate would go back into solution in 1M NaCl. This problem was partly overcome by diluting the fractions. The loss of activity encountered during ammonium sulphate precipitation could also be due to the activity precipitating at high protein concentration. Re-dissolving the 40% ammonium sulphate precipitate in a small volume of buffer and dialysing the protein suspension would result in high protein concentration that could lead to the resolvase activity precipitating.
Figure 5.3.1. Scheme for partial purification of a resolvase activity from haploid and diploid cells of *S. cerevisiae cce1Δ* strain SK19, SK20, and AK47. (A) Small scale purification scheme. (B) Large scale purification scheme.
Yeast cells

A

Resource S (0.6 - 0.8 M NaCl)

Fraction V

Superose 12 gel filtration

Fraction IV

Heparin-Sepharose (0.9 - 1.0 M NaCl)

Fraction III

Macro-Prep DEAE (0.6 - 1.0 M NaCl)

Fraction II

Crude cell-free extract

B

Mono Q FPLC

Superose 12 gel filtration

Source 15S

Heparin-Sepharose

Macro-Prep DEAE

Crude cell-free extract

Yeast cells
Growth conditions

The growth stage at which cells were harvested was important for detecting the resolvase activity. A large culture of cells was inoculated and growth was followed until stationary phase. One litre of cells was removed at early, mid and late log phases of growth. A cell free-extract was prepared for each sample using an equal number of cells and was fractionated by chromatography on DEAE Macro-Prep. Fractions tested for Holliday junction resolution showed the highest levels of activity when the number of cells was between 1-3x10^7/ml (Figure 5.3.2 and figure 5.3.3). This correlates with the cells being in early log phase of growth and when mitotic activity is high.

As the cells were auxotrophic for certain nutritional requirements, YPG media was supplemented with adenine sulphate, uracil, L-tryptophan, and L- histidine-HCl (all at 20 mg/litre) to improve the growth conditions. A cell-free extract was prepared, fractionated on DEAE and assayed. Very little activity was detected in the eluted fractions. A second extract showed similar results. The only difference from previous extractions was the addition of supplements to the growth media. A direct comparison of cells grown in YPG media with or without supplements confirmed these observations. The level of activity was higher in the unsupplemented media where the cells were growing at a slower rate (Figure 5.3.4). A possible explanation for this result is that the cells in unsupplemented media grow slowly enough to reveal the activity in its window of expression in the cell cycle. The idea of cellular regulation of the activity is appealing, as Holliday junctions have been shown to accumulate in S-phase of the cell cycle (Zou and Rothstein, 1997).

5.4 Holliday junction resolution activity from AK47 cells

A Holliday junction resolvase activity was detected initially in the SK20 cceIΔ strain. Cells from the opposite mating type SK19 and AK47 were also tested. All three strains were grown separately to a density of between 1 and 3x10^7 cells/ml. Individual 1 litre cultures were used to make cell-free extracts.
Figure 5.3.2. Elution profile of fractionated extracts from SK20 cells at early, mid and late log phase of growth. Elution of crude cell extract from Macro-Prep DEAE chromatography. Identical fractions were assayed using junction X12 as described in Methods. Products were analysed by denaturing PAGE. Elution profiles of the peak of activity are shown at (1) early log phase, (2) mid log phase and (3) late log phase of growth.
Figure 5.3.3. Growth curve of SK20 cells.
Figure 5.3.4. Comparison of activity from diploid AK47 cells grown in supplemented and unsupplemented media (A) Elution profile of crude cell extract from Macro-Prep DEAE chromatography, using cells grown in unsupplemented media and (B) supplemented media. Fractions were assayed using junction X12 as described in Methods and products were analysed by non-denaturing PAGE. Elution profiles of the peak of activity are shown in both cases.
Each extract was fractionated on a 0.5 ml DEAE Macro-Prep. Fractions of 0.5 ml were collected and assayed as described in Methods. For reliable identification of the activity, fractions from the initial DEAE chromatography step were concentrated 5 to 10-fold for the assays. All three strains, SK19, SK20, and AK47 were shown to contain a resolvase activity that cut a synthetic Holliday junction. The activity was identified following chromatography on a number of ion-exchange columns but could not be detected in crude extracts. Figure 5.4.1 shows a typical elution profile from DEAE chromatography. The cell-free extract was from diploid cells. The elution profile was from a large-scale preparation and is typical of both a small and large-scale preparation. The main peak of activity was preceded by a strong nuclease and the activity appeared to elute as two peaks. This could be the result of a nuclease co-eluting with the resolvase activity. The assays were performed in the presence of competitor duplex DNA. The use of competitor DNA in the assays was initially introduced to compete out the nuclease activity and later to demonstrate the high specificity of the resolvase for the junction.

The initial detection of a possible resolvase activity at this stage prompted its further purification. The products seen in non-denaturing PAGE needed to be analysed on a denaturing gel. Discrete cleavage site(s) would confirm that the activity was a true resolvase. Visualisation of cleavage products from Macro-Prep DEAE fractions on a denaturing gel would be inconclusive due to the presence of several nuclease activities in the eluted fractions that would cut unspecifically the labelled junction.

To confirm that the activity from DEAE chromatography produced true resolution products and not artefacts produced by nucleases, the cellular activity was partially purified using scheme A (Figure 5.3.1). Aliquots from fraction V, the final chromatography column, were used to show resolution of the synthetic junction X12 analysed by both neutral (Figure 5.4.2A.) and denaturing PAGE (Figure 5.4.2B).
Figure 5.4.1. Identification and initial fractionation of a resolvase activity from diploid cells of *S. cerevisiae* cce1Δ strain AK47. Elution of crude cell extract (fraction I) from Macro-Prep DEAE chromatography. Fractions were assayed using junction X12 as described in Methods and products were analysed by non-denaturing PAGE.
Figure 5.4.2. The activity from diploid *S. cerevisiae cce1Δ* cells shows specificity and selectivity for resolving four-way junctions. Reactions with 10 ng of junction X12 labelled on strand 2 were incubated in 20 μl reaction buffer with 0.5 μl CCE1, 0.5 μl YDC2 or 2 μl fraction V in the presence of a 500-fold molar excess of linear duplex DNA. Incubation was for 30 minutes at 30°C. Half the reaction was analysed by non-denaturing 6% PAGE followed by phosphorimager analysis (A), and the other half by denaturing 8% PAGE followed by phosphorimager analysis (B). Active fractions from Heparin-Sepharose were tested as described in Materials and Methods with junction X12 (C), and in parallel using junction X0 (D). Reactions using junction X12 and X0 were analysed on non-denaturing 6% PAGE followed by phosphorimager analysis.
A

<table>
<thead>
<tr>
<th>Ctrl</th>
<th>CCE1</th>
<th>Fr V</th>
<th>YDC2</th>
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B

<table>
<thead>
<tr>
<th>Ctrl</th>
<th>CCE1</th>
<th>Fr V</th>
<th>YDC2</th>
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C

Junction X12

<table>
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<th>RuvC</th>
<th>Heparin-Sepharose chromatography</th>
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D

Junction X0

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<tr>
<th>Control</th>
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<th>Heparin-Sepharose chromatography</th>
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Non-denaturing PAGE showed that incubation of Fraction V with synthetic junction X12 produced one labelled DNA species that was identical to the nicked duplex produced by YDC2, CCE1 (Figure 5.4.2A) and RuvC (Figure 5.4.2C). No other labelled products could be detected. The denaturing PAGE (Figure 5.4.2B) showed that strand 2 on junction X12 was cleaved by Fraction V at the same point as YDC2 and CCE1, but this site differed from that cut by RuvC (not shown). The cleavage site was situated near the point of crossover of this synthetic Holliday junction (Bennett et al., 1993) within the 12 bp region of homology (5' - ATGTCCT↓AGCAA-3'). The single cut seen on the denaturing gel and the production of a duplex product band on the non-denaturing gel confirmed that the resolvase was cutting across the junction and producing nicked duplex DNA. This property of the enzyme would be essential \textit{in vivo} to release the two duplex DNA molecules joined by a Holliday junction.

The activity in Fraction V was highly specific for the junction, as it could not be competed out by 500-fold molar excess of linear duplex DNA and no cleavage of a labelled single-stranded 62-mer oligonucleotide could be detected (data not shown). The resolvase required 10–15 mM Mg\(^{2+}\) ions for optimum activity but no cleavage could be detected in the presence of Ca\(^{2+}\).

Fractions from Heparin-Sepharose chromatography were used to compare cutting of junction X12 (Figure 5.4.2C), which contains a homologous core, with cutting of junction X0 (Figure 5.4.2D), which does not contain a core of homology or cleavage sites symmetric to the point of crossover. Although some cleavage of junction X0 could be observed, the efficiency of cutting was very low (Figure 5.4.2D) compared to cutting of junction X12 (Figure 5.4.2C). This indicates that the resolvase has sequence selectivity for cleavage of a four-way synthetic junction.

5.5 The cleavage sites of the activity differ from those of CCE1

The cleavage sites of the partially purified yeast resolvase were mapped at high resolution on all four strands of junction X12 (Figure 5.5.1, kindly produced by Dr. M. Oram). As initial results from denaturing gels revealed that the
resolvase activity from AK47 cells had a similar cleavage site on one arm of junction X12 as YDC2, we sought a comparison with the cleavage patterns produced by the activity, YDC2 and CCE1 on all four strands. The *S. cerevisiae cce1Δ* cellular activity, YDC2, and CCE1 were incubated with four preparations of junction X12 each labelled at the 3′ end of a different strand (Figure 5.5.1). The junction was cleaved at identical sites on strands of like polarity (1 and 3, and 2 and 4) and these sites were symmetric to the point of crossover. The cleavage sites, which all mapped within the region of homology of junction X12, are shown schematically in figure 5.5.2. Positions of cleavage were determined by comparison with sequencing ladders for each strand of junction X12 cleaved specifically for purines or pyrimidines, by the method of Maxam and Gilbert (Sambrook et al., 1989) (data not shown). Although minor sites were observed one to two nucleotides below the main cut sites, these were seen with all the enzymes used, including RuvC, and were attributed to the synthetic oligonucleotide preparations rather than an activity intrinsic to the resolvases. RuvC cleavage sites were also mapped as shown on the diagram, and were in agreement with sites mapped previously on this junction (Bennett et al., 1993; Oram et al., 1998).

The cleavage pattern of *S. cerevisiae cce1Δ* cellular activity was different from that of the mitochondrial resolvase CCE1. This clearly establishes that the partially purified *S. cerevisiae cce1Δ* activity has different sequence preferences for cleavage compared with CCE1. The activity cut at the same sites as YDC2 on all four strands of junction X12 (Figure 5.5.1). This was somewhat unexpected as the pattern of cleavage sites produced by a particular Holliday junction resolvase has been considered to be a unique signature mark for an individual enzyme (Oram et al., 1998; Whitby and Dixon, 1997). However, the use of a synthetic junction with one particular sequence combination presents only a limited number of cleavage sites and may not reveal more subtle differences in sequence preference. Junctions containing other sequences would need to be tested to reveal if the two resolvases show identical sequence specificity for junction cleavage.
<table>
<thead>
<tr>
<th>Strand 1</th>
<th>Strand 2</th>
<th>Strand 3</th>
<th>Strand 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Fr V</td>
<td>CCE1</td>
<td>YDC2</td>
</tr>
<tr>
<td>Control</td>
<td>Fr V</td>
<td>CCE1</td>
<td>YDC2</td>
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<tr>
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<td>YDC2</td>
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<tr>
<td>Control</td>
<td>Fr V</td>
<td>CCE1</td>
<td>YDC2</td>
</tr>
</tbody>
</table>

**Figure 5.5.1. S. cerevisiae cce1Δ activity, CCE1 and YDC2 cut each strand of junction X12 at sites symmetric to the point of crossover.** Denaturing PAGE analysis of cleavage products. Junction X12 was labelled separately on all four strands and incubated with fraction V, CCE1, YDC2, or RuvC. Cleavage products were visualised by phosphorimager.
Figure 5.5.2. *S. cerevisiae* cce1Δ activity, CCE1 and YDC2 cut each strand of junction X12 at sites symmetric to the point of crossover. Schematic of junction X12, showing Holliday junction cleavage sites. Arrows indicate sites of cleavage determined by comparison with sequencing ladders for each strand of junction X12.
5.6 Cleavage sites are nicks that can be ligated *in vitro* to restore a continuous DNA backbone

Resolution of the synthetic Holliday junction produced DNA duplexes containing nicks. When these products were incubated with T4 DNA ligase the nicks were efficiently ligated (Figure 5.6). The yeast resolvase thus produced single nicks with 3'-OH termini that could be repaired by ligation, which is characteristic of all resolvases studied so far. The restoration of full length labelled oligonucleotide demonstrated that the resolution sites were symmetric on strands of like polarity and the partially purified activity did not exhibit non-specific nuclease activity.

5.7 Fractions containing Holliday junction cleavage activity bind synthetic Holliday junctions

To test for specific Holliday junction binding, fractions containing the resolvase activity were incubated with labelled synthetic junction in the absence of Mg²⁺. Formation of specific protein-DNA complexes was tested by electrophoretic mobility shift analysis. Peak fractions from the Heparin-Sepharose chromatography were assayed for Holliday junction resolution and, in parallel, were used in band shift experiments (Figure 5.7). Both experiments were done in the presence of excess competitor DNA. Fractions that cut the synthetic Holliday junction (Figure 5.7A, fractions 10 and 11) produced a band shift (Figure 5.7B, fractions 10 and 11). The mobility of the protein-junction complex was similar to the band produced by RuvC. Upon careful examination of the autoradiographs at lower exposure it appeared slightly more retarded than the RuvC complex. The resolvase activity therefore co-eluted with a specific Holliday junction-binding activity. It is most likely that the resolvase in these fractions binds specifically to the synthetic Holliday junctions.
**Table:**

<table>
<thead>
<tr>
<th>Control</th>
<th>Fraction V</th>
<th>T4 DNA ligase</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**Figure 5.6. T4 DNA ligase repairs nicked DNA.** (A) Junction X12 was incubated with 2 µl fraction V and after 30 minutes half the reaction was stopped. The other half of the reaction was incubated with T4 DNA ligase as described in Methods. Products were analysed by denaturing PAGE and visualised by phosphorimager. (B) Schematic of the assay.
Figure 5.7 Activity from diploid *S. cerevisiae cce1Δ* cells cuts and binds specifically to the four-way junction X12. (A) Peak fractions from Heparin-Sepharose chromatography were incubated with 5 ng of X12 DNA in 10 μl reaction buffer. Reactions also contained a 500-fold molar excess of linear duplex DNA. Control reactions contained either no protein (labelled Control) or 5 ng RuvC (labelled RuvC). Incubation was for 30 minutes at 30°C (37°C for RuvC). Analysis was by non-denaturing 6% polyacrylamide gel electrophoresis followed by autoradiography. (B) In parallel, fractions were incubated on ice with junction X12, pre-mixed with a 500-fold excess of linear duplex DNA, as described in Methods. After 20 minutes, gel-loading buffer was added and samples were separated on a 6% polyacrylamide gel, run with TBE at 4°C with continuous buffer re-circulation. The gel was dried and bands visualised by autoradiography.
A

<table>
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Heparin-Sepharose
0.1-1M NaCl gradient

B

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Heparin-Sepharose
0.1-1M NaCl gradient

Junction-RuvC complex

Junction-protein complex

Junction protein
5.8 Native molecular mass of the Holliday junction resolvase

During the purification process, active fractions from ion-exchange chromatography were concentrated and fractionated by FPLC gel filtration on a Superose 12 column, in buffer A containing high salt (1 M NaCl). The native molecular mass of the activity was determined relative to protein standards ran under identical conditions (Figure 5.8A). A single peak of activity was observed and its relative molecular mass was determined as approximately 24 kDa. Repeating the gel filtration chromatography in buffer A at low salt (0.1 M NaCl) again resulted in a single peak of activity. However, in this case the relative molecular mass of the active fractions was about 50 kDa (Figure 5.8B), approximately twice the size determined in high salt (1 M NaCl) conditions. A likely explanation for this apparent near doubling in mass is that in the high salt buffer the resolvase is a monomer, which forms a dimer at low salt concentrations. All Holliday junction-specific endonucleases studied so far have been shown to bind and cleave the junction as dimers (reviewed in (White et al., 1997)). A dimer of about 50 kDa bound to the Holliday junction would be consistent with the band shifts observed in binding experiments (Figure 5.7B). The complexes produced by fractions containing the partially purified yeast resolvase were retarded slightly more than complexes formed by RuvC, which binds Holliday junctions as a 38 kDa dimer (Iwasaki et al., 1991; Shah et al., 1997).

5.9 RuvB-mediated branch migration

Although no co-operation was seen between RuvB and YDC2 on a Holliday junction, the possibility of the activity from AK47 working with RuvB was still considered, for the same reasons as mentioned in chapter 4. Also the activity, if nuclear, could be expected to work in conjunction with other proteins involved in DNA processing. Fractions IV and V were assayed with RuvB for branch migration of a synthetic Holliday junction (Figure 5.9).
Figure 5.8. Determination of the molecular weight of the Holliday junction specific endonuclease. Active fractions from the heparin-Sepharose column chromatography were concentrated and applied to the Superose 12 gel filtration column in buffer A containing (A) 1M NaCl and (B) 0.1M NaCl. Protein Standards (Bio-Rad) were run under identical conditions in each case. $K_{av} = (\text{elution volume - void volume})/\text{total volume of packed bed - void volume}$). Blue arrow indicates the peak of activity.
A

1.00 - 0.75 - 0.50 - 0.25 - 0.00

Vitamin B-12

Myoglobin

24000 Da

Ovalbumin

Gamma globulin

Thyroglobulin

1000 10000 100000 1000000

Molecular weight Da

B

1.00 - 0.75 - 0.50 - 0.25 - 0.00

Vitamin B-12

Myoglobin

50000 Da

Ovalbumin

Gamma globulin

Thyroglobulin

1000 10000 100000 1000000

Molecular weight Da
Figure 5.9. RuvB-mediated branch migration. (A) Reactions with 10 ng of junction X12 labelled on strand 2 were incubated in 10 μl calcium branch migration buffer with or without ATPγS, and with or without ATP in the presence of a 500-fold molar excess of linear duplex DNA. RuvB (600nM) was pre-incubated with junction X12 for 5 minutes followed by the addition of 2 μl fraction V. Incubation was for 30 minutes at 34°C. Control reactions were junction alone, RuvAB, and RuvB alone. (B). Reactions were also conducted in cutting buffer and calcium branch migration buffer. (C) All reactions were conducted in calcium branch migration buffer. Reactions were analysed by non-denaturing 6% PAGE followed by phosphorimager analysis.
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The results obtained showed the formation of branch migration products. Branch migration required both fraction V and RuvB, since neither RuvB alone nor fraction V gave branch migration products (Figure 5.9A and C). Assay conditions did not require the presence of ATPγS used in RuvBC branch migration assays (van Gool et al., 1998) (Figure 5.9A) and the resolvase-RuvB branch migration worked under the same conditions as RuvAB (Figure 5.9A and B). These experiments were conducted using a calcium assay buffer that allows branch migration by RuvAB but does not support resolution. Both branch migration and resolution could be seen when experiments were conducted in the presence of magnesium (Figure 5.9B). Fractions from the previous chromatography step that would resolve the synthetic junction would not co-operate with RuvB to give branch migration products (Figure 5.9B).

It should be noted that these experiments are preliminary. The branch migration activity was demonstrated in several experiments, including some experiments using fractions that has been stored at -20°C in glycerol for one month (data not shown). The fractions used in these experiments were obtained from the last batch of cells and have therefore not been repeated with activity from another batch of fractionated cells. Although these results need to be repeated they are considered important enough to be included here. This work is being actively pursued in the laboratory.

5.10 Conclusion

These results report the presence of a novel Holliday junction-specific resolvase in *S. cerevisiae*, which is different to the mitochondrial-specific resolvase encoded by *CCE1*. Although resolvase activities from *S. cerevisiae* have been previously identified and characterised (Evans and Kolodner, 1987; Jensch et al., 1989; Parsons et al., 1989; Parsons and West, 1988; Symington and Kolodner, 1985; West and Korner, 1985; West et al., 1987) these studies pre-dated the identification of CCE1 and all employed strains that were *CCE1*+. The
only study to use a cce1Δ null strain did report some residual activity (Kleff et al., 1992), but this activity was not characterised. This work presents the first unequivocal biochemical evidence for a resolvase activity in yeast that is not the mitochondrial-specific activity of CCE1.

The following properties were proposed to be diagnostic of a true Holliday junction resolvase (Hyde et al., 1994): the introduction of specific nicks at or close to the junction point; the introduction of symmetrically related nicks in strands of like polarity; and the generation of single nicks which can be ligated by DNA ligase. The resolvase activity characterised here fulfilled these criteria and displayed similar characteristics as CCE1, YDC2, and RuvC: the resolvase activity introduced nicks symmetrically on two strands of like polarity at the point of crossover of a Holliday junction, which produced two DNA duplex products. Similar to other resolvases, resolution was dependent on the presence of Mg²⁺, but did not occur in the presence of Ca²⁺ (data not shown).

The partially purified resolvase was highly specific for binding and cutting Holliday junctions. During homologous recombination a Holliday junction-specific endonuclease would have to distinguish a Holliday junction from an excess of duplex DNA. The activity from cce1Δ cells bound to the junction even in the presence of 500-fold excess of competitor duplex DNA. The gel filtration data and binding data strongly suggest the activity can bind as a dimer, and probably functions as a dimer. All reactions for characterising the enzyme contained a final NaCl concentration of 0.1 M or less. The gel filtration data suggest that these conditions favour dimer formation.

The resolvase activity showed sequence specificity of cleavage that is clearly different compared to CCE1 and to the S. cerevisiae resolvase reported previously (Jensch et al., 1989; Parsons and West, 1988). The molecular mass of the resolvase as determined by gel filtration was 24 kDa which was different from that reported for CCE1 (40 kDa) (Kupfer and Kemper, 1996), or for another characterised activity from S. cerevisiae (200 kDa) (West and Korner, 1985; West et al., 1987). The relative molecular mass of YDC2 was 35 kDa as
determined by gel filtration under identical conditions (M. Oram and I. Tsaneva, unpublished).

The cleavage sequence specificity of the Holliday junction resolvase poses the question of how the enzyme reaches a cleavable sequence if the only requisite for binding is structure? RuvC has been demonstrated to work coupled to branch migration as part of a RuvABC resolvasome complex (Eggleston et al., 1997; Zerbib et al., 1998), as well as in conjunction with RuvB alone (van Gool et al., 1998). Although the RuvB/resolvase experiments are preliminary, it is conceivable that the resolvase seen in AK47 cells could hold a junction in conformation that would allow a RuvB-like helicase to branch migrate a resolvase bound to the Holliday junction until a cleavable sequence is found, a molecular mechanism similar to the one used by RuvBC mediated branch migration. These results, if correct, could be an in vitro reaction that does not occur in vivo, but could also provide a clue to understanding branch migration in eukaryotes.

We cannot rule out the possibility that the partially purified activity from cce1Δ cells was yet another mitochondrial activity. However, no homologue of CCE1 has been found in the S. cerevisiae genomic sequence. In addition, deletion of CCE1 results in a petite phenotype and abnormal segregation of mtDNA, phenotypes which would not be expected if CCE1 were redundant. This suggests that the new resolvase activity is likely to function on nuclear DNA and that the late stages of recombination in eukaryotes may be functionally analogous to prokaryotes. Identification of the protein and its encoding gene would greatly enhance our current understanding of the molecular mechanisms of homologous recombination in eukaryotes and probably enable identification of nuclear resolvases in higher eukaryotes.
6. MAMMALIAN EXTRACTS

Holliday junction-specific endonucleases have been detected in several mammalian cell types: calf thymus, CHO cells, human placenta and HeLa cells. Some of these activities have been characterised to varying degrees but never identified. As branch migration enzymes have only been identified in bacterial systems the use of RecA-made alpha-structures was considered a suitable substrate to test mammalian cell-free extracts for branch migration activity. RecA-made alpha-structures are much larger than a synthetic junction. The long arms of alpha-structures could be more suitable for large proteins or a complex to attach to. Although detection of branch migration was the primary reason for using the assay, detection of a resolvase activity or any other enzymatic activity could also be pursued if revealed.

6.1 The search for activities: assays of partially fractionated cell-free extracts

Extracts from rat testis were fractionated on a variety of ion-exchange chromatography columns including Resource S, MacroPrep DEAE, heparin-Sepharose, and phosphocellulose, as described in Methods, and all fractions assayed for Holliday junction processing activity using RecA-made alpha-structures.

Initial assays did not detect branch migration products under conditions where using purified proteins RuvA and RuvB from *E. coli* as a control did produce the expected nicked circle branch migration product.

The RecA-made alpha structures could also be used to detect resolution of the Holliday junction. Some fractions from rat testis extracts showed signs of apparent resolvase activity. Figure 6.1 shows assays of fractions obtained from rat testis extracts fractionated by chromatography on a Resource S column eluted with a 0.1-1.0 M NaCl gradient. The appearance of linear dimer and nicked circle products could be observed in lanes 11 and 12 in this figure.
Figure 6.1. Rat testis cell-free extract fractionated on Resource S. Crude cell-free extract was applied to a 1 ml pre-packed Resource S column. The column was eluted with a 0.1 - 1.0 M NaCl gradient. Control assays were incubated with no protein or RuvAB. 2 μl of each fraction or column flow-through (FT) were incubated with substrate for 30 minutes at 37°C. Reaction products were deproteinised and separated by agarose gel electrophoresis as described in Methods. Bands were visualised by autoradiography.
Resolution of the alpha-structures in both orientations would give linear dimer and nicked circle products as seen in control reactions with RuvC, which also shows a pronounced cleavage preference resulting in the production of more linear dimer (Dunderdale et al., 1991) (Figure 3.2.1 and figure 6.2A)

6.2 HeLa cell fractionation

HeLa whole cell extracts were also prepared, applied to a phosphocellulose column, fractionated by a 0.1 - 1.0 M NaCl gradient and assayed for branch migration and resolution using RecA-made alpha-structures as described in Methods (Figure 6.2A). Branch migration was not detected but possible resolution products were seen in lanes 4-6 and 9-11, similar to those observed in fractions from rat testis. Figure 6.2B shows an enlargement of fraction 6. The arrow points to an intense band in the zone of alpha-structures. This band is referred to as a sigma-structure and can be formed by the combined action of an annealing activity and a nuclease.

6.3 ATP independent reactions

Routinely the fractions were assayed in the presence of ATP to detect either or both Holliday junction resolution and branch migration. All resolvases to date cleave the Holliday junction in an ATP-independent manner. To check if the possible resolution activity would be revealed in the absence of ATP, rat testis fraction 12 from Resource S (Figure 6.1) was assayed without ATP or in the presence of ATPγS, the non-hydrolyzable analogue of ATP (Figure 6.3A). The linear dimer and nicked circle products were again observed, suggesting an ATP-independent formation. The intensity of the bands in lanes 4 and 5 is lower compared to those seen before in figure 6.1, lanes 11 and 12. This difference appeared to be due to a nuclease activity that remained active on storage and seemed to increase after freeze-thaw action. This phenomenon was also seen in HeLa extracts (data not shown). The activity in HeLa cells was also shown to be ATP independent (Figure 6.3B)
Figure 6.2. HeLa cell extract fractionated on phosphocellulose. (A) Crude cell-free extract was applied to a 1 ml phosphocellulose column. The column was eluted with a 0.1 - 1.0 M NaCl gradient. Control assays were incubated with no protein, RuvAB, or RuvC. 2 μl of each fraction or column flow-through (FT) were incubated with substrate for 30 minutes at 37°C. Reaction products were deproteinised and separated by agarose gel electrophoresis as described in Methods. Bands were visualised by autoradiography. (B) Enlargement of fraction 6. The arrow points to an additional band in the intermediates which runs as a sigma-structure.
Figure 6.3. Dimer products form without the addition of ATP. Fraction 12 from Resource S (rat testis) (A) and fraction 6 (HeLa) (B) were assayed using alpha-structures in reactions to test for the requirement of ATP for the formation of dimer products. Control assays were incubated with no protein, RuvAB, or RuvC. Reactions contained 2 µl of each fraction and were incubated with substrate for 30 minutes at 37°C. Reactions were with ATP (+ATP), without ATP (-ATP), or with the non-hydrolysable analogue of ATP (ATP$_7$S). Reaction products were deproteinised and separated by agarose gel electrophoresis as described in Methods. Bands were visualised by autoradiography.
### A

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- Linear dimer
- Nicked circle

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- Linear dimer
6.4 Assays to test for ligation

The RecA pairing reaction is timed to maximise production of Holliday junction-containing alpha-structures without the production of nicked circle DNA. A significant fraction of the labelled linear duplex DNA therefore remains unpaired and is present in the alpha-structure preparations. The linear duplex DNA could act as a substrate for other cellular activities and in particular could be a possible substrate for a DNA ligase. A DNA ligase could ligate two linear duplex DNA molecules to form a dimer which could be extended to a trimer, etc. A DNA ligase could also ligate the ends of the linear DNA duplex to form a duplex DNA circle. These products would be very difficult to distinguish from the products of a resolvase acting on RecA-made alpha-structures.

The production of linear dimer products in our assays could be due to the presence of a DNA ligase acting on the linear DNA, or a Holliday junction resolvase acting on the alpha-structures. To differentiate between the two activities, ligation and resolution, fractions were incubated with different substrates. Chi DNA and a synthetic Holliday junction were used to detect resolution, and labelled linear duplex DNA was used to test for ligation. As shown in figure 6.4.1, a fraction incubated with labelled linear duplex DNA alone produced ligation products indicating that a DNA ligase activity was present in the fraction from the Resource S column (lane 3). Control reactions showed that RecA did pair ssDNA and labelled duplex DNA and produced nicked circle DNA in the presence of ATP (lane 5) but not in a reaction without ATP (lane 6). The ligation products however formed in the absence of ATP (lane 4). This can be explained by the presence of endogenous enzyme-adenylate complexes. The presence of these complexes in HeLa cell nuclear extracts was demonstrated at the time when these experiments were conducted (Robins and Lindahl, 1996). Similar ligation products were seen in fractions from HeLa extracts detailed below.
Figure 6.4.1. Fraction 12 incubated with labelled linear DNA. Rat testis, fraction 12 from Resource S was incubated with labelled linear duplex DNA in the presence or absence of ATP, as indicated. Control reactions contained no protein (lane 1), and RecA incubated with labelled linear duplex DNA plus ssDNA (lanes 5 and 6). Arrows point to the ligation products (dimer trimer tetramer), the substrate (labelled linear duplex DNA) and nicked circle DNA (RecA strand exchange products). Reaction products were deproteinised and separated by agarose gel electrophoresis as described in Methods. Bands were visualised by autoradiography.
Figure 6.4.2. Chi DNA reactions. (A) Schematic presentation of Chi DNA structures, labelled at the Sty I ends. Resolution of the Holliday junction in two orientations (North-South and East-West) produces four nicked linear DNA species (three of them labelled) of the sizes indicated. (B) Reactions contained $^{32}$P-labelled Chi DNA with either 5 ng RuvC, or 2 µl fraction 11 and 12 from Resource S in 20 µl reaction buffer. After incubation for 30 minutes at 37°C the reactions were deproteinised. Products were analysed by agarose gel electrophoresis and visualised by autoradiography.
**A**

- Diagram showing a cross with 'Sty', 'N', 'E', 'W', 'S', and 'Scal' labels.
- 'Resolution' arrow pointing downwards.
- 'W/E:' section with 'Sty', 'Scal', and 'Scal' labels.
- 'N/S:' section with 'Sty', 'Scal', and 'Scal' labels.
- Measurements: **2.06 kb**, **1.89 kb**, **2.50 kb**.

**B**

- Table showing 'Junction', 'RuvC', 'Fraction 11', and 'Fraction 12' columns.
- Diagram of Chi DNA and Cleavage products.
- Numbers 1, 2, 3, 4 at the bottom.
Assays using Chi DNA were used to test for Holliday junction resolution (Figure 6.4.2). Fractions that would ligate the labelled linear duplex DNA did not cut the Chi DNA structures, and did not cut a synthetic Holliday junction (data not shown).

The results shown in figure 6.4.1 and 6.4.2 allow us to conclude that the activities first seen in the fractionated cell-free extracts from rat testis and HeLa cells and also seen in yeast, were not a resolvase but a DNA ligase. The presence of endogenous enzyme-adenylate complexes in the extracts would result in the ATP-independent formation of ligation products from the labelled linear DNA present within the preparation of alpha-structures.

6.5 Further investigation of the activities from rat testis and HeLa cells

The production of prominent bands within the diffuse zone of alpha-structures (Figure 6.2B) and the large amounts of DNA trapped in the loading wells suggested that perhaps additional activities were present in these fractions. The results seen in both systems prompted further investigation.

A cell-free extract was prepared from rat testis and applied to a Superdex 200 gel filtration column. Fractions were incubated with alpha-structures to see what other activities could be revealed. Strong ligase activity was detected in fractions from the column eluting with an apparent molecular mass of approximately 630 kDa (Figure 6.5.1). The large molecular mass suggests that a protein complex was responsible for the products seen. The same experiment was repeated with HeLa cell extracts and the ligation activity seen eluted with an apparent molecular mass of greater than 670 kDa (data not shown).
Figure 6.5.1 Rat testis cell-free extract fractionated by gel filtration on Superdex 200. Crude cell-free extract was applied to a Superdex 200 gel filtration column. Control assays were incubated with no protein or RuvAB. 2 μl of each fraction was incubated with alpha-structures for 30 minutes at 37°C. Gel filtration standards were run under identical conditions for comparison. The void volume of the column was determined using Blue Dextran 2000. Reaction products were deproteinised and separated by agarose gel electrophoresis as described in Methods. Bands were visualised by autoradiography.
6.6 Homology dependant ligation and pairing?

HeLa extract fraction 8 from the phosphocellulose chromatography was tested for DNA pairing activity as described in Methods using RecA as a control (Figure 6.6.1). Lane 1 shows pairing of labelled linear duplex DNA and gDNA by RecA, producing the expected intermediates and some nicked circular product. No pairing or strand exchange products could be observed in RecA reactions containing labelled linear φX174 DNA (lane 3), which has no homology to the gDNA. The homology-dependent RecA reaction was not effected by the presence of the heterologous φX174 DNA (lane 2). When fraction 8 was incubated with gDNA and homologous labelled linear DNA, the formation of a linear dimer product was observed plus a faint higher molecular weight product (lane 4). No products were observed when the heterologous linear duplex φX174 DNA was used (lane 6), and no additional product bands were formed when both pDEA7z-f(+) and φX174 DNA were used (lane 5). The reaction required the presence of ATP (lane 7). These results suggest the possibility of a pairing and ligation activity in this fraction.

Further experiments showed that the pairing/ligation reactions depended on the presence of gDNA (Figure 6.6.2 lane 2 and 4). Pairing and strand exchange reactions with RecA produced the expected alpha-structures in reactions with gDNA and labelled linear duplex DNA (lane 1) and also with homologous blocked linear DNA (lane 3). No ligation products were seen with fraction 8 in reactions containing linear duplex DNA or supercoiled plasmid (lanes 6 and 8). Control reactions with RecA incubated with linear duplex DNA or supercoiled plasmid DNA also failed to produce products as expected (lanes 5 and 7).
### Table 6.6.1

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**Figure 6.6.1. Pairing reactions.** Fraction 8 from phosphocellulose (HeLa) was incubated with substrates required to produce alpha-structures. All reactions contained labelled linear duplex DNA and gDNA. Some reactions also contained labelled linear phi X174 DNA as indicated. Reactions contained RecA as a control or 2 μl fraction 8. Pairing reactions were for 10 minutes at 37°C as described in Methods. Markers were lambda DNA-Hind III digest. Reaction products were deproteinised and separated by agarose gel electrophoresis as described in Methods. Bands were visualised by autoradiography.
Figure 6.6.2. gDNA dependency of products: (A) Pairing reactions were set up with RecA and fraction 8 from phosphocellulose (HeLa). Pairing reactions were between gDNA and homologous labelled linear duplex DNA (H), gDNA and homologous blocked labelled linear duplex DNA (H/B), homologous labelled linear duplex DNA (H) and homologous blocked labelled linear duplex DNA (H H/B), and homologous labelled linear duplex DNA and homologous supercoiled plasmid pDEA7z-f(+) (scc). Reactions contained RecA as a control or 2 μl fraction 8. Pairing reactions were for 4 or 10 minutes at 37°C, as indicated. Reaction products were deproteinised and separated by agarose gel electrophoresis as described in Methods. Bands were visualised by autoradiography. (B) Schematic of formation of blocked alpha-structures. The region of heterology that blocks complete strand exchange is shown in bold.
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6.7 Separation of activities

The experiments with the HeLa cell-free extracts revealed that: (1) fractions produced both dimer products and sigma structures, (2) similar activities eluted from gel filtration with a high molecular weight, and (3) the formation of some bands was dependent on the presence of gDNA in the reaction. This suggests that more than one activity was processing the substrates. To define more specifically what activities were present in the fractionated extracts, fractions 6-9 from phosphocellulose chromatography were pooled, dialysed and applied to a 1 ml DEAE Macro-Prep anion-exchange column and fractionated with a 0.1 - 1.0 M NaCl gradient. Fractions were assayed using RecA-made alpha structures (Figure 6.7). Activities that produced the apparent dimer product could be separated from the activities that produced the sigma structure. The dimer products in lanes 7 and 8 (D) have higher intensity than the equivalent band in lanes 9, 10, and 11. The bands that run as a sigma-structure, in lanes 9, 10 and 11 (S) are of greater intensity than the equivalent bands in lanes 7 and 8. An additional band that runs in between the sigma-structure and dimer product is only present in lane 7 (→). We can conclude that our initial assumption of more than one activity processing the substrate was correct.

These results suggested that some of these activities could be part of a complex and ligation was stimulated when linear DNA sequences contained regions of homology with the gDNA. These findings could be explained if a homologous pairing activity was promoting ligation by bringing homologous DNA sequences into close proximity.
Figure 6.7. Further fractionation of partially purified HeLa cell extract. Fractions 6 - 9 eluted from phosphocellulose were dialysed and applied to MacroPrep DEAE. The column was eluted with a 0.1 - 1.0 M NaCl gradient. Control assays were incubated with no protein and RuvC. 2 μl of each fraction was incubated with substrate for 30 minutes at 37°C. Reaction products were deproteinised and separated by agarose gel electrophoresis as described in Methods. Bands were visualised by autoradiography. The protein peak is shown only.
6.8 Active HeLa fractions contain Rad51

To investigate if a known recombination protein was producing any of the product bands, more experiments were conducted with fractions from the DEAE chromatography. Fractions were incubated with labelled linear duplex DNA alone, linear and gDNA, and linear and ssDNA. In parallel reactions antibodies raised against human Rad51 (hRad51) were added to the reaction mixtures, which were pre-incubated for 15 minutes before the addition of substrates. Pre-incubation with hRad51 antibodies appeared to have reduced the intensity of bands and in some cases product bands were not produced (data not shown). However the results from these experiments were not conclusive as pre-immune serum was not available and it could not be ruled out that the inhibition observed was the result of inhibition by other proteins present rather than the antibodies raised to hRad51.

To explore the possible presence of a pairing activity in the fractions from the DEAE column, Western blotting analysis was performed on fraction 8 and 9, using antibodies raised against human RAD51 (hRAD51), and using hRAD51 as a control. Fractions 8 and 9 were blotted onto nitrocellulose and probed with hRAD51 antibodies (Figure 6.8). The results show that Rad51 was present in fractions that produced dimer and sigma products. Mammalian systems revealed a much more complex picture of activities compared to bacterial systems.
Figure 6.8. The presence of RAD51 in fractions from HeLa cells. Fractions 8 and 9 were resolved by SDS PAGE and hRad51 was detected by western blotting with Rad51 antibodies. hRad51 was used as a control.
RecA-made intermediates have been used successfully before to identify Holliday junction-processing activities in bacterial cell-free extracts (Connolly and West, 1990). Our use in this study has been both problematic, and successful. The initial use of alpha-structures revealed numerous activities. These activities needed to be defined using a simpler and homogeneous substrate. Activities from both mammalian systems were shown to produce ligation products from linear duplex DNA. These ligation activities eluted from the gel filtration column with an apparent molecular mass greater than 600 kDa suggesting a large protein complex containing numerous activities.

Experiments with extracts from HeLa cells provided additional evidence that the observed activities were part of a complex. The different product bands could be separated in fractions from ion-exchange chromatography. One activity seemed to be a ligation activity stimulated by homologous gDNA in the reaction. These activities co-purified with Rad51.

As a more general observation the DNA substrates were processed in different ways depending on which substrate was available. This probably reflects complex systems present in eukaryotic cells and suggests that a substrate can be processed by several mechanisms. The substrate availability and competition between enzymes could regulate these mechanisms.

Both RAD51 and RAD52 have been shown to promote ligation in vitro (Baumann and West, 1997; Van Dyck et al., 1999). Recent experimental evidence has demonstrated that RAD52 has DNA end-binding activity and has led to a proposed model that RAD52 binds DNA ends protecting them from nuclease activity and recruits RAD51 protein leading to strand invasion (Figure 6.9). It could be that the ligation and possibly homology dependent ligation seen in fractions from HeLa cells are revealed as a predominant activity due to the presence of RAD51 stimulating ligation.
Figure 6.9. A possible model for double strand break repair.
7. GENERAL DISCUSSION

The aim of this project was to investigate the molecular mechanisms of homologous recombination in eukaryotic cells and particularly the mechanisms associated with processing of Holliday junctions. The identification of novel Holliday junction-specific enzymes has allowed a comparison to be made between known enzymes that process Holliday junctions and the newly identified activities.

Two approaches were taken to discover Holliday junction processing activities. The first was to look for putative homologues by searching available databases of DNA with sequences of known Holliday junction processing enzymes. This led to the discovery of a novel Holliday junction resolvase from *S. pombe*, YDC2, a homologue of CCE1.

The second approach involved biochemical screening for Holliday junction-processing activities from various crude cell extracts fractionated by ion-exchange chromatography. This approach led to the identification of a potential nuclear resolvase in budding yeast and a ligation activity in mammalian cells. Both results are extremely interesting.

7.1 YDC2

The open reading frame of YDC2 was over-expressed in *E. coli* and the protein product purified to near homogeneity. This allowed the biochemical characterisation of the protein. This enzyme behaved in a manner similar to other resolvases characterised, such as RuvC and CCE1.

Two other groups also reported YDC2 from *Schizosaccharomyces pombe* at the same time that our results were being published. (Oram et al., 1998; Whitby and Dixon, 1997; White and Lilley, 1997). Our data are in agreement with all the results published in these and subsequent papers on YDC2 (Whitby and Dixon, 1998; White and Lilley, 1998). The main differences between our work and the
work of other groups were firstly, our purification protocol to identify YDC2 in cellular extracts used different chromatography columns, and secondly, our plasmid construct produced native protein, while the other two groups used cleavable fusion proteins to produce YDC2 protein for characterisation.

YDC2 binds to Holliday junctions as a dimer and resolves the junction by introducing nicks into DNA at sites symmetric to the crossover on strands of like polarity. YDC2, like CCE1 and RuvC (Dunderdale and West, 1994; Shida et al., 1996; White and Lilley, 1997), has a high degree of structural specificity for DNA binding and a sequence-specific cleavage activity (Oram et al., 1998; Whitby and Dixon, 1998; White and Lilley, 1998). YDC2 cleaves a junction 3' of a thymine nucleotide. YDC2 will cleave an Y junction providing the correct sequence is present within the 3-way junction (Oram et al., 1998; Whitby and Dixon, 1998).

Binding of YDC2 to the junction is independent of divalent metal cations but cleavage of YDC2 requires the presence of magnesium or manganese ions. The precise chemistry between the divalent metal cations and the protein tertiary structure is unclear. The metal ion probably alters the conformation of the junction and/or the protein, and promotes interaction with the junction.

The conservation of CCE1 between the two yeast species is evidence for a conserved pathway playing an important biological role. In S. cerevisiae CCE1 is localised in mitochondria and appears to be involved only in the metabolism of the mitochondrial genome. This is most likely to be the case in S. pombe and we are undertaking further experiments in this regard. CCE1 homologues are likely to be present in other eukaryotes, especially plants which harbour both mitochondrial and chloroplast genomes. It is of particular interest to search for a human CCE1 homologue and to study its effect on the mitochondrial genome stability, as mutations and deletions in mtDNA are the molecular basis of some human diseases and may be one of the mechanisms of ageing (reviewed in (Wallace, 1992)).
The role for a Holliday junction resolvase in the maintenance of the mitochondrial genome is not fully understood. Clearly, CCE1 of *S. cerevisiae* resolves mtDNA recombination junctions *in vivo*, as shown directly by the accumulation of mtDNA junctions in *ccel (mgtl)* mutants (Lockshon et al., 1995). It has been proposed that several mitochondrial genomes linked via recombination junctions constitute the mtDNA heritable unit in *S. cerevisiae*, whose size is affected directly by CCE1 (Lockshon et al., 1995). This may be a specialised adaptation of a more general role played by junction resolvases during DNA replication, as Holliday junctions were recently shown to accumulate in replication mutants of *S. cerevisiae* (Zou and Rothstein, 1997). A YDC2 deletion strain of *S. pombe* should throw more light on the biological role of this protein.

A YDC2 deficient strain will also open the way to look for nuclear Holliday junction-processing activities in *S. pombe* which are likely to be masked by the mitochondrial activity. The search for enzymes involved in the late stages of recombination in eukaryotes is particularly important as no identifiable RuvA, RuvB or RuvC homologues can be found in the eukaryotic DNA sequences available at present. It may turn out that Holliday junctions are processed in eukaryotes via novel mechanisms, distinct from the mechanisms and proteins identified in prokaryotes.

YDC2 was able to partially complement a UV sensitive resolvase deficient strain of *E. coli* (Whitby and Dixon, 1997). The ability of YDC2 to complement the prokaryotic resolvase function suggests some conservation of function.

### 7.2 Characterisation of a possible nuclear resolvase

The identification of a possible nuclear resolvase suggests that Holliday junction resolution may indeed be a conserved function present in both prokaryotes and eukaryotes. Holliday junction-specific endonucleases have been demonstrated
in higher eukaryotes (Couture and Chow, 1992; Elborough and West, 1990; Hyde et al., 1994; Jeyaseelan and Shanmugam, 1988; Waldman and Liskay, 1988), although some of these activities have not been characterised in detail and have yet to be conclusively demonstrated as true Holliday junction resolvases. Two groups have reported activities from yeast (Jensch et al., 1989; Parsons and West, 1988). Endo X1 from *S. cerevisiae* was partially purified from a strain that most likely contained the *CCE1* gene. However, the activity is unlikely to be CCE1. CCE1 cleaves a Holliday junction with sequence specificity, cleavage is symmetric to the point of crossover and 3' to a thymine (White and Lilley, 1996; White and Lilley, 1997). Endo X1 would cleave a junction with no sequence specificity and although it would cleave a junction at sites that are homologous, the activity would also cleave a junction at sites that were not homologous and at consecutive nucleotide sites. The activity did not show characteristics of currently known resolvases. Endo X3 was also partially purified from a *S. cerevisiae* strain that contained the *CCE1* gene. This activity did show characteristics of a resolvase. Endo X3 was remarkably similar to T4 endo VII. The location, number and usage of cleavage sites were almost identical and the partially purified activity would also cleave the highly branched replicative DNA from bacteriophage T4. The protein even had the same molecular weight as T4 endo VII (Jensch et al., 1989). The activity reported in chapter 5 is the first Holliday junction resolvase demonstrated to be from a *ccelA* strain.

The activity purified from *ccelA* AK47 cells showed similar characteristics as CCE1, YDC2, and RuvC, group one resolvases. The activity bound to the Holliday junction in a structure specific manner and introduced nicks into DNA strands of like polarity, at points symmetric to the crossover, in a manner similar to RuvC, CCE1 and YDC2 reported in chapter 4 (Dunderdale and West, 1994; Shida et al., 1996; White and Lilley, 1997). Our data is consistent with the activity binding to Holliday junctions as a dimer in a structure specific manner like other resolvases identified. The activity also demonstrated a high degree of structural specificity for DNA binding and sequence-specific cleavage specificity activity similar to YDC2, which also cleaves a junction 3' of a
thymine nucleotide. The cleavage sites of YDC2 and fraction 5 from AK47 cells on junction X12 were the same. Mapping of cleavage sites of both the *S. cerevisiae* activity and YDC2 on different junctions could demonstrate differences. A suitable junction for this experiment could be junction X0 that contains no region of homology. Both resolvase activities would cleave this junction with greatly reduced efficiency. Junction X0 appeared to be cut with greater efficiency by the activity in *S. cerevisiae* than YDC2 (Figures 4.13A and 5.4.2D). As this junction does not contain cleavage sites that are symmetric to the point of crossover, resolvase activities have to cleave a site that best fits their sequence requirements. Under these conditions differences of cleavage may be revealed. Presenting the resolvases with a consensus sequence, which may be similar is unlikely to reveal differences. The use of junction X0 may reveal subtle differences in the way the resolvases mould the junction for cleavage. Resolvases and junction binding proteins have been shown to change junction conformation on binding.

As junction X0 does not contain cleavage sites symmetric to the point of crossover, the cleavage site on one strand may be preferred to the cleavage site on the other strand. Mapping of cleavage sites could reveal a cleavage preference for one of the two strands. In addition, this could reveal if the resolvase cleaves the junction by a dual incision mechanism or by, two consecutive nicks. Cleavage of a Holliday junction by a dual incision mechanism to release both DNA molecules would be advantageous. Cleavage of one strand by one dimer (partial cleavage) followed by second dimer binding and cleavage, to release the two DNA molecules, could be an alternative mechanism. Co-ordinated cleavage of both DNA strands of the Holliday junction by one dimer would seem the most probable mechanism to ensure release of the two DNA molecules. However, in order to cleave both strands the resolvase must bind long enough to ensure complete cleavage of the junction. If this is the case the question arises how does the resolvase remain bound to the junction long enough to cleave both strands of the junction and yet release itself from a junction that does not contain its preferred cleavage sequence? Branch migration of a cleavable site to the resolvase could be a possibility.
The branch migration data obtained for the *S. cerevisiae* enzyme need to be confirmed. However, the results do suggest the possibility of a RuvABC-like complex functioning in lower eukaryotes. CCE1 and YDC2 have been shown to change the conformation of the stacked junction to an open square planar conformation, similar to RuvA. The resolvase from AK47 cells could instil a similar conformational change to a junction on binding leading to an open square planar junction-resolvase complex. This structure would be similar to the structure imposed by RuvA on a four-way junction. It is now easier to imagine the resolvase activity from yeast holding the junction in a manner similar to RuvA, allowing hexameric rings of RuvB to branch migrate the DNA strands. The addition of divalent ions inhibits spontaneous branch migration of Holliday junctions in solution, while the open structure adopted by the junction on binding of RuvA facilitates branch migration. Perhaps an open structure adopted by the junction on resolvase binding also facilitates branch migration and the search for a cleavable sequence. This would overcome the binding question mentioned above. The resolvase could bind tightly to the junction to ensure cleavage of both DNA strands and if a cleavable sequence was not found, branch migration could allow the resolvase to scan for such a sequence. YDC2 branch migration with RuvB was not observed. This could be due to the shape of the protein not being suitable to accommodate RuvB hexamers on the junction at the same time. The buffer conditions may not have been appropriate for both proteins to work together, or the two proteins cannot work in unison. This is not to say that a similar mechanism of branch migration and cleavage cannot happen. YDC2 could rely on the high likelihood of binding a consensus sequence due to a higher AT content in mtDNA. This might not be the case for a nuclear resolvase. The apparent lack of RuvB-mediated branch migration with YDC2 may be due to binding affinities. Binding experiments suggest that RuvA can displace a dimer of YDC2 but a dimer of YDC2 cannot bind to an octamer of RuvA bound to the junction (Whitby and Dixon, 1998).

The partial purification of the activity enabled bands from SDS-PAGE to be sent for sequencing. The results from sequencing protein bands purified from SDS-PAGE may provide a gene sequence for the resolvase characterised. This
would obviously enable a thorough in-depth characterisation of the activity at the molecular level and enable structural studies to be undertaken as well. Confirmation changes in the protein structure on binding DNA could also be determined from neutron scattering. If a knockout gene was not lethal the requirement of the gene product could be studied from knockout experiments. Rescue of wild type phenotype could be used to clone similar enzymes from other eukaryotes. Screening available mammalian genomic sequence database libraries could reveal a mammalian resolvase homologue; alternatively degenerate primers could be used to amplify by PCR mammalian genomic sequence.

It would be very interesting to investigate levels of expression of the activity from AK47 cells during the cell cycle and following DNA damage. This could be achieved by monitoring resolvase activity from synchronised cells as they pass through cell cycle checkpoints. Cells were synchronised, as mentioned in section 5.2 to see if the resolvase activity could be enriched in SK20 cells at a specific stage of the cell cycle. Cells were synchronised at G1 and S phase of the cell cycle were checked but no activity was seen. This was unexpected as Holliday junctions had been shown to accumulate in S phase of the cell cycle (Zou and Rothstein, 1997). However, recent experiments in the laboratory have suggested that cells need to go through a G2 checkpoint to initiate recombination. Cells in G2 were not tested, these experiments will be pursued in the future.

The results from the activities identified allow us to make a comparison with known Holliday junction resolvases (Table 7.2).
Table 7.2. Comparison of known Holliday junction resolvases with recently identified activities.

<table>
<thead>
<tr>
<th>RuvC</th>
<th>CCE1</th>
<th>Fr V (AK47)</th>
<th>YDC2</th>
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<tr>
<td>Structure selective</td>
<td>Structure selective</td>
<td>Structure selective</td>
<td>Structure selective</td>
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<tr>
<td>Binding is metal ion independent</td>
<td>Binding is metal ion independent</td>
<td>Binding is metal ion independent</td>
<td>Binding is metal ion independent</td>
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<tr>
<td>Alters junction structure on binding</td>
<td>Alters junction structure on binding</td>
<td>May alter junction structure on binding</td>
<td>Alters junction structure on binding</td>
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<tr>
<td>Functions as a dimer</td>
<td>Functions as a dimer</td>
<td>Evidence suggests formation of a dimer</td>
<td>Functions as a dimer</td>
</tr>
<tr>
<td>Requires divalent metal ion for cleavage</td>
<td>Requires divalent metal ion for cleavage</td>
<td>Requires divalent metal ion for cleavage</td>
<td>Requires divalent metal ion for cleavage</td>
</tr>
<tr>
<td>Cleaves strands of like polarity</td>
<td>Cleaves strands of like polarity</td>
<td>Cleaves strands of like polarity</td>
<td>Cleaves strands of like polarity</td>
</tr>
<tr>
<td>Cleavage at or near point of strand exchange</td>
<td>Cleavage at or near point of strand exchange</td>
<td>Cleavage at or near point of strand exchange</td>
<td>Cleavage at or near point of strand exchange</td>
</tr>
<tr>
<td>Cleaves 3’ of thymine</td>
<td>Cleaves 3’ of thymine</td>
<td>Cleaves 3’ of thymine</td>
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</tr>
<tr>
<td>Preference for continuous strand</td>
<td>Preference for continuous strand</td>
<td>Both strands cleaved on junction X12</td>
<td>Both strands cleaved on junction X12</td>
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</table>
7.3 Activities in fractionated HeLa cell extracts

The experiments with fractionated mammalian cell extracts demonstrated that numerous activities were present and suggest a complex picture of activities that can function together. The initial screen, primarily aimed to detect branch migration, revealed other activities. Using a homogenous substrate for assays could have produced results that were easier to understand. However, the use of the alpha-structure preparations revealed activities that would not have been seen using a single substrate. The probable homology dependent ligation that was seen would not have been revealed without the presence of both gDNA and linear duplex DNA. The results agree with the proposed DSBR model discussed in section 6.8. This model speculates about in vivo mechanisms of DNA processing. The methods used, discussed in chapter 6, provide a possible tool to test biochemically the current ideas about DSBR. Fractionation of extracts by gel filtration revealed the ligation activity to have a large molecular weight. This probable complex could be further fractionated by ion-exchange chromatography, as demonstrated by the results from DEAE chromatography. Fractions could be probed with antibodies raised against proteins proposed to function in the DSBR mechanism. Positive results from western blotting could be confirmed by demonstrating inhibition of activities by pre-incubation of fractions with antibodies before the addition of substrate.

Further experiments could determine which ligation products predominate (head to tail, tail to tail or head to head products) and in what ratio, could also help to characterise the ligation activity.

The activities detailed in chapters 4-6 and in particular the resolvase activity in cce1Δ AK47 cells will be part of the main projects in the laboratory.
Bibliography


237


Appendix

The DNA sequence of the putative *S. pombe* CCE1 homologue is 777 bases and located on chromosome I.

ATGGCTACTGTGAAACTTTAGTTTTTTACAGCACATCTGTAAGTTAACT
GGATTGTCAAGAAGTGGAAGAAAGGATGAGTTGCTTCGAAAGAATTG
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CCGCATGTCATTTTTATGGAACGCAGCAGTTATCGTCCCGGTATTGCT
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Symbols for amino acids.

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<td>Cysteine</td>
<td>C</td>
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240
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