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by

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Declaration

The work reported in this thesis was performed by the author in the Department of Genetics, University of Leicester.

Except where acknowledgement is made, the work is my own and has not been submitted for any other degree in this or any other University or Institute of learning.

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Contents

List of Tables

Table of Figures

Chapter 6: Processing of the *HIS4* Double-Strand Break by Exo1p

Chapter 7: Discussion

Abbreviations

A	-	Alanine
Ade	-	Adenine
AICAR	-	5-amino-1-(5′-phosphoribosyl)-imidazole-4-carboxyamide
AIR	-	1-(5′-phosphoribosyl)-5-aminoimidazole
ATP	-	Adenosine Triphosphate
BD	-	Becton Dickinson
BER	-	Base Excision Repair
βME	-	β-mercaptoethanol
bp	-	Base pair
CAIR	-	1-(5′-phosphoribosyl)-5-aminoimidazole-4-carboxylate
cM	-	CentiMorgans
CO	-	Crossover
D	-	Aspartic Acid
dHJ	-	Double Holliday Junction
DNA	-	Deoxyribonucleic Acid
dNTP	-	Deoxynucleoside Triphosphate
DSB	-	Double-strand break
dsDNA	-	Double-Stranded Deoxyribonucleic Acid
E	-	Glutamic Acid
EDTA	-	Ethylenediamine Tetra-acetic Acid
FOA	-	Fluoro-orotic Acid
G	-	Glycine
GC	-	Gene Conversion
HCl	-	Hydrochloric Acid
hDNA	-	Heteroduplex Deoxyribonucleic Acid
His	-	Histidine
HNPCC	-	Hereditary Non-Polyposis Colorectal Cancer
Hyg	-	Hygromycin
KAc	-	Potassium Acetate
kb	-	Kilobase
LB	-	Luria Bertani
Leu	-	Leucine
LiAc	-	Lithium Acetate
Lys	-	Lysine
M	-	Molar
Met	-	Methionine
mg	-	Milligram
ml	-	Millilitre
mM	-	MilliMolar
MMR	-	Mismatch Repair
MMS	-	Methylmethane Sulfonate
MSI	-	Microsatellite Instability
MutLα	-	Complex of Mlh1p and Pms1p (Pms2p in humans)
MutSα	-	Complex of Msh2p and Msh6p
MutSβ	-	Complex of Msh2p and Msh3p

µg	-	Microgram
µl	-	Microlitre
µM	-	MicroMolar
MMR	-	Mismatch Repair
MRDS	-	Meiotic Recombination-related DNA Synthesis
MRX	-	Complex of Mre11p, Rad50p and Xrs2p
NaCl	-	Sodium Chloride
NaOAc	-	Sodium Acetate
NaOH	-	Sodium Hydroxide
$NaPO_4$	-	Sodium Phosphate
Nat	-	Nourseothricin
NCO	-	Non-Crossover
ng	-	Nanogram
NMS	-	Non-Mendelian Segregation
NPD	-	Non-parental Ditype
ORF	-	Open Reading Frame
PCR	-	Polymerase Chain Reaction
PD	-	Parental Ditype
PEG	-	Polyethylene Glycol
pmole	-	Picomole
PMS	-	Post-Meiotic Segregation
PNACL	-	Protein and Nucleic Acid Chemistry Laboratory
PRR	-	Post-Replicative Repair
PSSC	-	Precocious Separation of Sister Chromatids
rpm	-	Revolutions Per Minute
SAICAR	-	1-(5′-phosphoribosyl)-4-(*N*-succinocarboxyamido)-5-aminoimidazole
SC	-	Synaptonemal Complex
SDSA	-	Synthesis-Dependent Strand Annealing
SEI	-	Single-End Invasion
SIC	-	Synapsis Initiation Complex
ssDNA	-	Single-Stranded Deoxyribonucleic Acid
TBE	-	Tris-Borate, EDTA
TE	-	Tris-EDTA
TF	-	Transcription Factor
Trp	-	Trytophan
TT	-	Tetratype
Ura	-	Uracil
V	-	Valine
v/v	-	Volume/Volume
w/v	-	Weight/Volume
YEPD	-	Yeast Extract, Peptone, Dextrose
YEPEG	-	Yeast Extract, Peptone, Ethanol, Glycerol

A Structural and Functional Analysis of Mismatch Repair Proteins in Meiosis

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Abstract

A number of the mismatch repair proteins facilitate recombination during meiosis. Among these are the MutS homologues, Msh4p and Msh5p, the MutL homologues Mlh1p and Mlh3p, and the exonuclease Exo1p. Msh4p and Msh5p are meiosis specific proteins, which function to promote crossing over, although the mechanism is unknown. Mlh1p and Mlh3p are involved in the same pathway, but are thought to act later during meiosis as the meiotic phenotypes of *mlh1*Δ and *mlh3*Δ are less severe than those of *msh4*Δ or *msh5*Δ. Structure and function studies of Mlh1p have begun to elucidate its roles in mitosis and meiosis. In particular it has been shown that ATP binding by Mlh1p is important for both its mitotic and meiotic roles, whereas ATP hydrolysis is only partially required for mitotic mismatch repair (MMR) and not at all for meiotic recombination.

In this study it has been demonstrated that ATP binding by Mlh3p is essential for its meiotic crossover function, suggesting that the ATP-mediated conformational change is essential for function and/or interactions. In contrast, ATP hydrolysis is not required for crossing over. However, when both Mlh1p and Mlh3p are unable to hydrolyse ATP, a defect was observed in crossing over suggesting that some recycling of the Mlh1p-Mlh3p complex is necessary for functionality.

The loss of Exo1p reduces processing of the *HIS4* DSB and also crossing over to approximately 50% of the levels observed in a wild-type. Here we show that both the 5′-3′ exonuclease and the 5′ flap endonuclease activities of Exo1p are required to maintain crossing over. We propose that reduced resection of DSBs produces less stable strand invasions, which consequently reduces crossing over. However, not all DSBs are dependent on Exo1 for processing. Additionally, Exo1p is required for the removal of DNA flaps produced from over-replication of DNA during meiotic recombination.

Chapter 1: Introduction

Chapter 1: Introduction

1.1 Mismatch Repair Proteins

Mismatch repair proteins are highly conserved from prokaryotes to eukaryotes, and they play a number of roles in both mitosis and meiosis. The process of mismatch repair involves recognition and removal of DNA mismatches that arise during replication and recombination. This is essential for maintaining genome stability (reviewed in Harfe and Jinks-Robertson, 2000 and in Kolodner, 1996). Defects in this process cause an increase in spontaneous mutation rates, characterised by forward mutations, base-base transitions and microsatellite instability (MSI) in mitotic cells (Aaltonen *et al.*, 1993; Ionov *et al.*, 1993; Thibodeau *et al.*, 1993) and post-meiotic segregation (PMS) in meiotic cells (reviewed in Borts *et al.*, 2000 and in Surtees *et al.*, 2004). In humans, this confers a predisposition to hereditary non-polyposis colorectal cancer (HNPCC; reviewed in Peltomaki and Vasen, 1997).

In meiosis, some of the mismatch repair proteins are involved in the promotion of crossing over between homologous chromosomes and regulation of their distribution (Hollingsworth *et al.*, 1995; Hunter and Borts, 1997; Khazanehdari and Borts, 2000; Ross-Macdonald and Roeder, 1994; Wang *et al.*, 1999). Meiotic recombination generates genetic diversity and creates the necessary physical interactions to ensure correct segregation of the chromosomes at division I of meiosis (reviewed in Roeder, 1997). Defects in crossing over result in chromosome non-disjunction, which can have serious implications for fertility in humans.

1.2 Mismatch Repair

1.2.1 Bacterial Mismatch Repair

The mismatch repair (MMR) system is most well characterised in *Escherichia coli*, where only a small number of proteins are involved (Figure 1.1; reviewed in Jiricny, 2006). MutS and MutL are both ATPases that function as homodimers. MutS recognises and binds to mismatched DNA, which can consist of either a mispaired base, or insertions/deletions that create loops in the DNA. MutL is then recruited and coordinates downstream events, including activation of MutH and

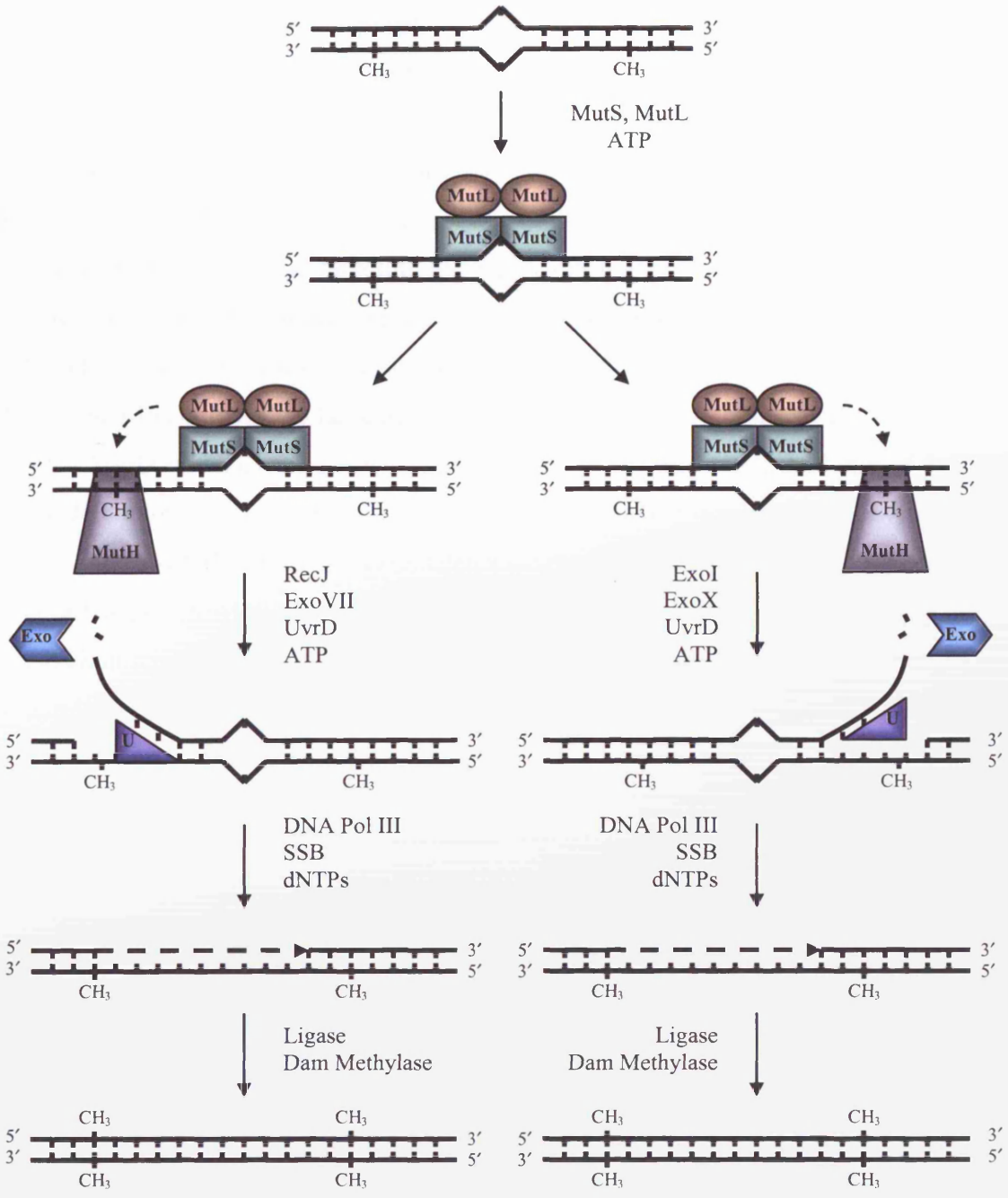


Figure 1.1 Mismatch Repair in *Escherichia coli*
MutS recognises the mismatch, and recruits MutL in an ATP-dependent manner. These proteins locate the nearest hemimethylated site, which can be 5′ or 3′ to the mismatch. Activated MutH nicks the unmethylated DNA strand, targeting repair to the newly synthesised strand containing the mismatch. UvrD unwinds the DNA, and depending on the position of the nick relative to the mismatch, one of four exonucleases is recruited, to remove the DNA from the nick to beyond the mismatch. DNA polymerase III resynthesises the DNA and DNA ligase seals the remaining nick. Exo = ExoI/ExoVII/ExoX/RecJ; U = UvrD.
Redrawn from Matson and Robertson (2006).

UvrD. Following DNA replication, the newly synthesised DNA strand is transiently unmethylated, and this provides a mechanism for strand discrimination. The MutS-MutL complex locates the d(GATC) methylation site nearest to the mismatch by an as yet unknown mechanism. MutH, a latent endonuclease, is targeted to this site, which can be either 5′ or 3′ to the mismatch, where it nicks the unmethylated DNA strand targeting the repair machinery to the newly synthesised strand. The nick that directs repair and the mismatch can be separated by up to 2000 bp, but the efficiency of repair decreases the further away the signal is located. MutL then loads the 3′-5′ helicase, UvrD (also called DNA helicase II), onto the DNA. UvrD unwinds the DNA to allow access to the mismatch, and one of four redundant exonucleases (ExoI, ExoVII, ExoX and RecJ) is involved in its excision. RecJ and ExoVII are 5′-3′ exonucleases whereas ExoI and ExoX are 3′-5′ exonucleases, allowing excision from either direction depending on the position of the nick in relation to the mismatch. DNA polymerase III resynthesises the DNA to repair the gap, and DNA ligase anneals the ends of the DNA strand back together to complete the repair process (reviewed in Modrich, 1991 and in Matson and Robertson, 2006).

1.2.2 Eukaryotic Mismatch Repair

In *Saccharomyces cerevisiae* there are six **MutS** **H**omologues (*MSH1-6*) and four **MutL** **H**omologues (*MLH1-3* and *PMS1*; **P**ost-**M**eiotic **S**egregation), although they are not all involved in MMR. Msh1p is involved in the repair of mitochondrial DNA (Reenan and Kolodner, 1992), whereas Msh4p and Msh5p are meiosis specific proteins (Hollingsworth *et al.*, 1995; Pochart *et al.*, 1997; Ross-Macdonald and Roeder, 1994). In humans, *PMS2* is the functional homologue of yeast *PMS1* (reviewed in Kunkel and Erie, 2005).

Whilst MutS in *E. coli* recognises both frameshifts and insertions or deletions, in *S. cerevisiae* different complexes are involved in the recognition and processing depending on the type of mismatch present (Figure 1.2). Msh2p forms the basis of all of the mismatch-recognition complexes, and can recognise mispaired bases alone (Alani *et al.*, 1995). Msh2p forms heterodimers with Msh3p and Msh6p, and each of these complexes are involved in the recognition of a subset of mismatches. This implies that Msh3p and Msh6p provide the specificity to bind to the different types

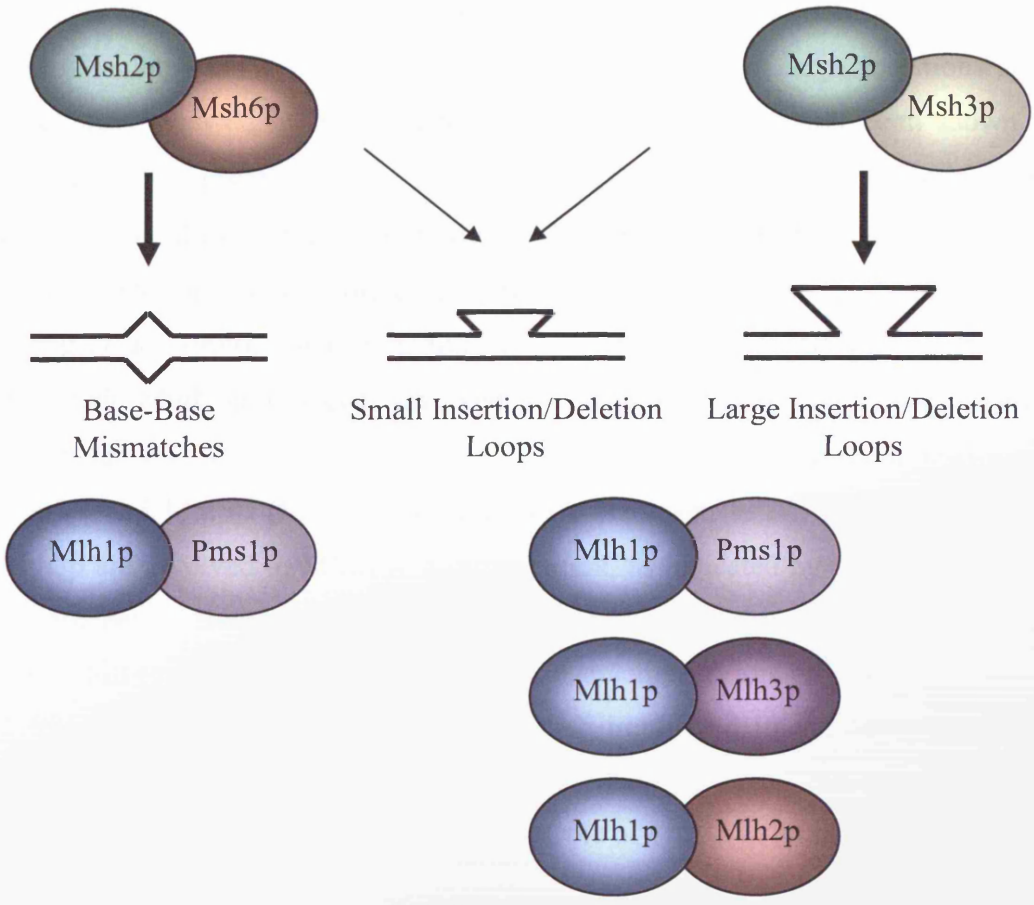


Figure 1.2 Mismatch Repair Complexes in *Saccharomyces cerevisiae*
MutS and MutL homologues form multiple complexes in *S. cerevisiae* during MMR, to repair different types of mismatches. Msh2p and Mlh1p are common to all MutS and MutL complexes respectively. The complexes involved in the repair of each type of mismatch are shown. Adapted from Marti *et al.* (2002).

of mismatches (Alani *et al.*, 1997). A heterodimer of Msh2p-Msh6p (MutSα) is involved in the repair of base-base mismatches and small insertions and/or deletions whilst Msh2p-Msh3p (MutSβ) is involved in the repair of large insertions and/or deletions (reviewed in Jiricny, 2006). However, it has recently been shown that MutSβ is also involved in the repair of a small subset of base-base mismatches, distinct from those recognised by MutSα (Harrington and Kolodner, 2007). Mlh1p forms heterodimers with the other three MutL homologues (Prolla *et al.*, 1994; Wang *et al.*, 1999), but their individual contributions to MMR vary (Figure 1.2). The main MutL homologues involved in MMR are Mlh1p and Pms1p (MutLα), which are involved in the repair of all types of mismatches in combination with MutSα and MutSβ (Habraken *et al.*, 1997; Habraken *et al.*, 1998). Mlh2p and Mlh3p are involved in MMR to a lesser extent, as indicated by their weak mutator phenotypes. Both function in the *MSH2-MSH3* pathway, which recognises frameshift mutations caused by insertions or deletions (Flores-Rozas and Kolodner, 1998; Harfe *et al.*, 2000). Mlh3p was also implicated in the Msh3p-dependent repair of a minority of base-base mismatches (Harrington and Kolodner, 2007; See also Chapter 3 for more information on the role of Mlh3p in mutation repair).

In humans, germline mutations in *MLH1* and *MSH2* account for the majority of cases of HNPCC. Germline mutations of *MLH3* have also been found in patients with atypical HNPCC (Wu *et al.*, 2001b) although the association is small. However, overexpression of *MLH3* in mammalian cell culture has been shown to induce MSI, possibly by sequestering the functional Mlh1p (Lipkin *et al.*, 2000). Also, in patients at an increased genetic risk of HNPCC that have no detectable mutation in *hMLH1* or *hMSH2*, *hMLH3* is frequently inactivated (Lipkin *et al.*, 2001). Subsequent studies have either found no evidence for the involvement of *hMLH3* in HNPCC (Hienonen *et al.*, 2003), or that mutations in *hMLH3* are only present in low-risk patients and are not associated with MSI, indicating that defective mismatch repair function was not the cause of the disease (Liu *et al.*, 2003). Therefore the precise contribution of hMLH3 to HNPCC remains undetermined.

The MutL homologues are considered to be the molecular 'matchmakers' of the

MMR process, being involved in the recruitment and coordination of downstream factors. How strand discrimination is achieved is not well understood. However, as no homologue to MutH has been identified and additionally DNA is unmethylated in yeast (Proffitt *et al.*, 1984) the mechanism is clearly distinct to that observed in *E. coli*. Recent data suggests a role for PCNA (**P**roliferating **C**ell **N**uclear **A**ntigen). PCNA, the processivity factor for DNA replication, interacts with a number of MMR proteins, including Mlh1p, Msh2p, Msh3p and Msh6p (Clark *et al.*, 2000; Flores-Rozas *et al.*, 2000; Umar *et al.*, 1996). Colocalisation of PCNA with hEXO1 at sites of DNA replication has also been observed (Nielsen *et al.*, 2004), indicating an involvement with MMR. There is also evidence to suggest that the 5′ ends of Okazaki fragments may provide a signal for strand discrimination, and the role of PCNA in the processing of Okazaki fragments (reviewed in Moldovan *et al.*, 2007) provides a means to link the two processes together (Umar *et al.*, 1996). It has been demonstrated that repair of the lagging strand during DNA replication is more efficient than repair of the leading strand, and mutating a number of MMR genes results in an increase in the amount of mutations arising from lagging strand synthesis (Pavlov *et al.*, 2003; reviewed in Schofield and Hsieh, 2003). This may be due to the presence of a large number of smaller fragments during lagging strand synthesis, as this means a mismatch will always be closer to a 5′ end compared to during leading strand synthesis (Pavlov *et al.*, 2003). Additionally a higher concentration of PCNA is present on the lagging strand (Pavlov *et al.*, 2003).

One exonuclease that functions in the removal of mismatches is Exo1p (**Exo**nuclease **I**), which has 5′-3′ exonuclease activity (Lee and Wilson, 1999; Szankasi and Smith, 1992; Tran *et al.*, 2002). Exo1p was implicated in this process due to its interaction with Msh2p and Mlh1p (Tishkoff *et al.*, 1997a; Tran *et al.*, 1997). However, strains deleted for *EXO1* only exhibit a low mutation rate. This indicates that Exo1p is functionally redundant with other exonucleases. An *exo1*Δ strain and an *exo1* mutant defective in exonuclease activity have identical phenotypes for MMR, indicating that it is the catalytic activity of Exo1p that is important for its MMR function (Sokolsky and Alani, 2000). The interaction of Mlh1p and Exo1p is not dependent on ATP binding by Mlh1p, but the additional deletion of *EXO1* in an *mlh1* ATP binding or hydrolysis mutant did increase the

mutation rate (Tran *et al.*, 2001). Genschel *et al.* (2002) found that hEXO1 is required for both 5′ and 3′ directed mismatch repair, which was unexpected as Exo1p only possesses a 5′-3′ exonuclease activity (Lee and Wilson, 1999; Szankasi and Smith, 1992; Tran *et al.*, 2002). This activity of hEXO1 is activated in the presence of MutSα and a mismatch (Genschel *et al.*, 2002). Additionally, DNA polymerases δ and ε (encoded by *POL3* and *POL2* respectively) have been suggested to function in MMR (Longley *et al.*, 1997; Tran *et al.*, 1999). Both of these polymerases have 3′-5′ proofreading abilities and function in DNA replication in conjunction with PCNA, which enhances their processivity (reviewed in Moldovan *et al.*, 2007). Nuclear extracts of HeLa cells that were defective for mismatch repair activity *in vitro* were utilised to identify components of the mismatch repair machinery (Longley *et al.*, 1997). The extracts used were functional for known components of the MMR process, as processing of hDNA was observed, indicating that MMR was blocked after recognition of the mismatch and recruitment of other factors. Cell fractions were added to the MMR defective extracts and then tested for MMR activity. The fraction that restored MMR activity was found to contain DNA polymerase δ whereas the MMR defective nuclear extracts did not (Longley *et al.*, 1997). Mutating the exonuclease domains of *POL2* and *POL3*, resulting in loss of their proofreading activity (*pol3-01* and *pol2-4* mutants) only results in a moderate increase in mutation rate (Tran *et al.*, 1999). The increase was greatest in *pol3-01*, as Polε is inefficient at proofreading long homonucleotide runs. However, when *pol3-01* or *pol2-4* mutants were combined with *exo1*Δ a large increase is observed, indicative of synergy (Tran *et al.*, 1999). It is difficult to distinguish the difference a defect in proofreading and a defect in mismatch repair would have on mutation rate. However, Polε does not effectively proofread long runs of homonucleotides, and therefore the authors claim that the synergy observed in *exo1 pol2-4* mutants in such an assay is due to a combined inability to repair these mismatches (Tran *et al.*, 1999). In haploids, combining *exo1* with *pol3-01* results in lethality (Tran *et al.*, 1999), which might indicate that these two nucleases are normally responsible for the majority of the repair. This provides more evidence for coordination of DNA replication and repair by PCNA on the lagging strand (Umar *et al.*, 1996)

Purified human proteins have also been used to reconstitute bidirectional MMR systems *in vitro*. MutSα, hEXO1 and RPA are sufficient to maintain 5′ directed excision of mismatches (Constantin *et al.*, 2005; Dzantiev *et al.*, 2004; Genschel and Modrich, 2003), although MutLα confers additional specificity by preventing excision of homoduplex DNA (Genschel and Modrich, 2003). However, it has also been suggested that MutLα and RPA function together to stop excision once the mismatch has been removed (Zhang *et al.*, 2005). RPA has been shown to reduce the processivity of the MutSα-EXO1 complex to prevent extensive excision beyond the mismatch (Genschel and Modrich, 2003). For 3′ directed excision, MutLα, PCNA and RFC are absolutely required in addition to MutSα, hEXO1 and RPA (Constantin *et al.*, 2005; Dzantiev *et al.*, 2004). The involvement of hEXO1 in 3′ directed excision was explained with the discovery that MutLα possesses a latent endonuclease activity (Kadyrov *et al.*, 2006). When the nearest nick to direct mismatch repair is located 3′ to the mismatch, MutLα introduces a nick distal to the mismatch, producing a 5′ end which can be processed by hEXO1 (Kadyrov *et al.*, 2006). RFC also represses the activity of hEXO1 at nicks 3′ to a mismatch to prevent excision in the wrong direction (reviewed in Modrich, 2006). Although hEXO1 clearly has roles in mismatch removal, the weak mutator phenotype observed in yeast indicates that there is at least one other nuclease involved in this process. Additionally, if it were the main nuclease involved in human systems, then a larger number of HNPCC cases would be expected due to mutations in *hEXO1*. Although a number of studies have been performed to identify mutations in *hEXO1* associated with HNPCC, varying results have been obtained (reviewed in Liberti and Rasmussen, 2004). One study identified eight missense mutations in patients with atypical HNPCC (Wu *et al.*, 2001a), of which only one had associated MSI, which is a hallmark of defective MMR and of HNPCC. Functional analysis of these found that some of the mutations had reduced 5′-3′ processing whereas others affected the interaction of Exo1p with Msh2p (Sun *et al.*, 2002). However, a subsequent study identified four of these mutations in a control group, indicating that they are normal rather than mutagenic variants (Jagmohan-Changur *et al.*, 2003). Additionally, two individuals identified carrying a deletion of both copies of *EXO1* exhibited no MSI and there was no history of HNPCC within their family (Alam *et al.*, 2003). However, *Exo1*$^{-/-}$ mice show reduced survival rates, increased

MSI, and increased presence of lymphomas although other types of tumours normally associated with HNPCC are not significantly more prevalent than in wild-type mice (Wei *et al.*, 2003). The phenotypes of *Exo1*$^{-/-}$ mice are also not as severe as those in *Msh2*$^{-/-}$ mice (Wei *et al.*, 2003). Together these suggest that there is some functional redundancy in the removal of mismatches. The majority of studies conclude that the inactivation of *hEXO1* is responsible for very few cases of HNPCC. When *hEXO1* is responsible, it causes late onset of the disease, exhibits incomplete penetrance and is often associated with atypical HNPCC.

1.3 Meiosis

Meiosis produces haploid gametes from diploid cells (Figure 1.3). The meiotic cell division is distinct from mitosis as it consists of a single round of DNA replication followed by two rounds of division. Meiosis I is the reductional division, where the homologous chromosomes are separated, and meiosis II is the equational division, with separation of the sister chromatids. This results in the production of four non-identical haploid gametes. Restoration of the haploid chromosome number is essential for the production of viable gametes and to allow subsequent mating without doubling this number. The reciprocal exchange of DNA (recombination) between homologous chromosomes occurs during prophase of meiosis I, and is essential to ensure correct segregation of homologous chromosomes at division I of meiosis (reviewed in Kleckner, 1996 and in Roeder, 1997). Whereas in mitosis the small amount of recombination that does occur is between sister chromatids (reviewed in Prado *et al.*, 2003), during meiosis the bias is towards recombination between non-sister chromatids, as only the latter results in stable interactions to promote accurate segregation of chromosomes. In the absence of crossing over, non-disjunction occurs resulting in aneuploidy that can lead to disease and/or reduced fertility. Consequently, mechanisms are in place to ensure that at least one crossover event occurs per chromosome (discussed below), which is referred to as the obligate crossover. The physical connections between homologues are called chiasmata.

Incorrect segregation during meiosis in yeast causes spore death (Figure 1.4). Non-disjunction of homologous chromosomes at meiosis I results in the death of two spores, and the two viable spores are sisters, that are disomic for the non-disjoining

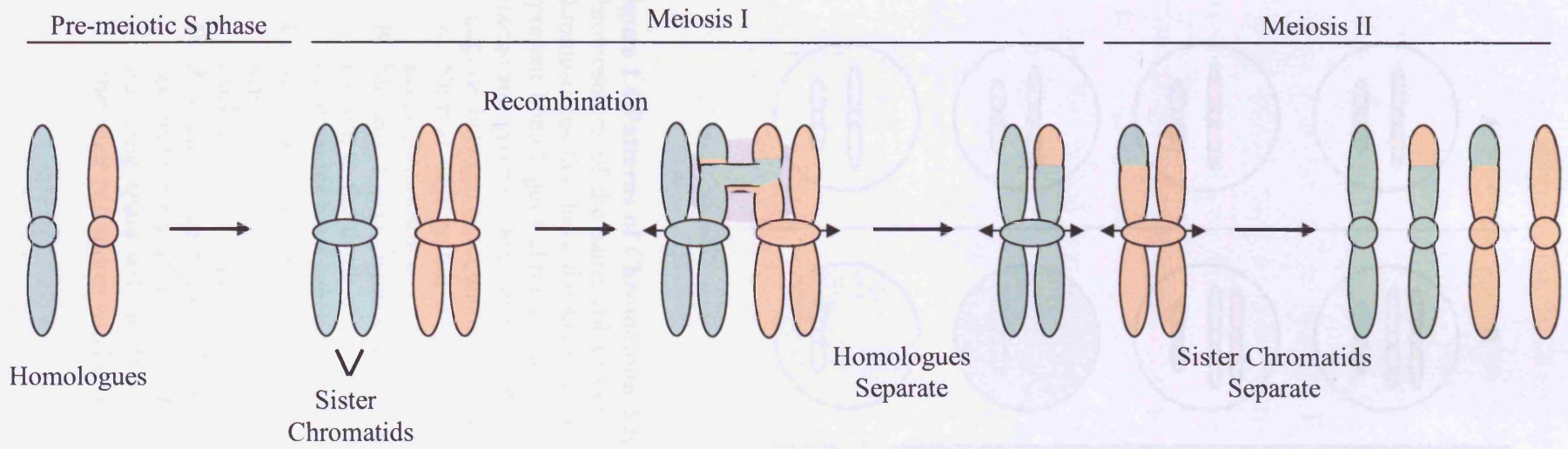


Figure 1.3 Chromosome Dynamics During Meiosis
A single round of DNA replication produces two copies of every chromosome, termed sister chromatids. Homologous chromosomes (one from each parent) align, and can exchange genetic information. Recombination provides stable interactions between homologous chromosomes to ensure they segregate correctly at the first division of meiosis. At meiosis I, the homologous chromosomes segregate, and then at meiosis II the sister chromatids segregate, producing four haploid gametes.
Adapted from Whitby, 2005.

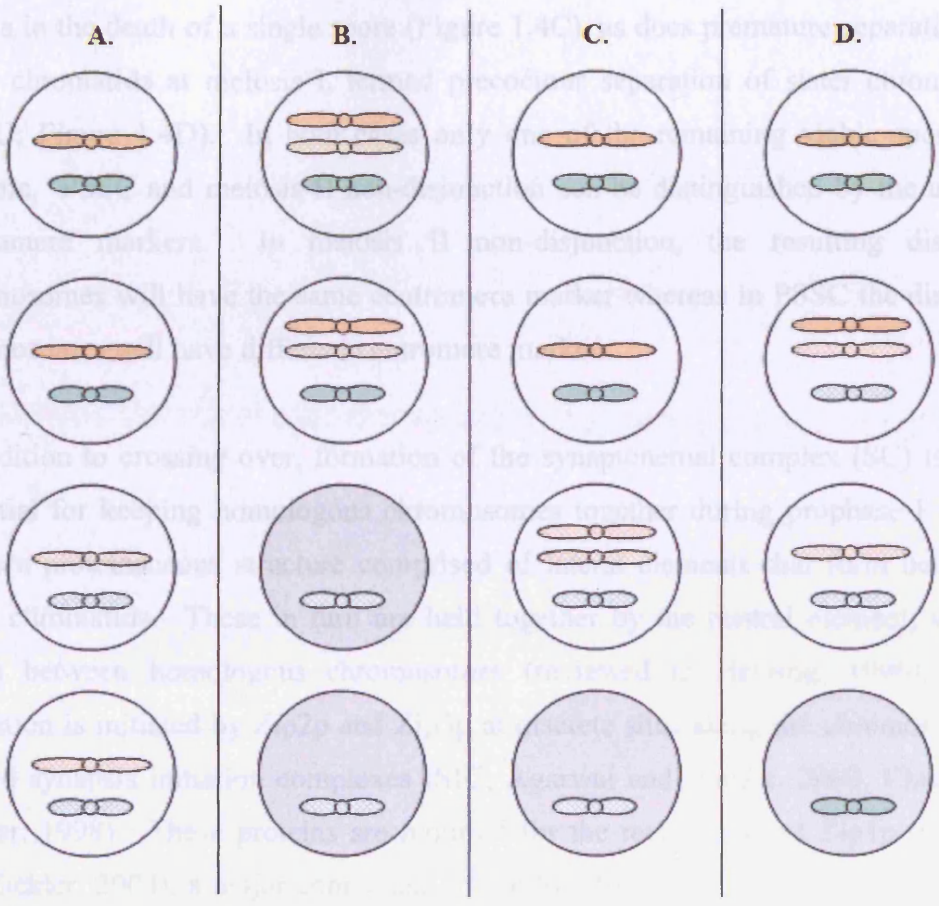


Figure 1.4 Patterns of Chromosome Segregation
Chromosomes of the same colour (and same pattern) represent sister chromatids. Chromosomes that have the same colour but different patterns (solid and hatched) represent homologous chromosomes. Two different chromosomes are represented (orange and green). Dead spores are shaded grey. Centromere markers are used to distinguish between sister and non-sister chromatids.

- **A.** Normal segregation of chromosomes. Every spore receives one copy of every chromosome.
- **B.** Meiosis I non-disjunction. Homologous chromosomes fail to segregate leading to the death of two spores. The surviving spores will be sisters but the disomes have genetic markers from both parents.
- **C.** Meiosis II non-disjunction. Sister chromatids fail to segregate leading to the death of one spore. One surviving spore will be disomic, with genetic markers from one parent. The other two spores will be sisters.
- **D.** Precocious separation of sister chromatids (PSSC). Sister chromatids segregate early at meiosis I leading to the death of a single spore. One surviving spore will be disomic, with genetic markers from both parents. The other two spores will be non-sisters.

chromosome (Figure 1.4B). Incorrect segregation of sister chromatids at meiosis II results in the death of a single spore (Figure 1.4C), as does premature separation of sister chromatids at meiosis I, termed precocious separation of sister chromatids (PSSC; Figure 1.4D). In both cases only one of the remaining viable spores is disomic. PSSC and meiosis II non-disjunction can be distinguished by the use of centromere markers. In meiosis II non-disjunction, the resulting disomic chromosomes will have the same centromere marker whereas in PSSC the disomic chromosomes will have different centromere markers.

In addition to crossing over, formation of the synaptonemal complex (SC) is also essential for keeping homologous chromosomes together during prophase I. The SC is a proteinaceous structure comprised of lateral elements that form between sister chromatids. These in turn are held together by the central element, which forms between homologous chromosomes (reviewed in Heyting, 1996). SC formation is initiated by Zip2p and Zip3p at discrete sites along the chromosomes, termed synapsis initiation complexes (SIC; Agarwal and Roeder, 2000; Chua and Roeder, 1998). These proteins are required for the recruitment of Zip1p (Bishop and Zickler, 2004), a major component of the SC (Sym *et al.*, 1993), to these sites and then Zip1p polymerises along the entire length of the chromosomes (synapsis). Zip4p also forms part of the SIC, and functions with Zip2p to promote meiotic progression (Tsubouchi *et al.*, 2006). Mutants of all four of these proteins result in reduced crossing over and spore viability, and also affect the distribution of crossovers (Agarwal and Roeder, 2000; Chua and Roeder, 1998; Tsubouchi *et al.*, 2006). SC formation is initiated during the zygotene stage of prophase I (Figure 1.5), and is dependent on the production of double-strand breaks (DSBs) that initiate recombination (Agarwal and Roeder, 2000; Chua and Roeder, 1998). Msh4p is involved in the distribution and promotion of crossover events (Novak *et al.*, 2001; Ross-Macdonald and Roeder, 1994) and is also recruited to SICs (Novak *et al.*, 2001). Localisation of Msh4p to SICs requires Zip1p and Zip2p, as in their absence weaker foci of Msh4p are observed (Novak *et al.*, 2001). The SC persists throughout pachytene whilst interactions between homologous chromosomes occur, and disassembly occurs during the diffuse stage before diplotene (reviewed in Zickler and Kleckner, 1999).

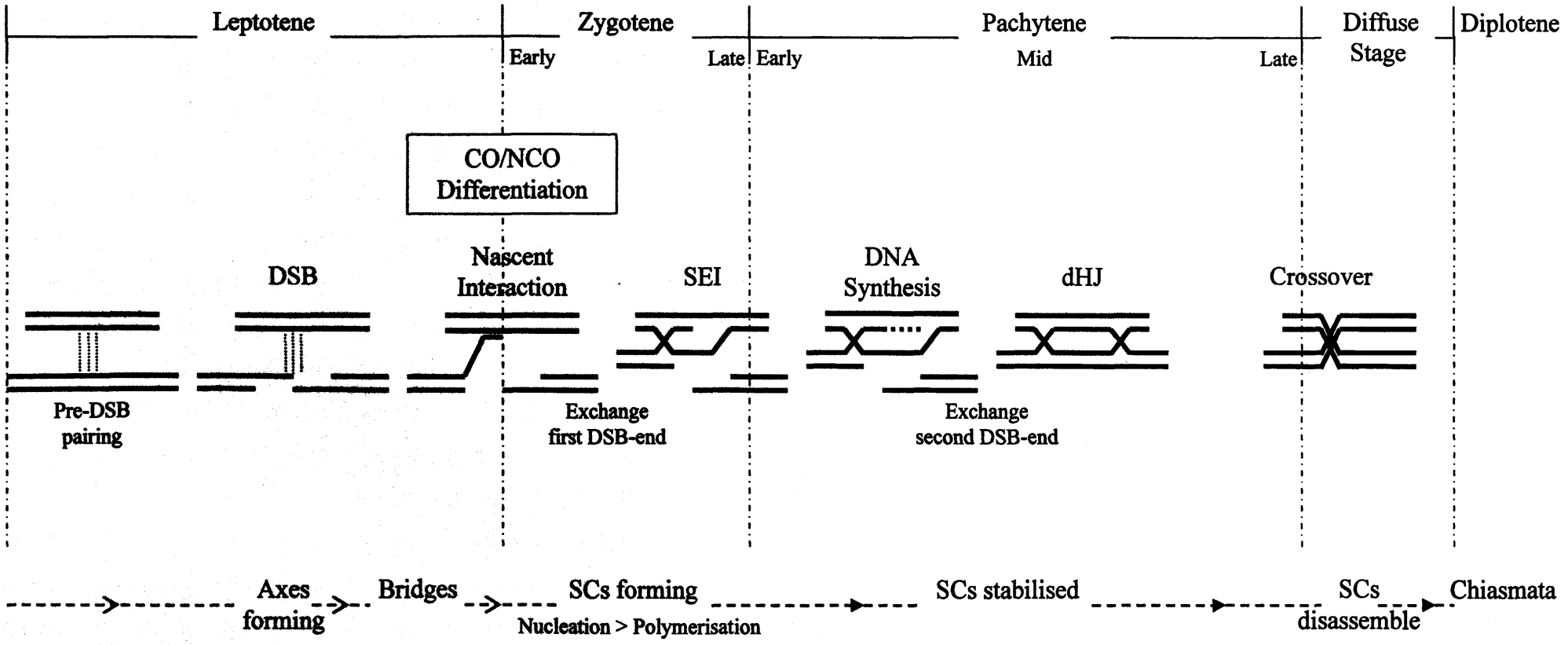

Leptotene
Zygotene
Pachytene
Diffuse Stage
Diplotene
Early
Late
Early
Mid
Late
CO/NCO Differentiation
DSB
Nascent Interaction
SEI
DNA Synthesis
dHJ
Crossover
Pre-DSB pairing
Exchange first DSB-end
Exchange second DSB-end
Axes forming
Bridges
SCs forming
SCs stabilised
SCs disassemble
Chiasmata
Nucleation > Polymerisation

Figure 1.5 Coordination of Events During Prophase I of Meiosis
The four distinct stages of prophase I are shown (Top). Relative to this, the approximate timings of the steps in the production of crossovers (Middle), and the assembly and disassembly of the synaptonemal complex (Bottom) are also indicated. During leptotene, meiotic recombination is initiated by the production of DSBs. Nascent interactions are formed between homologous chromosomes leading to the formation of single strand invasion intermediates during zygotene. It is at this stage that the synaptonemal complex (SC) is assembled. During pachytene DNA synthesis occurs to fill in the gaps produced by resection of DSBs, and double Holliday Junctions are produced. At late pachytene and during the diffuse stage, stable recombination intermediates are formed and chiasmata are visible by diplotene. Concomitantly the SC is disassembled. The crossover/non-crossover decision is thought to occur at the leptotene to zygotene transition.
Redrawn from Hunter and Kleckner (2001).

1.3.1 Double-Strand Break Repair

Recombination is initiated by the introduction of double-strand breaks (DSBs) at recombination hotspots (see Chapter 5 for more information), which then must be repaired, as breaks in DNA are otherwise lethal. These breaks can be repaired as crossovers, where exchange of DNA between interacting chromatids occurs, or non-crossovers where no exchange of DNA occurs. DSBs are made at much higher frequencies than crossovers, indicating the presence of a mechanism to designate some precursors as crossovers whilst the remaining precursors are fated to become non-crossovers. A modified version of the original double-strand break repair (DSBR) model (Resnick and Martin, 1976) was proposed by Szostak *et al.* (1983), in which both crossover and non-crossover products arise through the resolution of a common intermediate (Figure 1.6; Szostak *et al.*, 1983). A number of the physical intermediates and proteins involved in this pathway have been identified in *S. cerevisiae*, and in other organisms (reviewed in Svetlanov and Cohen, 2004 and in Baudat and de Massy, 2007), and these will be discussed below. DSBs are catalysed by covalently attached Spo11p (Bergerat *et al.*, 1997; Keeney *et al.*, 1997), aided by a number of proteins: Mre11p, Rad50p, Xrs2p, Ski8p, Rec102p, Rec104p, Rec114p, Mei4p, and Mer2p (reviewed in Keeney, 2001). All of these accessory factors are required for the production of DSBs, and although their precise roles are unknown some functional interactions have been determined.

Mre11p, Rad50p and Xrs2p comprise the MRX complex (Johzuka and Ogawa, 1995; Usui *et al.*, 1998). A number of mutants of these components block the production of DSBs and/or their subsequent processing indicating this complex has multiple roles (reviewed in Haber, 1998). Mre11p is recruited to the site of DSB formation even if the DSB is not made, indicating that its presence is required even before DSB formation (Borde *et al.*, 2004). For processing of DSBs to occur, Spo11p must be removed from the DSB ends. Neale *et al.* (2005) demonstrated that endonucleolytic cleavage released Spo11p bound to a small oligonucleotide with a free 3′ OH. However, the Spo11p bound DNA was of two different sizes and present in equal amounts, suggesting that cleavage occurs asymmetrically. Mre11p possesses a single-stranded endonuclease activity (Paull and Gellert, 1998), and therefore it is hypothesised that the MRX complex is responsible for removing

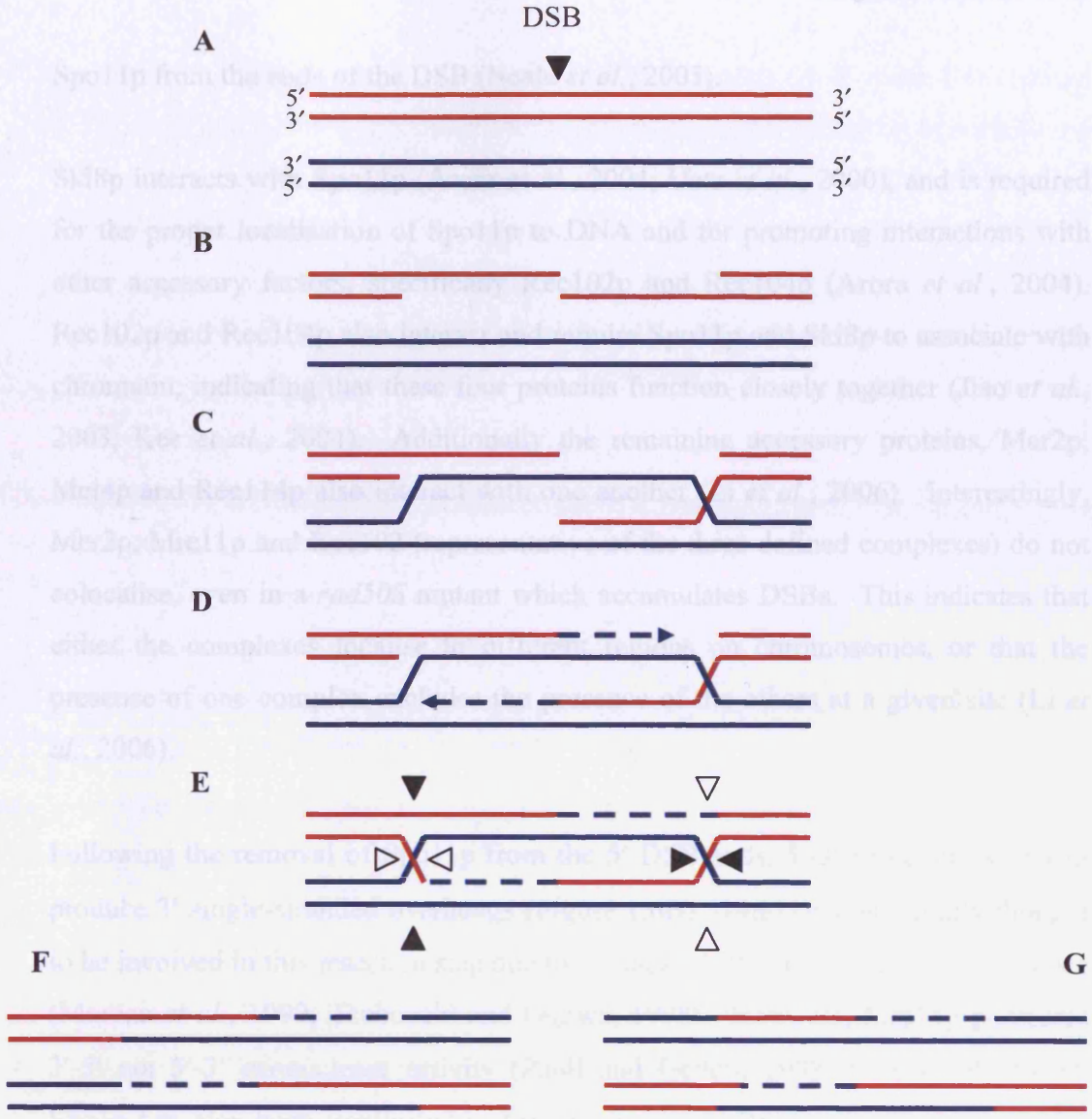


Figure 1.6 The Szostak/Resnick Model of Double-Strand Break Repair

A. DSBs are made during meiosis to initiate stable interactions between homologous chromosomes and generate diversity through exchange of DNA.
B. The 5′ ends of the DSB are resected to produce 3′ single-stranded overhangs.
C. The 3′ end is capable of invading the double-stranded DNA homologue, forming a single-end invasion (SEI) intermediate and a D-loop.
D. The 3′ ends prime DNA synthesis using the homologue as a template. The dashed lines indicate newly synthesised DNA.
E. Subsequent capture of the 5′ end results in the formation of a double Holliday Junction (dHJ). Resolution will give rise to a crossover (**F**) when cutting occurs at arrowheads of the same colour, or a non-crossover product (**G**) when cutting occurs at one black and one white pair of arrowheads. Cutting at the second junction must occur perpendicular to cutting at the first junction to give rise to a crossover. See text for details of the proteins involved. Adapted from Paques and Haber (1999).

Spo11p from the ends of the DSB (Neale *et al.*, 2005).

Ski8p interacts with Spo11p (Arora *et al.*, 2004; Uetz *et al.*, 2000), and is required for the proper localisation of Spo11p to DNA and for promoting interactions with other accessory factors, specifically Rec102p and Rec104p (Arora *et al.*, 2004). Rec102p and Rec104p also interact and require Spo11p and Ski8p to associate with chromatin, indicating that these four proteins function closely together (Jiao *et al.*, 2003; Kee *et al.*, 2004). Additionally the remaining accessory proteins, Mer2p, Mei4p and Rec114p also interact with one another (Li *et al.*, 2006). Interestingly, Mer2p, Mre11p and Rec102 (representative of the three defined complexes) do not colocalise, even in a *rad50S* mutant which accumulates DSBs. This indicates that either the complexes localise to different regions on chromosomes, or that the presence of one complex excludes the presence of the others at a given site (Li *et al.*, 2006).

Following the removal of Spo11p from the 5′ DSB ends, 5′-3′ resection occurs to produce 3′ single-stranded overhangs (Figure 1.6B). Mre11p was initially thought to be involved in this resection step due to the lack of DSB processing in its absence (Moreau *et al.*, 1999; Tsubouchi and Ogawa, 1998). However, Mre11p possesses 3′-5′ not 5′-3′ exonuclease activity (Paull and Gellert, 1998; Usui *et al.*, 1998). Exo1p has also been implicated in the processing of DSBs. Exo1p in yeast and humans has 5′-3′ double-strand DNA exonuclease activity (Fiorentini *et al.*, 1997; Lee and Wilson, 1999; Szankasi and Smith, 1992; Tran *et al.*, 2002), and *exo1*Δ mutants exhibit delayed processing of DSBs (Tsubouchi and Ogawa, 2000). A *dmc1*Δ (**D**isrupted **M**eiotic **c**DNA) mutant blocks DSB processing at the strand invasion stage (Bishop *et al.*, 1992), and by using an *exo1*Δ*dmc1*Δ double mutant, a defect in the rate of exposure of ssDNA tails was revealed (Tsubouchi and Ogawa, 2000). Therefore, it is possible that Exo1p functions to resect the 5′ ends of DSBs to leave 3′ overhangs. As processing is only delayed not prevented, there must be other nucleases capable of performing this role. Another protein recently demonstrated to have 5′-3′ double-strand DNA exonuclease activity is Trm2p/Rnc1p (Choudhury *et al.*, 2007b). Trm2p functions in the repair of DSBs during mitosis, as the *trm2* mutants exhibit increased sensitivity to DNA damaging

agents. Combined with reduced levels of single-stranded DNA at the HO-induced DSB at the *MAT* locus, this indicates that there is a defect in the processing of the DSB (Choudhury *et al.*, 2007a; Choudhury *et al.*, 2007b). Analysis of the *trm2 exo1* double mutant revealed a synergistic relationship, as sensitivity to DNA damaging agents was increased and long term DSB induction by the HO endonuclease severely decreased cell survival compared to either single mutant (Choudhury *et al.*, 2007a; Choudhury *et al.*, 2007b). This suggests that Trm2p is involved in the processing of mitotic DSBs, and can perform some of the same functions as Exo1p. Therefore, Trm2p presents a potential candidate for a role in the processing of meiotic DSBs.

A single-stranded 3′ end invades the homologous chromosome and displacement of one DNA strand from the homologue creates a D-loop (Figure 1.6C). This is an asymmetric single-end invasion (SEI) intermediate which has been detected physically (Hunter and Kleckner, 2001). Two homologues of *E. coli* RecA, Rad51p and the meiosis-specific Dmc1p are required for strand invasion (Bishop *et al.*, 1992; Rockmill *et al.*, 1995). Both form nucleoprotein filaments on single-stranded DNA (reviewed in Shinohara and Shinohara, 2004 and in Neale and Keeney, 2006) although the presence of Dmc1p on chromosomes is dependent on the presence of Rad51p (reviewed in Shinohara and Shinohara, 2004). No interaction has been detected between these proteins, and colocalisation studies suggest that they are situated adjacent to one another as opposed to together in a complex. Therefore it has been suggested that Rad51p loads onto one 3′ end of the DNA whilst Dmc1p loads onto the other (reviewed in Shinohara and Shinohara, 2004). A number of additional proteins aid Rad51p and Dmc1p in this process, including Rad52p, Rad54p (reviewed in Masson and West, 2001) and the Rad55p-Rad57p complex (Sung, 1997). Rad51p and Dmc1p are also involved in the homology search to identify homologous sequences to pair with (Tsubouchi and Roeder, 2003), along with the Hop2p-Mnd1p complex. In the absence of Hop2p, an increase in SC formation is observed between non-homologous chromosomes rather than homologous chromosomes (Leu *et al.*, 1998; Tsubouchi and Roeder, 2002; Tsubouchi and Roeder, 2003). This complex may stabilise SEI intermediates after homologous sequences have been identified (Petukhova *et al.*, 2005; Pezza *et al.*,

2007). In the absence of Dmc1p and Rad51p, DSBs are hyper-resected and strand invasion cannot occur (reviewed in Masson and West, 2001). Therefore the nuclease(s) involved in resection, in the absence of loading of Rad51p and/or Dmc1p continue to resect, indicating that there may be some coordination between these proteins (Pezza *et al.*, 2007). Mer3p is a 3′-5′ DNA helicase that is thought to stabilise SEI intermediates (Mazina *et al.*, 2004; Nakagawa *et al.*, 2001). Mer3p promotes branch migration and heteroduplex extension in a 3′-5′ direction, which increases interactions between the invading single-strand DNA and the invaded duplex DNA (Mazina *et al.*, 2004). In the absence of Mer3p, reduced levels of SEI intermediates are formed (Borner *et al.*, 2004) and foci of Dmc1p and Rad51p accumulate, indicating that Mer3p is required after the formation of nucleoprotein filaments on the 3′ single-stranded DNA tails, which is consistent with a role in promoting stable strand invasions (Mazina *et al.*, 2004).

DNA synthesis using the homologue as a template completes the formation of a double Holliday Junction (dHJ; Figure 1.6D and E; Schwacha and Kleckner, 1995). A protein with the ability to resolve these intermediates has not been identified. It was originally thought that the alternative resolution of dHJs, by cutting the DNA strands in different orientations, would give rise to both crossover and non-crossover products (Szostak *et al.*, 1983). A prediction of this is that both crossover and non-crossover products should arise concurrently. This was observed by Storlazzi *et al.* (1995) using physical analysis of meiotic recombination products. However, there is increasing amounts of evidence to suggest that crossovers and non-crossovers arise from different recombination intermediates, via different pathways. Ndt80p is a meiosis-specific transcription factor that activates genes involved in sporulation, and *ndt80* mutants cause pachytene arrest (Chu and Herskowitz, 1998). It was also observed that an *ndt80* mutant reduced the amount of crossover events, without affecting non-crossover events, indicating that they arise from different pathways (Allers and Lichten, 2001). This crossover-specific effect has also been observed for a number of other proteins, which will be discussed below. Additionally, Gilbertson and Stahl (1996) tested the predictions of the DSBR model genetically, by assessing the configuration of heteroduplex DNA (hDNA) produced by poorly repaired palindromic insertions. The configurations

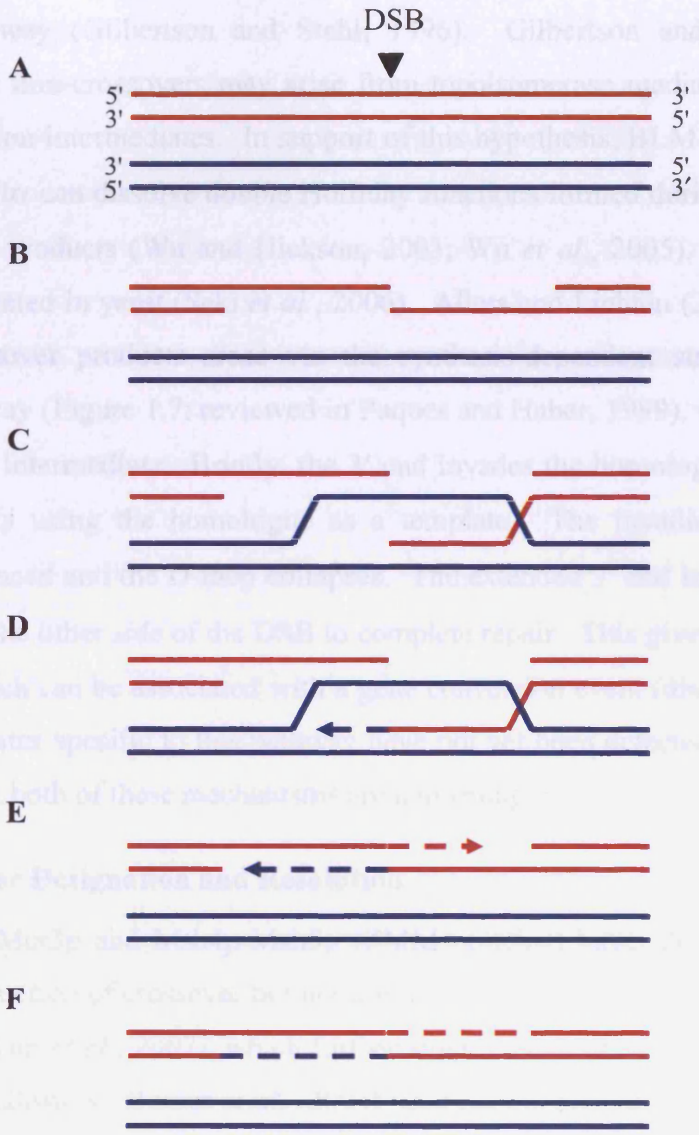


Figure 1.7 Synthesis-Dependent Strand Annealing

A. – **D.** These steps are the same as for the double-strand break repair model (Figure 1.6). Briefly, events are initiated by a DSB which is processed to leave 3′ single-stranded overhangs which can invade the homologue. DNA synthesis occurs using the homologue as a template, represented by the dashed lines.

E. Displacement of the invading strand occurs, resulting in collapse of the D-loop.

F. The newly synthesised strand anneals with the free end of the recipient to form a non-crossover product.

DNA synthesis is indicated by the dashed lines.
Adapted from Paques and Haber (1999).

predicted by the production of non-crossover events from double Holliday Junction cleavage were not observed. This also indicates that non-crossovers arise from a different pathway (Gilbertson and Stahl, 1996). Gilbertson and Stahl (1996) suggested that non-crossovers may arise from topoisomerase mediated unwinding of recombination intermediates. In support of this hypothesis, BLM in conjunction with hTOPOIIIα can dissolve double Holliday Junctions formed during mitosis into non-crossover products (Wu and Hickson, 2003; Wu *et al.*, 2005). This has also been demonstrated in yeast (Seki *et al.*, 2006). Allers and Lichten (2001) proposed that non-crossover products arose via the synthesis-dependent strand annealing (SDSA) pathway (Figure 1.7; reviewed in Paques and Haber, 1999), which does not contain a dHJ intermediate. Briefly, the 3′ end invades the homologue and primes DNA synthesis using the homologue as a template. The invading strand then becomes displaced and the D-loop collapses. The extended 3′ end is annealed with the free 5′ on the other side of the DSB to complete repair. This gives rise to a non-crossover, which can be associated with a gene conversion event (discussed below). The intermediates specific to this pathway have not yet been detected. Therefore it is possible that both of these mechanisms are happening.

1.3.2 Crossover Designation and Resolution

Zip1p-Zip4p, Mer3p and Msh4p-Msh5p (ZMM proteins) have all been shown to affect the production of crossover but not non-crossover events (Borner *et al.*, 2004; reviewed in Lynn *et al.*, 2007), which further suggests that these products form via independent pathways. Borner *et al.* (2004) used 2D gel electrophoresis to analyse the recombination intermediates formed in these mutants in an SK1 background but when sporulated at 33°C. All of the *zmm* mutants had undetectable SEI intermediates except for *zip1*Δ, which produced normal levels of SEI intermediates although these were considerably delayed. This indicates that *zmm* mutants are defective in the transition from DSB processing to production of a SEI intermediate at 33°C (Borner *et al.*, 2004). As non-crossovers form at normal levels and with normal timing, but SEI and consequently dHJ intermediates are reduced, this suggests that these intermediates are specific to the crossover pathway. This also implies that the crossover/non-crossover decision is made prior to the formation of SEI intermediates (Borner *et al.*, 2004).

Msh4p and Msh5p are meiosis specific proteins that form heterodimers (Pochart *et al.*, 1997) and are involved in the promotion of crossing over (Hollingsworth *et al.*, 1995; Ross-Macdonald and Roeder, 1994). It is unknown whether this occurs through stabilisation of recombination intermediates or through the physical resolution of such intermediates. Furthermore, Msh4p also plays a major role in establishing interference (Novak *et al.*, 2001). These proteins do not play a role in meiotic mismatch repair, as gene conversion and PMS events are unaffected in *msh4* and *msh5* mutants (Hollingsworth *et al.*, 1995; Ross-Macdonald and Roeder, 1994). The crystal structure of *Thermus aquaticus* (TAQ) MutS has been solved (Obmolova *et al.*, 2000). As the general structure of the protein is conserved among eukaryotic MutS homologues, sequence comparisons of TAQ MutS to the MutS homologues have allowed structural and functional comparisons. TAQ MutS consists of five structural domains, and sequences in domain I are important for mismatch recognition. Both Msh4p and Msh5p lack domain I, and instead the Msh4p-Msh5p heterodimer is thought to contain a hole which is large enough for two duplex DNA molecules (Obmolova *et al.*, 2000). Msh4p-Msh5p specifically bind to HJs and the complex forms a sliding clamp when bound to ATP (Snowden *et al.*, 2004). This is consistent with a role that involves binding of HJs but not mismatches.

Mlh1p also functions in the Msh4p-Msh5p pathway for crossing over, as shown by analysis of an *mlh1*Δ*msh4*Δ mutant (Hunter and Borts, 1997). However, the *msh4*Δ single mutant is more defective in crossing over, chromosome disjunction, and has lower spore viability than an *mlh1*Δ single mutant. Therefore it is postulated that Mlh1p functions downstream of Msh4p-Msh5p (Hunter and Borts, 1997).

Evidence for the involvement of Mlh3p in meiotic recombination has been shown by the reduction in levels of crossing over in *mlh3*Δ, which are similar to those seen in an *mlh1*Δ mutant (Abdullah *et al.*, 2004; Wang *et al.*, 1999; this study, Chapter 3). There is also an associated increase in levels of non-disjunction (Wang *et al.*, 1999). As Mlh1p and Mlh3p interact during mitotic mismatch repair, it is also likely that they function together in meiosis. Supporting this, it has been shown in mouse spermatocytes that Mlh1p/Mlh3p cololocalise at sites believed to be crossovers (Marcon and Moens, 2003). *MLH3*-deficient mice are infertile, and fail

to recruit Mlh1p to meiotic chromosomes (Lipkin *et al.*, 2002; reviewed in Svetlanov and Cohen, 2004). This is consistent with the observation that MLH1 and MLH3 are not always found together at foci. Therefore either Mlh3p recruits Mlh1p to chromosomes, or Mlh3p has a function independent of Mlh1p (reviewed in Svetlanov and Cohen, 2004). Also in mammalian cells, MLH3 and MSH4 interact, indicating that MLH3 is involved in the Msh4p-Msh5p pathway (Santucci-Darmanin *et al.*, 2002), as is Mlh1p (Santucci-Darmanin *et al.*, 2000).

It is possible that Mlh1p-Mlh3p are required to stabilise recombination intermediates to ensure the production of crossovers, or that they are involved in the recruitment of factors that are involved in the resolution process similar to the role of MutL homologues in mitotic MMR. Recently it has been demonstrated that hPMS2 possesses latent endonuclease activity (Kadyrov *et al.*, 2006). The active site contains a motif that is highly conserved in homologues of hPMS2 and hMLH3, and therefore it is possible that Mlh1p-Mlh3p are directly involved in the resolution of these intermediates. It is unclear whether Mlh1p and Mlh3p contribute to interference (discussed further in Chapter 3; Abdullah *et al.*, 2004; Argueso *et al.*, 2002; Hoffmann *et al.*, 2003).

Although the search for a Holliday Junction resolvase in yeast has proved elusive, components of the Holliday Junction resolvase complex have been identified in humans. Cell fractions lacking Holliday Junction and branch migration activities were also found to be deficient for a number of proteins including RAD51C (Liu *et al.*, 2004). The addition of purified complexes containing RAD51C to cell fractions lacking RAD51C restored the missing activities. RAD51 paralogues also appear to be involved in branch migration. As using the purified RAD51C complexes alone did not result in Holliday Junction resolution or branch migration, further unidentified proteins must also be involved (Liu *et al.*, 2004). The RAD51B-RAD51C complex is thought to be the functional equivalent of the Rad55p-Rad57p complex in yeast (Dosanjh *et al.*, 1998; Sigurdsson *et al.*, 2001), as both of these complexes are important for the loading of Rad51p onto ssDNA to form the nucleoprotein filament required for strand invasion (Sigurdsson *et al.*, 2001; Sung, 1997).

1.3.3 Distribution of Crossovers Within and Between Chromosomes

Crossovers occur in close proximity less frequently than would be predicted if they were completely independent of one another. Additionally, smaller chromosomes appear to undergo higher frequencies of crossing over compared to larger chromosomes (Kaback *et al.*, 1989; Kaback *et al.*, 1999). If the distribution of crossover events was random, then the smaller chromosomes would sometimes fail to receive any crossovers and consequently they would not segregate properly. However, non-disjunction of the smallest chromosome, chromosome I, occurs very infrequently (Kaback *et al.*, 1989). This non-random distribution of crossovers along and between chromosomes is called positive interference (reviewed in Hillers, 2004; Kleckner *et al.*, 2004), and implies that there is some means of 'communication' between DSB initiation sites (reviewed in Zickler and Kleckner, 1999). Interference is stronger on larger chromosomes than on smaller chromosomes, which also ensures every chromosome receives at least one crossover. Weaker interference on a small chromosome will allow more crossovers to occur within a given distance than stronger interference would. Two main models have been developed to explain this distribution. The 'counting model' suggests that two crossover events are separated by a set number of non-crossover events (Foss *et al.*, 1993; Foss *et al.*, 1999; Stahl *et al.*, 2004). The 'mechanical stress' model proposes that chromosomes are under tension, and increasing tension concomitantly increases the need for a release (Borner *et al.*, 2004; Kleckner *et al.*, 2004). Designation of a site as a future crossover results in localised relief of this tension, and removes the requirement of a second crossover site in that region. With increasing distance, the tension will build again until another crossover is necessary (Borner *et al.*, 2004; Kleckner *et al.*, 2004).

Recently it has been shown using alleles of *SPO11* with varying levels of activity, that reducing the number of DSBs does not proportionally reduce the number of crossovers that occur (Martini *et al.*, 2006). Therefore, under conditions of reduced initiation of recombination, crossovers are maintained at the expense of non-crossovers, a mechanism termed crossover homeostasis (Martini *et al.*, 2006). Interestingly, interference was also unaffected. This provides support for the second model of interference, for two reasons. Firstly, the ratio of crossovers to non-

crossovers is not fixed in this situation, as predicted by the counting model. Secondly, if fewer DSBs are produced on a given chromosome a larger distance would have to be covered before enough DSBs were encountered to allow a second crossover. This would increase the relative strength of interference. In the second model the tension will persist for the same distance regardless of the number of DSBs produced and therefore interference should remain the same, which is consistent with the observations made (Martini *et al.*, 2006).

It was originally thought that the SC was responsible for the transmission of the 'signal' between recombination initiation sites. However, recruitment of SICs (consisting of Zip2p and Zip3p; Agarwal and Roeder, 2000; Chua and Roeder, 1998) is dependent on the formation of DSBs. SICs also localise with crossover-designated sites and show interference, although they arise prior to SC formation (reviewed in Bishop and Zickler, 2004). This indicates that the crossover/non-crossover decision has been made before SICs are localised to sites on the chromosomes. Mutants of Spo11p are defective in SC formation, and when less than 20 % of DSBs are made the number of Zip3p foci decreases substantially (Henderson and Keeney, 2004). The number of Zip3p foci does not show a linear relationship with the number of DSBs, but rather are consistent with the number of crossovers being formed, indicating that they are associated with these crossover sites (Henderson and Keeney, 2004). Consistent with this, *zmm* mutants either fail and/or are delayed in the formation of SEI intermediates at high temperatures, yet non-crossovers form normally indicating that the crossover/non-crossover decision has already been made (Borner *et al.*, 2004). This suggests that SC formation occurs after the crossover/non-crossover decision has been made, as the *zmm* mutants still make non-crossover products yet *zip1*Δ fails to make SC (Sym *et al.*, 1993; Sym and Roeder, 1995), and the other *zmm* mutants also exhibit defects in SC formation (Agarwal and Roeder, 2000; Chua and Roeder, 1998; Novak *et al.*, 2001; Tsubouchi *et al.*, 2006). Additionally, although the crossovers produced in *msh4*Δ and *zip1*Δ fail to exhibit interference (Novak *et al.*, 2001; Sym and Roeder, 1994) the SICs that form in these mutants do exhibit interference (Fung *et al.*, 2004). Therefore, interference is still established in the absence of SC formation (Borner *et al.*, 2004). This suggests that the establishment of interference is independent of the

SC, although the SC may be required for execution of interference at the crossover level.

1.3.4 A Second Pathway for Crossing Over

Msh4p-Msh5p and Mlh1p-Mlh3p promote crossing over (Hollingsworth *et al.*, 1995; Ross-Macdonald and Roeder, 1994; Wang *et al.*, 1999). However, in the absence of these proteins a substantial number of crossovers are still produced. This indicates that crossovers are also produced via a different pathway, which is independent of Msh4p-Msh5p and M1h1p-Mlh3p.

Two further proteins, Mus81p and Mms4p, have been shown to exhibit defects in meiosis (de los Santos *et al.*, 2001; de los Santos *et al.*, 2003). Mus81p and Mms4p physically interact (Mullen *et al.*, 2001), and mutants of either protein result in reduced crossing over, chromosome segregation and spore viability (de los Santos *et al.*, 2001; de los Santos *et al.*, 2003). The initiation and processing of DSBs occurs normally in *mus81* and *mms4* mutants, as DSBs do not accumulate. However, the majority of cells arrest at prophase before the first meiotic division occurs. The meiotic recombination checkpoint is activated at this stage by Mek1p, in the presence of inappropriate recombination intermediates (reviewed in Roeder and Bailis, 2000). Combining *mek1* with either *mms4* or *mus81* allowed progression of the cells through prophase, indicating that these mutants have a defect in processing recombination intermediates (de los Santos *et al.*, 2001; de los Santos *et al.*, 2003).

The Mus81-Mms4p complex was proposed to be a Holliday Junction resolvase (de los Santos *et al.*, 2001). However, Mus81p-Mms4p is a structure specific endonuclease that exhibits a preference for 3′ single-stranded flaps and replication forks over Holliday Junctions (Kaliraman *et al.*, 2001). Additionally, the reduction in crossing over in *mms4* and *mus81* mutants is not as severe as would be expected if this was their function, indicating that the majority of resolution is independent of this complex at least in *S. cerevisiae* (de los Santos *et al.*, 2001). When mutants of Mus81p/Mms4p are combined with either Msh4p/Msh5p, a further additive decrease in crossing over and spore viability is observed, indicating that these proteins function independently in separate pathways (Abdullah *et al.*, 2004; de los Santos *et al.*, 2001; de los Santos *et al.*, 2003). As Mus81p-Mms4p do not have a

preference for Holliday Junctions, the recombination intermediates that they are responsible for are not likely to consist of double Holliday Junctions (de los Santos *et al.*, 2003). A number of observations are consistent with this. In the fission yeast, *Schizosaccharomyces pombe*, the majority of crossovers are produced by the Mus81p-Eme1p (the homologue of *S. cerevisiae* Mms4p) pathway. There is also no synaptonemal complex formation (reviewed in Wells *et al.*, 2006) and no known homologue of Msh4p in *Sz. pombe*. Additionally, no double Holliday Junction intermediates have been detected. A model has been proposed whereby Mus81p-Eme1p/Mms4p cleaves D-loops and half-crossover structures (Osman *et al*, 2003; reviewed in Hollingsworth and Brill, 2004; Figure 1.8). This is supported by the recent identification of single Holliday Junction intermediates in *Sz. pombe* (Cromie *et al.*, 2006).

In the absence of Msh4p-Msh5p, crossovers do not display interference (Novak *et al.*, 2001). In contrast, the residual crossovers in the *mus81/mms4* mutants do display interference (de los Santos *et al.*, 2003). This indicates that Mus81p-Mms4p dependent crossovers do not display interference. Crossover events on smaller chromosomes are also affected more in the absence of Mus81p/Mms4p, indicating that their role is more important for these chromosomes (de los Santos *et al.*, 2003). Conversely, crossing over is affected more substantially on larger chromosomes in the absence of Msh4p (Abdullah *et al.*, 2004; Khazanehdari and Borts, 2000; Stahl *et al.*, 2004). As interference is dependent on the length of the chromosome, it was predicted that in the absence of Msh4p, this dependence would be removed (Stahl *et al.*, 2004). However, the opposite was observed as the length dependence increased. From these data it was proposed that the Mus81p-Mms4p designated crossovers occur at a roughly fixed number per kb (Stahl *et al.*, 2004). It is also possible that there is a third crossover pathway as there are residual crossovers in the *msh4Δmus81Δ* mutants (Abdullah *et al.*, 2004; Argueso *et al.*, 2004; de los Santos *et al.*, 2001; de los Santos *et al.*, 2003). However, the viability of an *mms4Δmsh5Δ* strain is only 21.8 % (de los Santos *et al.*, 2001), so detecting the presence of residual crossovers is difficult. It has been shown by physical analysis that both expected crossover products are produced in this double mutant, although at a low concentration (de los Santos *et al.*, 2003). However, these products could also arise through gene conversion.

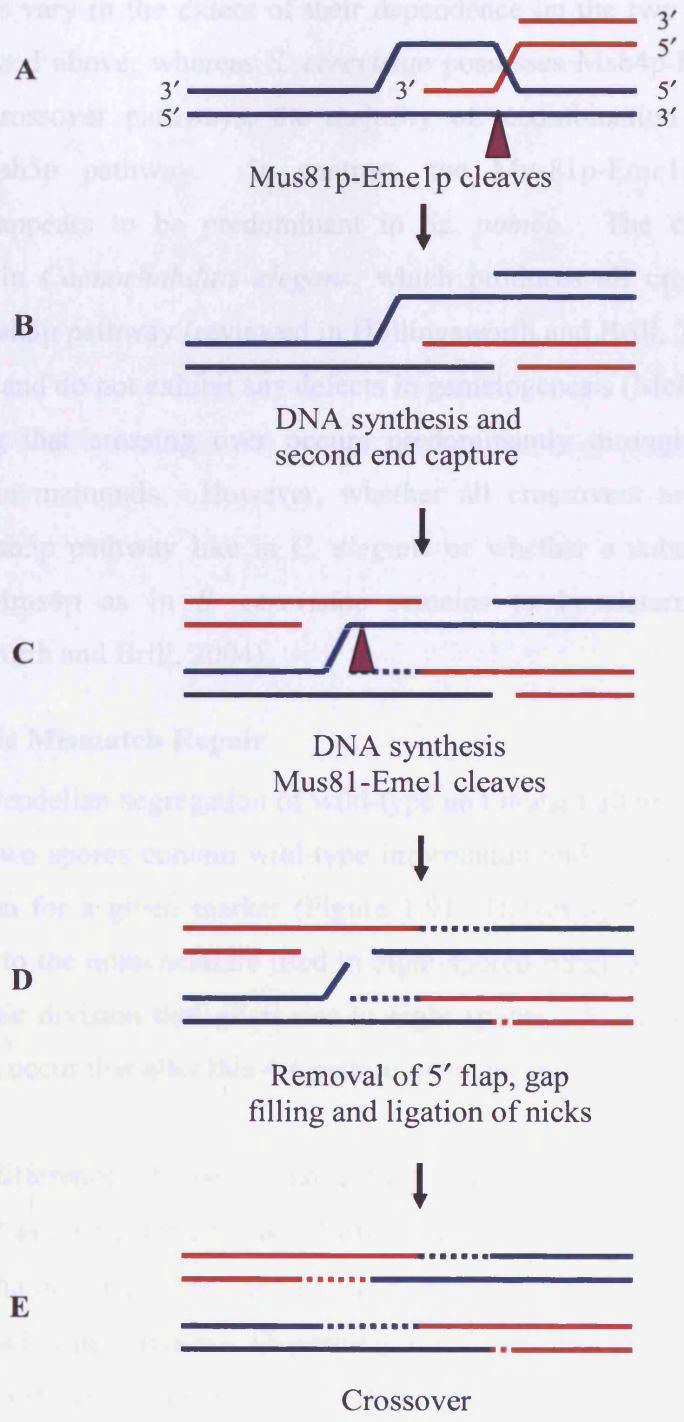


Figure 1.8 Model for Crossover Formation by Mus81p-Eme1p/Mms4p
DSB processing and 3′ strand invasion occurs as in the Msh4p-Msh5p pathway for crossing over, except Mus81p-Eme1p cleave the intermediate (A) before a dHJ is formed (**B**). DNA synthesis occurs followed by capture of the second end, and Mus81p-Eme1p/Mms4p cleave the intermediate again (**C**). DNA synthesis occurs to fill the gaps (**D**), the 5′ flap is removed and ligation seals the crossover product (**E**). The purple triangle represents Mus81p-Eme1p/Mms4p and newly synthesised DNA is represented by the dashed lines. Adapted from Osman *et al.*, 2003.

Organisms vary in the extent of their dependence on the two crossover pathways. As discussed above, whereas *S. cerevisiae* possesses Msh4p-Msh5p and Mus81p-Mms4p crossover pathways, the majority of recombination occurs through the Msh4p-Msh5p pathway. In contrast, the Mus81p-Eme1p/Mms4p crossover pathway appears to be predominant in *Sz. pombe*. The converse situation is observed in *Caenorhabditis elegans*, which produces all crossovers through the Msh4p-Msh5p pathway (reviewed in Hollingsworth and Brill, 2004). *Mus81*$^{-/-}$ mice are fertile and do not exhibit any defects in gametogenesis (McPherson *et al.*, 2004), suggesting that crossing over occurs predominantly through the Msh4p/Msh5p pathway in mammals. However, whether all crossovers are dependent on the Msh4p/Msh5p pathway like in *C. elegans* or whether a subset are processed by Mus81p/Mms4p as in *S. cerevisiae* remains to be determined (reviewed in Hollingsworth and Brill, 2004).

1.4 Meiotic Mismatch Repair

Normal Mendelian segregation of wild-type and mutant alleles exhibit a 2:2 pattern whereby two spores contain wild-type information and two spores contain mutant information for a given marker (Figure 1.9). However, this is referred to as 4:4 according to the nomenclature used in eight-spored fungi, as some fungi undergo a post-meiotic division that gives rise to eight spores. A number of recombination events can occur that alter this 4:4 pattern of segregation (Figure 1.9).

Genetic differences between interacting homologues during meiosis will be recognised as mismatches by the MMR system. After DSB formation and invasion of the homologous chromosome by the 3′ end, the resulting heteroduplex tract can contain such mismatches. Depending on the direction of repair, repair of a mismatch can result in either restoration of the original genotype, which is genetically undetectable, or a conversion can occur. Whereas crossing over is a reciprocal exchange of genetic information, gene conversions are non-reciprocal and result in three spores of one phenotype and one spore of the opposite phenotype (6:2 or 2:6). However, if the mismatch is not repaired, then a sectored colony arises, as the spore contains a section of DNA from both parents. Such an event is referred to as a post-meiotic segregation (PMS) event, as it is only when the two DNA strands are separated during mitotic cell growth after meiosis that the

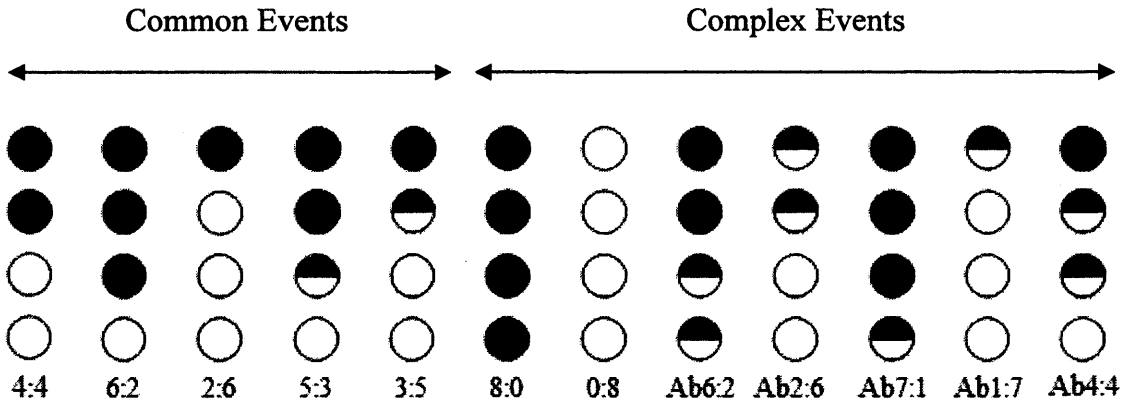


Figure 1.9 Patterns of Segregation
Normal Mendelian segregation gives rise to a 4:4 pattern, where two spores are wild-type and two are mutant. A restoration event of a mismatch contained in heteroduplex DNA will also give rise to this pattern, and the two are indistinguishable genetically. Repair of a mismatch (gene conversion) will result in either a 6:2 or a 2:6 segregation pattern, depending on which strand the DSB is made. Failure to repair a mismatch (PMS) gives rise to a 5:3 or 3:5 segregation pattern. Involvement of all four chromatids generates complex events. 8:0 or 0:8 segregation patterns arise from two gene conversion events, whereas two independent PMS events give rise to aberrant 6:2 and 2:6. If a gene conversion and a PMS event occur, aberrant 7:1 and 1:7 patterns are observed. Aberrant 4:4 can also arise from two independent PMS events, or from branch migration.

difference in phenotype is observed. Gene conversion and PMS events are collectively known as non-Mendelian segregation (NMS) events, and the ratio of gene conversion to PMS events reflects the efficiency of the MMR system (Holliday, 1964). As heteroduplex DNA is formed during the production of crossovers and non-crossovers, gene conversion and PMS events can be associated with either (reviewed in Harfe and Jinks-Robertson, 2000 and in Surtees *et al.*, 2004).

Pms1p was the first protein to be identified with a role in the repair of hDNA in eukaryotes (Kramer *et al.*, 1989; Williamson *et al.*, 1985). In the absence of Pms1p there is an increase in PMS events (Prolla *et al.*, 1994; Wang *et al.*, 1999), indicative of a failure to repair mismatches. This was also observed in *mlh1*Δ (Hunter and Borts, 1997; Prolla *et al.*, 1994; Wang *et al.*, 1999), and in *msh2*Δ (Reenan and Kolodner, 1992). A double *mlh1*Δ*pms1*Δ mutant has levels of PMS events that are equivalent to either single mutant indicating that they function in the same pathway for meiotic repair (Prolla *et al.*, 1994). An *msh2*Δ*pms1*Δ double mutant also disrupted the polarity gradient at *ARG4* to the same extent as either single mutant suggesting that Msh2p also functions in the same pathway as Pms1p (Alani *et al.*, 1994). In comparison to *msh2*Δ, *msh3*Δ and *msh6*Δ mutants only show a small increase in the number of PMS events (Chambers *et al.*, 1996; Stone and Petes, 2006). This suggests that as in mitotic MMR, Msh2p is the major component of the MutS complex involved in meiotic MMR.

Mlh2p is not involved in meiotic repair as PMS events are not observed in *mlh2*Δ mutants (Abdullah *et al.*, 2004). However, an increase in gene conversions is observed at all loci studied (Abdullah *et al.*, 2004). Therefore, it has been suggested that Mlh2p is involved in regulating the length of tracts of heteroduplex DNA, through termination of DNA synthesis by an as yet unknown mechanism (Abdullah *et al.*, 2004). At many loci, Mlh1p and Mlh2p appear to have distinctive roles in the repair of heteroduplex DNA as an *mlh1*Δ*mlh2*Δ mutant exhibits an increase in NMS greater than either single mutant. This indicates that they make independent contributions to this process (Abdullah *et al.*, 2004). At *HIS4* differential effects are seen depending on the allele (and corresponding distance from start codon) studied (Abdullah *et al.*, 2004).

The role of Mlh2p also appears to be distinct from Pms1p, as they have differential effects on segregation of the *his4-X/HIS4* and *MATa/α* alleles (Wang *et al.*, 1999). Strains deleted for *MLH2* had no effect on NMS at the *his4-X/HIS4* locus, whereas gene conversion events were significantly increased at the *MATa/α* locus. The *pms1Δ* mutant significantly increased NMS at both loci. Additionally, approximately equal numbers of 2:6 and 6:2 segregations were observed in the *mlh2Δ* mutant, which is expected as DSB formation can occur on either parental chromosome. In contrast, the *pms1Δ* mutant had significantly increased numbers of 6:2 events (Wang *et al.*, 1999). As double-strand breaks are known to be initiated with equal frequency on both parental chromosomes, this suggests that all mismatches are not dealt with equivalently (Hoffmann *et al.*, 2005; White *et al.*, 1993)

1.4.1 Gene Conversion Gradients

At some loci it has been observed that a gradient of gene conversion occurs. At *HIS4* and *ARG4*, high levels of gene conversion occur at the 5' end of the gene, which is closest to the site of the double-strand break hotspot, and these levels decrease with distance from this site towards the 3' end of the gene (Alani *et al.*, 1994; White *et al.*, 1992). This has been termed a polarity gradient, and a number of models have been proposed to explain its presence. Initially, this gradient was thought to reflect the length of the heteroduplex DNA tracts formed during meiotic recombination. Mismatches occurring closest to the DSB are more likely to be included in hDNA than those further away, and hence a gradient is produced. However, it has been argued that this alone cannot account for the gradient (reviewed in Borts *et al.*, 2000).

The conversion-restoration model was proposed by Detloff *et al* (1992). Poorly repaired mismatches were used to analyse the polarity gradient at *HIS4*. They postulated that if this gradient was a reflection of the length of the hDNA tract only, the gradient should look the same as for well repaired mismatches. However, although at the 5' end of *HIS4* the level of aberrant segregation was the same as for well repaired mismatches, at the 3' end the level was much higher, making a much shallower gradient. Therefore, they concluded that the gradient reflected the type of repair (conversion or restoration) that was occurring. Mismatches that occur in hDNA in close proximity to the DSB are repaired predominantly using the donor strand resulting in conversion type-repair. With increasing distance from the DSB,

mismatches are repaired using either the donor or the recipient strands, resulting in both conversion and restoration type repair events and these occur equally. Kirpatrick *et al.* (1998) found further evidence to support this model. Firstly they demonstrated that restoration type repair does occur in yeast, by using the *HIS4* gene containing three mutations; one well repaired mutation flanked by two poorly repaired mutations (Kirkpatrick *et al.*, 1998). In some cases the two poorly repaired markers exhibited a PMS event, whereas the well repaired marker exhibited normal Mendelian segregation. As the flanking markers exhibited PMS, this suggests that all of the mutations were included in a single heteroduplex, and that the middle mutation must have undergone restoration repair (Kirkpatrick *et al.*, 1998). Additional experiments were performed to determine if the frequency that mutations were restored changed with distance from the *HIS4* DSB. A poorly repaired marker positioned near to the 3′ end of *HIS4* was used in conjunction with a well repaired marker positioned at different distances from the 5′ end of *HIS4*. PMS or gene conversion of the 3′ marker was used to detect events where the hDNA had spanned the entire length of *HIS4*, and consequently would of included the mutation at the 5′ end of the gene. A higher frequency of PMS or gene conversion of the 3′ marker was associated with normal Mendelian segregation of the *his4-17* marker (positioned at +688 bp) than with normal Mendelian segregation of the *his4-ACG* marker (positioned at +2 bp in relation to the start of the *HIS4* ORF). Therefore it was concluded that markers further away from the DSB undergo more restoration repair (Kirkpatrick *et al.*, 1998). Foss *et al* (1999) expanded on this model suggesting that repair of mismatches close to the initiating DSB would occur early, and are directed by the DSB itself towards conversion type repair, whereas mismatches further away would escape early repair. Instead these would be repaired later, directed by the nicks produced during Holliday Junction resolution. Therefore, the direction of repair (conversion or restoration) would depend on the direction of resolution of these structures.

The second model is the heteroduplex rejection model. It has been demonstrated that in the absence of Msh2p, a mismatch at the 3' end of *HIS4* exhibits a much higher level of aberrant segregation compared to the wild-type, and this was similar to the level observed at the 5' end in the wild-type (Alani *et al.*, 1994; Reenan and Kolodner, 1992). Therefore, it was suggested that mismatch repair proteins function to control

the length of heteroduplex DNA formed, and that once a mismatch is detected, the heteroduplex tract is prevented from extending any further. Additionally, Hillers and Stahl (1999) used poorly repaired markers to demonstrate that hDNA length changes depending on the position of the mismatch. Less aberrant segregation of a poorly repaired marker near the 3′ end of *HIS4* occurred when there was a second mismatch present near the 5′ end of the gene, in comparison to when there was no other mutation present (Hillers and Stahl, 1999). Therefore, mismatches closer to the initiating DSB will be encountered first and limit the formation of hDNA beyond that point. Consequently any other mismatches distal to that region will not be included in the hDNA. Only in the absence of any other mismatches will those at a distance away become incorporated into hDNA (Alani *et al.*, 1994; Hillers and Stahl, 1999).

A key determining feature of both of these models is the length of the hDNA formed. However, variation amongst different strain backgrounds has been observed. In a *S. cerevisiae* S288c background, it has been shown that a proportion of the heteroduplex DNA formed spans the entire *HIS4* gene (Detloff *et al.*, 1992; Porter *et al.*, 1993). However, in an *S. cerevisiae* Y55 background, contrasting results have been observed. Firstly, at *HIS4* the length of heteroduplex DNA varies. However, a higher frequency of short gene conversion tracts were detected (Hoffmann *et al.*, 2005). This favours the explanation that the gradient is a function of heteroduplex length. It was also thought that defects in mismatch repair would uncover all NMS events, but in an *mlh1*Δ strain, a conversion gradient is still present (Hunter and Borts, 1997). As already mentioned an *msh2*Δ strain abolishes the conversion gradient, indicating that it is the recognition and not the repair of the mismatches that result in the gradient (Alani *et al.*, 1994).

In the absence of Exo1p, there is a reduction in gene conversion along the entire length of the gradient of approximately 2-fold compared to wild-type (Khazanehdari and Borts, 2000), although the gradient itself is maintained. PMS events are not observed in an *exo1*Δ strain indicating that Exo1p does not function in meiotic mismatch repair. From this it was concluded that Exo1p did not play a role in the removal of hDNA arising after strand invasion. However, Kirkpatrick *et al.* (2000) observed an increase in the frequency of PMS events in strains containing both

palindromic and non-palindromic insertions at *HIS4*. Therefore they suggested that Exo1p plays a minor role in the removal of large mismatches in hDNA. This effect is allele-specific as no increase in PMS events was observed at *ARG4*, which contained a point mutation. This observation is consistent with that of Khazanehdari and Borts (2000) who used small, well repaired insertions in *HIS4*.

The reduction in gene conversion observed provides further evidence of a role for Exo1p in the resection of DSBs, as reduced resection will limit the amount of heteroduplex DNA formed between homologues. Consequently fewer mismatches will be included in the hDNA reducing the opportunity for their repair. The effect of *exo1*Δ on conversion gradients is also locus-specific (see Chapter 4). However, there are two possible explanations for the effects observed. Either fewer molecules are resected overall, or all molecules are still resected, but that resection is shorter than in the wild-type (Khazanehdari and Borts, 2000). This would give a predicted effect on the gradient observed along *HIS4*. If half the number of molecules are resected (indicated by the 2-fold reduction in NMS), then the gradient will be shallower than in the wild-type, and any mismatches present have half the chance of being incorporated into the hDNA, at all points along the gradient. If resection is shorter, then there will be a shift in the gradient due to the reduced length of hDNA, although the slope of the gradient will remain the same as wild-type. Consequently mismatches further away will have less opportunity to become included in the hDNA. Genetic studies to date have been unable to distinguish between these possibilities.

1.5 Aims and Objectives

Although a large number of proteins are known to function during meiotic recombination, their precise functions are relatively poorly characterised. This study aims to investigate the roles of two proteins, Mlh3p and Exo1p in order to try and elucidate some of the features of their functions during meiotic recombination.

Mlh1p is involved in the production of crossovers in conjunction with Mlh3p, and ATP binding by Mlh1p has been shown to be critical for the crossover function of Mlh1p. A number of ATPase mutants of Mlh3p will be analysed to determine whether ATP binding and/or ATP hydrolysis by Mlh3p is important for the meiotic functions of this protein.

Exo1p is implicated in crossover promotion and the resection of the *HIS4* DSB. Exo1p possesses two nuclease activities, a 5′-3′ exonuclease and a flap endonuclease activity. To determine if either of these activities are important for the functions of Exo1p during meiotic recombination, mutants that are defective for one or both of the nuclease activities will be utilised. Additionally, we would like to analyse the role of Exo1p in the resection of DSBs.

Chapter 2: Materials and Methods

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Yeast Strains

All yeast strains used were derived from *Saccharomyces cerevisiae* Y55 unless otherwise stated. Strain genotypes are included in the relevant chapter.

2.1.2 Plasmids

Several plasmids were used in this study, containing selectable markers or genes of interest (mutated or wild-type). A brief description of these is given in Table 2.1.

Table 2.1 Plasmids

Plasmid		Reference
pMOSBlue	Amp^R, dephosphorylated blunt ended cloning vector	Amersham Life Sciences
pAG32	*hphMX4*	Goldstein and McCusker, 1999
pFA6-*kanMX4*	*kanMX4*	Wach *et al.*, 1994
pWJ716	*KlURA3*[1]	Erdeniz *et al.*, 1997
pCORE	*kanMX4- KlURA3*[1]	Storici *et al.*, 2001
pGSKU	*GAL1*-I-*Sce*I, *kanMX4-KlURA3*[1]	Storici *et al.*, 2003
pRHB12	*his4::ClaI*	Borts and Haber, 1989
pEAM67	*EXO1* ORF	Sokolosky and Alani, 2000
pEAM69	*exo1-D171A*	Sokolosky and Alani, 2000
pEAM71	*exo1-D173A*	Sokolosky and Alani, 2000
pJM3	*exo1-E150D*	J. Meadows, unpublished
p93	Contains *lys2*::InsE-A_{14} allele, linearised using *Hpa*I	Tran *et al.*, 1997
pVC8	*exo1-S445A F447A F448A*	This study

[1] *KlURA3* is the *URA3* gene from *Kluyveromyces lactis*.

2.1.3 Oligonucleotides

All oligonucleotides used were purchased from Invitrogen. See Appendix 1 (Table A1.1) for a complete list of olignoucleotides used.

2.1.4 Media

Standard yeast media have been described previously (Abdullah and Borts, 2001; McCusker and Haber, 1988; Sherman *et al.*, 1986). Rich media, YEPD (Yeast

Extract, Peptone and Dextrose), consisted of 1% (w/v) Bacto yeast extract (Becton Dickinson; BD), 2% (w/v) Bacto peptone (BD), 2% (w/v) dextrose (D-glucose; Fisher Scientific) and 1% adenine solution (0.5% in 0.05 M HCl). YEPEG (Yeast Extract, Peptone, Ethanol and Glycerol) consisted of 1% succinic acid (BDH), 1% (w/v) Bacto yeast extract, 2% (w/v) Bacto peptone, 2% glycerol (ICN), 2% ethanol, 1% adenine solution (0.5% in 0.05 M HCl) and 2% Bacto agar (BD), corrected to pH 5.5 (McCusker and Haber, 1988).

Minimal media contained 0.68% (w/v) Difco yeast nitrogen base without amino acids (BD) and 2% (w/v) dextrose (Sherman *et al.*, 1986). Synthetic complete media was made as minimal media, with the addition of 875 mg nutrient mixture per litre (Table 2.2; Abdullah and Borts, 2001) and supplemented with 6.3 ml 1% leucine and 3 ml 1% lysine amino acid solutions. Omission media was made as synthetic complete media, with the appropriate amino acid or nutrient excluded (Abdullah and Borts, 2001). All nutrients and amino acids listed in Table 2.2 were purchased from Sigma.

Table 2.2 Composition of Nutrient Mixture

Nutrient/Amino acid	Amount (mg)
Adenine sulphate	800
L-arginine-HCl	800
L-aspartic Acid	4000
L-histidine-HCl	800
L-leucine	800
L-lysine-HCl	1200
Methionine	800
L-phenylalanine	2000
Threonine	8000
L-tryptophan	800
Tyrosine	1200
Uracil	800

Cycloheximide, G418 (geneticin; Wach *et al.*, 1994), hygromycin B and nourseothricin were added to YEPD after autoclaving (Table 2.3; Goldstein and McCusker, 1999). 5'-Fluoro-orotic acid (FOA; Boeke *et al.*, 1984) was added to uracil omission media, supplemented with 50 μg/ml uracil, and canavanine was

added to arginine omission media (Table 2.2).

For sporulation, synthetic complete media containing potassium acetate (KAc; 2% (w/v) potassium acetate (Fisher Scientific), 0.22% (w/v) Bacto yeast extract, 0.05% (w/v) dextrose, 0.09% complete nutrient mixture (Table 2.2)), adjusted to pH 7.0, was used (Hunter and Borts, 1997).

Table 2.3 Drugs

Drug	Concentration (μg/ml)	Supplier
Ampicillin	100	Sigma
Cycloheximide	10	Sigma
Canavanine	60	Sigma
5′-FOA	1000	Apollo Scientific
Geneticin (G418)	400 / 200*	Invitrogen
Hygromycin B	600	Invitrogen
Nourseothricin	100	Werner Bioagents

* Different concentrations used for selection and confirmation of G418 resistance.

Luria-Bertani (LB) medium contained 1% (w/v) Difco tryptone peptone (BD), 0.5% (w/v) Bacto yeast extract, 1% (w/v) NaCl (Sigma), corrected to pH 7.0 and supplemented with ampicillin (100 μg/ml; Sigma; Sherman *et al.*, 1986).

All were corrected to pH 6.0-6.5 unless otherwise stated. To make agar plates, 2.5% Bacto agar was added to all media (unless stated otherwise).

2.1.5 DNA Markers

λ DNA digested with *Bst*EII or *Hin*dIII (New England Biolabs; NEB) or 100 bp and 1 kb markers (Invitrogen) were used as standards at a concentration of 50 ng/μl.

2.2 Methods

2.2.1 Agarose Gel Electrophoresis

Agarose (Cambrex Bioscience) was melted in 1 x TBE (0.09 M Tris-Borate, 0.002 M EDTA, pH 8.0), at either a concentration of 0.8% or 2% depending on the size of fragments to be visualised (Sambrook *et al.*, 1989). 1 x TBE was also used as the electrophoresis running buffer. To stain the DNA, 0.2 μg/ml ethidium bromide (Sigma) was added per 100 ml agarose gel. 5 x loading dye (10% Ficoll type 400 (Sigma), 100 mM EDTA, 0.2% bromophenol blue (Sigma), 0.2% xylene cyanol (Sigma) and 0.5% SDS (Amersham Biosciences)) was added to samples, and molecular weight markers. Molecular weight markers were used to allow determination of the size (and concentration; Section 2.2.6) of fragments by comparison of the distance samples had travelled through the agarose gel. Visualisation of DNA under UV light was performed using a Gel Logic 200 Imaging System (Kodak).

2.2.2 Preparation of Genomic DNA

2.2.2.1 Potassium Acetate Method

Yeast cells were grown overnight in 5 ml liquid YEPD, at 30°C with shaking. The cells were centrifuged for 5 minutes at 3,600 rpm before being resuspended in 0.5 ml of spheroplasting solution (1.2 M sorbitol, 0.2 M Tris-HCl pH 8.5, 0.02 M EDTA, 0.1% β-mercaptoethanol (βME). 50 μl of 10 mg/ml zymolyase (made up in spheroplasting solution, without βME) was added and cells were incubated at 37°C for 20 minutes. The cells were centrifuged at 13,000 rpm for 30 seconds to confirm spheroplasting. This is the breakdown of the cell wall, and is apparent by the 'fluffy' appearance of the pellet after centrifugation as opposed to a compact appearance. If spheroplasted, the cells were centrifuged for a further minute. Otherwise they were incubated again at 37°C, and checked regularly. The supernatant was removed and the cells were gently resuspended in 50 μl 1 M sorbitol, and 0.5 ml lysis solution (50 mM Tris-HCl, 100 mM NaCl, 100 mM EDTA, 0.5% SDS) was added. In addition, 0.2 mg proteinase K (Roche Diagnostics) and 50 μl 1 mg/ml RNAse (Sigma) was added, and cells were incubated at 65°C for a minimum of 2 hours. After this time, the cells were chilled

on ice for 5 minutes, 20 µl of pre-chilled 5 M potassium acetate was added and they were left on ice for a further 15 minutes. After centrifugation at 4°C for 20 minutes at 13,000 rpm, the supernatant was removed to clean 1.5 ml Eppendorf tubes and an equal volume of isopropanol was added. This was followed by washing the cells in 1 ml 70% ethanol, and samples were left to air dry. DNA was resuspended in 100-200 µl 1 x TE (1 M Tris, 0.5 M EDTA, pH 8.0). This method was described previously by Sherman *et al.* (1986).

2.2.2.2 Phenol Chloroform Method

This protocol is the same as the potassium acetate method (Section 2.2.2.1), up to and including the incubation at 65°C. After chilling the cells on ice for 5 minutes, 0.5 ml phenol chloroform (25 units phenol : 24 units chloroform : 1 unit isoamyl alcohol; phenol and chloroform from Fisher Scientific) was added. Samples were centrifuged at 13,000 rpm for 10 minutes, and the aqueous phase removed to a clean 1.5 ml Eppendorf tube. The addition of phenol chloroform was repeated once more before adding 1 ml 100% ethanol and centrifuging for a further 10 minutes. The samples were washed in 70% ethanol, left to air dry and resuspended in 100-200 µl 1 x TE as above. This method was modified from Borts *et al.* (1986).

2.2.3 Preparation of Plasmid DNA

Three methods of plasmid DNA extraction from *E. coli* cells were used, depending on the quality of DNA required and the number of samples to be processed. The first method was a boiling 'miniprep' method, used to process a large number of samples, when checking for inserts in plasmids. 1.5 ml cultures of LB-ampicillin were inoculated and grown overnight at 37°C with shaking. The cells were centrifuged at 2,500 rpm in a table-top centrifuge. The supernatant was removed and the cells resuspended in 0.35 ml STET (8% sucrose (Sigma), 5% (v/v) Triton X-100 pH 8.0 (Sigma), 50 mM Tris-HCl pH 8.0, 50 mM EDTA), and transferred to 1.5 ml Eppendorf tubes. 25 µl lysozyme (10 mg/ml; Sigma) was added to each sample, followed by incubation at 95°C for 3 minutes. The samples were centrifuged for 10 minutes at 13,000 rpm. The pellet was removed using a toothpick, and 0.35 ml isopropanol was added. The centrifugation step above was repeated, the supernatant removed and the samples left to air dry. The plasmid DNA was resuspended in 50 µl 1 x TE. Plasmids containing the correct structure or

plasmids required as PCR templates for example, were cultured as above and processed using either a QIAprep Spin Miniprep Kit (Qiagen) or a ChargeSwitch Plasmid ER Mini Kit (Invitrogen), following the supplied instructions.

2.2.4 DNA Purification

To purify PCR products, the reactions were processed using either the MinElute PCR Purification Kit (Qiagen; for a final volume of 10 µl) or the ChargeSwitch PCR Clean-Up Kit (Invitrogen; for final volumes of 25-50 µl) depending on the output volume required. To extract PCR products from agarose gels, the required bands were excised and processed using the MinElute or QiaQuick Gel Extraction Kit (Qiagen; for final volumes of 10 µl or 25-50 µl respectively), again depending on the output volume required.

2.2.5 Polymerase Chain Reaction

2.2.5.1 Genomic DNA

Polymerase Chain Reaction (PCR) was used for the amplification of specific sequences required for transformation, cloning, labelling or sequencing. Typically, *Taq* Polymerase (ABgene) was used in reactions unless high fidelity was required. For sequencing or PCR-based mutagenesis, *Pfu* polymerase (Stratagene) or Phusion (New England Biolabs) were used. A typical reaction consisted of 1 x PCR buffer, from a stock of 11.1 x (45 mM Tris-HCl pH 8.8, 11 mM ammonium sulphate (Fisher Scientific), 4.5 mM magnesium chloride (BDH), 6.7 mM β-mercaptoethanol (Sigma), 4.4 µM EDTA pH 8.0, 1 mM of each dNTP (Gibco), and 113 µg/ml BSA) (Jeffreys *et al.*, 1990), 0.2 µM each oligonucleotide, approximately 50 ng DNA, 1-2.5 units *Taq* polymerase. *Pfu* polymerase and Phusion were used according to guidelines provided. Depending on the intended use for the PCR product, reaction volumes of 10 – 50 µl were used. The cycling steps of the PCR programme varied depending on the individual PCR. For genomic DNA, an initial denaturation step of 95°C for 1 minute was followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute and elongation at 72°C for 1 minute/kb of DNA to be amplified. A final step at 72°C for 10 minutes was included. The parameters varied in particular when using *Pfu* polymerase or Phusion, and the manufacturers guidelines were followed for these. All PCR was carried out using a PTC-225

Peltier Thermal Cycler (MJ Research).

2.2.5.2 Colony PCR

Colony PCR was performed by adding a yeast cell suspension directly to the reaction as opposed to genomic DNA. Approximately 0.2 mm^2 of cells were resuspended in 20 µl 0.02 M NaOH (Fisher Scientific), and 2 µl used in the subsequent reaction. Reactions had a final volume of 10 µl, with concentrations of reagents the same as for PCR from genomic DNA (Section 2.2.5.1), except for the oligonucleotides, which were used at a concentration of 1.5 µM. Cycling was as described above except for using an extended initial denaturation step of 3 minutes at 95°C.

2.2.6 DNA Quantification

To quantify DNA for use in downstream applications, agarose gel electrophoresis was used. The samples were run alongside a defined molecular weight marker, which was used as a comparison. Kodak ID Image Analysis Software was used to compare the intensities of the sample bands to the intensities of the bands of the marker, which are of a known concentration. This was performed following the software instructions.

2.2.7 Restriction Enzyme Digestion of DNA

Restriction enzymes were purchased from NEB, and used with buffers supplied, following the suppliers guidelines.

2.2.8 DNA Sequencing

50-100 ng of DNA was used for sequencing (Sanger *et al.*, 1977), with templates generally being 500-800 bp. Two protocols for sequencing were followed. For the first protocol, reactions consisted of 2 µl BigDye (v3.0) reaction mix (ABI Ltd), 2 µl HalfDye solution (Bioline), 1 µl oligonucleotide (3.2 pmoles/µl) and a maximum of 5 µl DNA. The following programme was used for cycling: 96°C for 10 seconds, followed by 25 cycles of 98°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes. To precipitate samples, 2 µl 1.5 M NaOAc and 250 mM EDTA, pH 8.0, and 50 µl 95% ethanol was added. The samples were vortexed and chilled on ice for 15 minutes, followed by centrifugation for 40 minutes at

13,000 rpm. The supernatant was removed, the samples washed in 70% ethanol, and air-dried. The samples were sent to the DNA Sequencing Facility, Department of Biochemistry, Oxford.

To prepare sequencing reactions for PNACL, University of Leicester, a different protocol was used. Reactions consisted of 8 µl 1:4 dilution of BigDye solution (v3.0), 8 µl template DNA and 4 µl oligonucleotide (1 pmole/µl). The programme used for cycling was as follows: 96°C for 1 minute, with 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. The reactions were purified using the DyeEx 2.0 Spin Column kit (Qiagen).

2.2.9 Genetic Procedures

The following methods have been previously described (Abdullah and Borts, 2001; Hunter and Borts, 1997; Sherman *et al.*, 1986).

2.2.9.1 Mating Type Testing

To test mating type, strains were replicated to YEPD plates with *MAT*a and *MAT*α tester strains present. After incubation at 30°C overnight, these were replicated to minimal media and grown again at 30°C overnight. Due to auxotrophies present in the tester strains (*ura2*, *tyr1*), the strains being tested can only grow if mating has occurred resulting in complementation of the auxotrophic genotypes. Strains used were also grown on YEPEG to ensure they were not petite (Section 2.1.4). Petite strains have lost all or part of their mitochondrial genome, and therefore have a deficiency in respiration (Hampsey, 1997). Petite cells cannot utilise ethanol or glycerol as a carbon source.

2.2.9.2 Sporulation

Sporulation was induced by starving diploid cells of nitrogen. Two haploid strains of opposite mating type were mated for 4 hours at 30°C on YEPD, and transferred to sporulation media containing KAc (Section 2.1.4; Abdullah and Borts, 2001). Cells were left at 23°C (unless otherwise stated) for 3-5 days to sporulate. Sporulation of yeast cells was determined by visualising the cells on a Swift M400D phase contrast microscope (Swift Instruments).

2.2.9.3 Selection for Diploids

After mating of cells, selection can be used to enrich for diploids. Cells were transferred to minimal media, supplemented only with any nutrients or amino acids that neither haploid can synthesise. All diploid strains used in this study were heterozygous for a number of markers, and therefore in their haploid states would be unable to synthesise some amino acids. Consequently, only cells that have mated to form diploids can grow on specifically supplemented minimal media.

2.2.9.4 Tetrad Dissection

To prepare the cells for dissection, they were resuspended in 100 µl dissecting buffer (1 M sorbitol (Sigma), 10 µM EDTA, 10 µM sodium phosphate ($NaPO_4$; BDH)), and 5 µl zymolyase (5 mg/ml in dissecting buffer; Seikagaku Corporation). The cells were incubated at 37°C for 30 minutes to digest the ascal walls. A further 400 µl dissecting buffer was added, and the preparation stored at 4°C for up to two weeks. For dissection, the prepared cells were spread along the centre of YEPD plates. A Zeiss Axioscope microscope and micromanipulator were used for dissecting yeast ascospores (Sherman *et al.*, 1986).

2.2.9.5 Recombination Analysis

The diploid strains used were heterozygous for markers at a number of loci on chromosomes III and VII, allowing meiotic recombination to be monitored at intervals on these chromosomes. Dissected tetrads were replicated to omission media or drug-containing media as required, to allow scoring of segregation of the markers. Mating-type was also tested. This also enabled selection of desired segregants from a given cross. The software programme Linkage Macro 6.9 (Greene, 1994) was used to analyse the data, by calculating map distances as described below, and determining the numbers of non-Mendelian segregation events (NMS). NMS includes gene conversion (6:2/2:6) and post-meiotic segregation (PMS) events (5:3/3:5). Only tetrads consisting of four viable spores were used for analysis of recombination. Map distances in centiMorgans (cM) were calculated using the formula:

$$cM = ½(TT + 6NPD) / (TT + NPD + PD)$$

where TT, NPD and PD are the numbers of tetratypes, non-parental ditypes and parental ditypes respectively (Perkins, 1949).

The number of viable spores within each tetrad dissected was also recorded, and used to calculate the viability (%) of the strain as follows:

Spore viability = ((4 x no. 4-spore tetrads + 3 x no. 3-spore tetrads + 2 x no. 2-spore tetrads + no. 1-spore tetrads) / (4 x total no. of tetrads)) x 100

Tetrads with only two viable spores were used to analyse the degree to which non-disjunction occurred, and to determine whether a defect in meiosis I was responsible. By following the segregation of *ADE1*, which is adjacent to the centromere on chromosome I, the surviving spores can be analysed to determine if they are sister spores or non-sister spores. Sister spores have the same phenotype, either both being auxotrophic or prototrophic for adenine. The *MAT* locus is present on chromosome III, and can be used to determine if non-disjunction of chromosome III has occurred. The presence of both *MATa* and *MATα* alleles in a single spore gives rise to non-maters.

2.2.10 Random Spore Assays

Pairs of haploid strains were used that had different alleles of *HIS4* (Chapter 6, 2.1) and recessive drug resistance markers *can1* and *cyh2*. These strains can be used to measure rates of gene conversion at *HIS4*. Diploids heteroallelic for *his4* and drug resistance were mated together as previously described (Section 2.2.9.1), and sporulated for five days at 23°C (unless stated otherwise). Once sporulation was confirmed, cells were resuspended in 500 µl dissecting buffer with 50 µl zymolyase, and incubated at 37°C in a heating block for 3 hours.

After heating, the cells were vortexed for 10 minutes using a Vortex Genie 2 with a microtube foam insert (Scientific Industries Inc), followed by 2 minutes on ice. This cycle was repeated four times, or until the spores were sufficiently separated, as determined by visualisation under the Swift M400D phase contrast microscope (Protocol from E. Hoffmann, personal communication). Six 1:10 serial dilutions were made in sterile distilled water, and 200 µl of the 10^{-2} to 10^{-6} dilutions were

plated on complete media lacking arginine, and containing canavanine and cycloheximide (Section 2.1.4; Hunter and Borts, 1997). This allowed determination of the overall cell number. To select for histidine positive cells, 100 µl of each of the neat solution and 10^{-1} to 10^{-2} dilutions were plated onto two histidine and arginine dropout plates, containing canavanine and cycloheximide. These were grown for three days at 30°C, and the colonies were counted using a colony counter (Stuart Scientific). Growth on media containing canavanine and cycloheximide allowed selection for true meiotic progeny, as only colonies that have undergone meiosis, and subsequent haploidisation, can have resistance to both. Between two and four independent experiments were carried out for each strain or condition tested.

These experiments were used to give a measure of gene conversion of different alleles of *HIS4*, at different distances from the initiating meiotic double-strand break (DSB). Due to the selection, only one in four of all meiotic products were analysed. The total number of colonies and histidine prototrophs were calculated, taking into account the dilutions made and the amount plated. Weighted averages were used to obtain a single value from the different dilutions. From this, the frequency of conversion was calculated by:

Frequency = (number of histidine prototrophs / total number of colonies) x 100.

2.2.11 Yeast Transformation

Transformations were performed using a modified version of the lithium acetate method (Gietz *et al.*, 1992). Yeast cells were grown overnight in 5 ml liquid YEPD, at 30°C with shaking. A 1/10 dilution of cells was made in fresh YEPD and grown for 3 hours under the same conditions. The cells were centrifuged for 5 minutes at 3,600 rpm and washed twice in 1 ml sterile distilled water. The cells were resuspended in 1 ml 0.1 M lithium acetate (LiAc; Sigma) and split into two aliquots, one of which was used as a negative control, containing no DNA. After centrifugation, the following components were added to the pellet: 240 µl (w/v) PEG 3350 (50%; Sigma), 36 µl 1 M LiAc, 50 µl 2 mg/ml denatured salmon sperm DNA and 34 µl DNA for transformation or sterile distilled water for the control. The mixture was vortexed until cells were completely resuspended, and incubated at

30°C for 30 minutes, followed by 40 minutes at 42°C. The cells were washed twice more before plating onto the appropriate selection media. When selecting for drug resistance, the cells were grown in 1 ml liquid YEPD with shaking for 4 hours before plating.

2.2.12 Site Directed Mutagenesis

2.2.12.1 Gene Disruption

Genes of interest were disrupted using a PCR-based method (Wach *et al.*, 1994). Oligonucleotides with homology to a selectable cassette at the 3′ end, and the upstream and downstream regions of the gene of interest at the 5′ end, were used to allow amplification and insertion of the cassette into the genome at the required location (See Appendix 1, Table A1.1, for sequences of oligonucleotides used).

2.2.12.2 PCR-Based Allele Replacement

One method used to introduce point mutations was the PCR-based allele replacement method (Erdeniz *et al.*, 1997; Figure 2.1). The first step of this two-step process required amplification of the desired allele and amplification of the 5′ and 3′ ends of the *Kluyveromyces lactis URA3* gene (*KlURA3* from pWJ716; Table 2.1) with oligonucleotides containing complementary sequences (Figure 2.1). If the required mutation existed on a plasmid, then the relevant plasmid was used as a template for PCR. If the mutation did not pre-exist, it was incorporated into an oligonucleotide which was used to amplify the ORF of interest. The second step involved combining the amplified allele in separate reactions with each amplified end of the *KlURA3* gene to produce fusion products. Transformation of the two fusion products into yeast cells and *in vivo* recombination produces a duplication of the mutation. These events are selected for by plating on uracil omission media. Ura^+ transformants were replica plated to 5′-FOA, which is toxic to cells expressing *URA3*. This selects for cells which have undergone a "pop-out" event, removing the *URA3* gene and leaving only one copy of the desired mutant allele. Sequences of all oligonucleotides used are listed in Appendix 1, Table A1.1.

2.2.12.3 *delitto perfetto*

Another method used to create point mutants was "*delitto perfetto*" (Storici *et al.*, 2001). This is a two-step transformation procedure used to modify, replace or insert

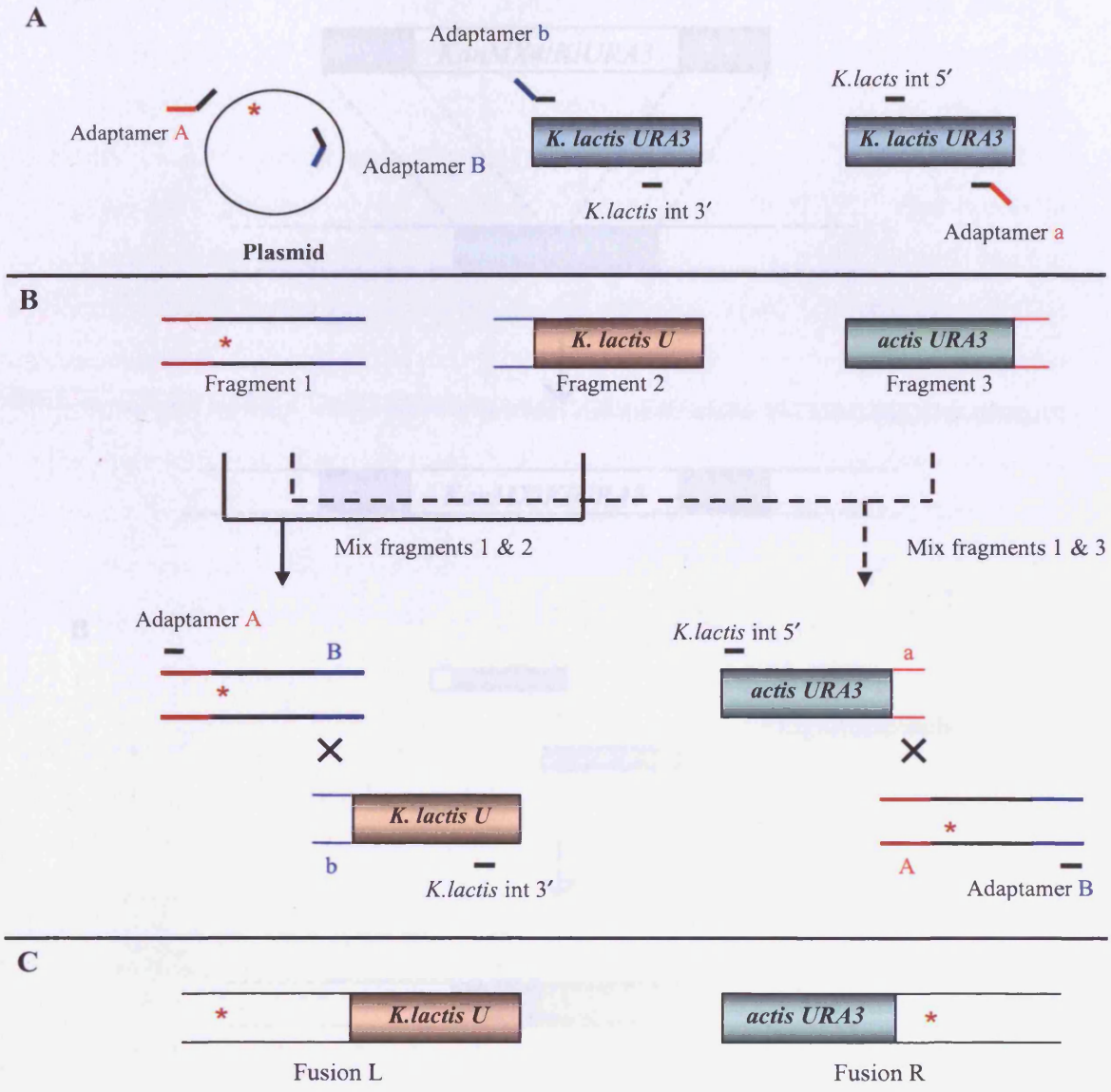


Figure 2.1. PCR-Based Allele Replacement

A. Amplification of the desired mutant allele from a plasmid, and PCR of the 5′ and 3′ ends of *KlURA3* to produce overlapping fragments.

B. The mutant allele was combined with the two *KlURA3* fragments independently, followed by PCR.

C. The two final products, used for transformation, consist of the mutant allele tagged with the 5′ and 3′ ends of *KlURA3*.

— Black lines indicate oligonucleotides that are entirely homolgous to the target sequence. Coloured lines indicate oligonucleotides with homologous tags (adaptamer A with a, and B with b), used to create the fusion products.

* Represents the mutation. For further details, see section 2.2.12.2.

Adapted from Erdeniz *et al.*, 1997.

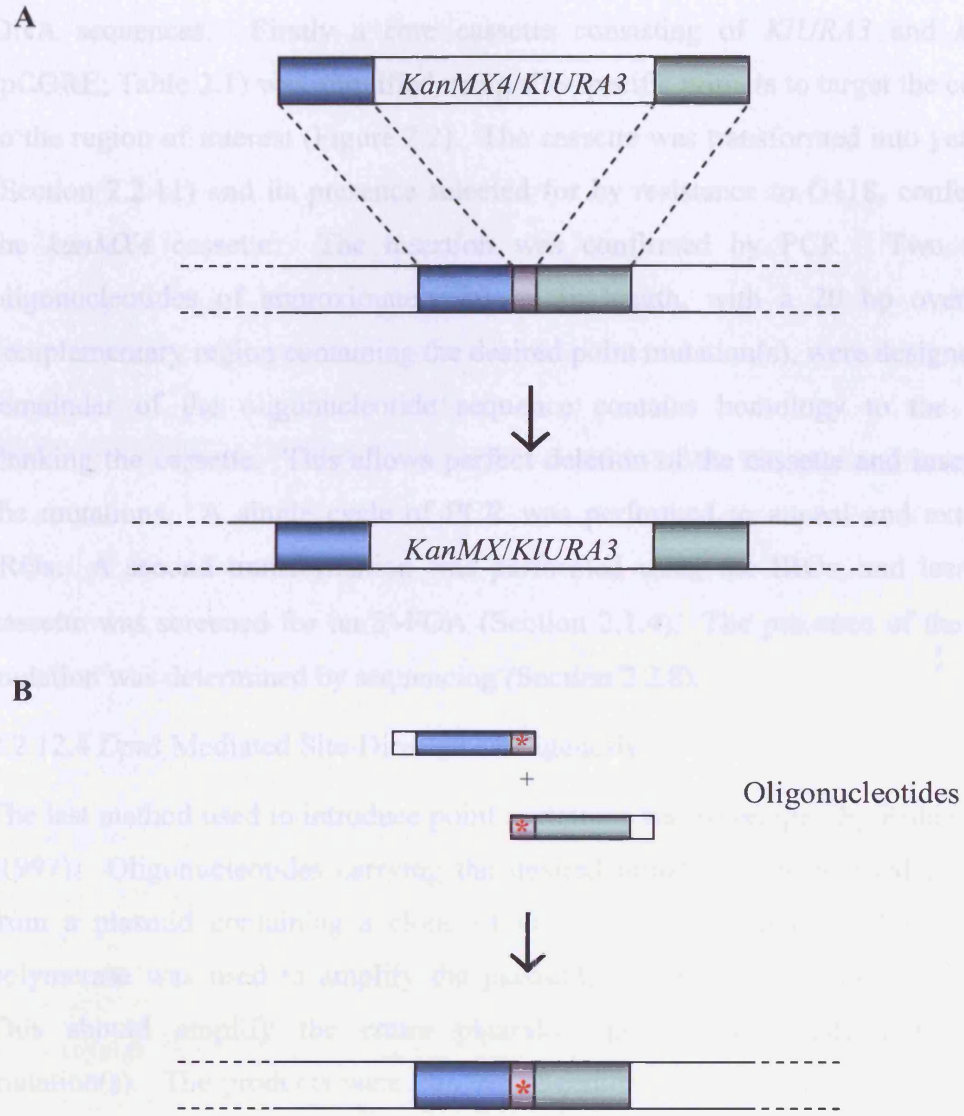


Figure 2.2 The *delitto perfetto* Technique

A. Insertion of a CORE cassette, containing *kanMX4* (selectable/reporter) and *KlURA3* markers (counterselectable), via homologous recombination, resulting in deletion of the region to be mutated.

B. Transformation of cells with oligonucleotides containing homology to the target area and the mutation to be introduced. This results in deletion of the CORE cassette and insertion of the desired mutation(s).

Green and blue boxes mark regions of homology, the grey box indicates the region to be mutated.

* : Represents the mutation

Adapted from Storici *et al.*, 2001.

DNA sequences. Firstly a core cassette consisting of *KlURA3* and *kanMX4* (pCORE; Table 2.1) was amplified using site-specific primers to target the construct to the region of interest (Figure 2.2). The cassette was transformed into yeast cells (Section 2.2.11) and its presence selected for by resistance to G418, conferred by the *kanMX4* cassette. The insertion was confirmed by PCR. Two specific oligonucleotides of approximately 80 bp in length, with a 20 bp overlapping complementary region containing the desired point mutation(s), were designed. The remainder of the oligonucleotide sequence contains homology to the regions flanking the cassette. This allows perfect deletion of the cassette and insertion of the mutations. A single cycle of PCR was performed to anneal and extend the IROs. A second transformation was performed using the IROs, and loss of the cassette was screened for on 5′-FOA (Section 2.1.4). The presence of the correct mutation was determined by sequencing (Section 2.2.8).

2.2.12.4 *Dpn*I Mediated Site-Directed Mutagenesis

The last method used to introduce point mutations was developed by Fisher and Pei (1997). Oligonucleotides carrying the desired mutation(s) were used to amplify from a plasmid containing a clone of the gene to be mutated. Phusion DNA polymerase was used to amplify the plasmid, following the guidelines provided. This should amplify the entire plasmid, and simultaneously introduce the mutation(s). The products were purified (Section 2.2.3), and digested using *Dpn*I, which will digest the parental, methylated plasmid but not the newly synthesised plasmid. The resulting plasmids were transformed into library efficiency *E. coli* DH5α competent cells (Invitrogen), following the suppliers instructions. Transformants were selected for on LB-ampicillin media (Section 2.1.4). Plasmid DNA recovered from transformants was analysed by digestion (Section 2.2.7) and sequencing (Section 2.2.8) to confirm the presence of the required mutation(s). Once a correct plasmid had been constructed, the mutated region was digested out and inserted into the relevant yeast strain using the "*delitto perfetto*" method (Section 2.2.12.3).

2.2.13 Measuring Mutation Rates

Mutation rates were determined using fluctuation tests (Lea and Coulson, 1949) modified by Reenan and Kolodner (1992; method of the median). Two different

alleles were used to test for different types of mutations. The *lys2*::InsE-A_{14} allele is an insertion of 14 adenine residues in the *LYS2* gene, resulting in a frameshift that renders it inactive. Insertions or deletions which restore the reading frame also restore the function of the gene (Tran *et al.*, 1997). The second allele is *CAN1*, an arginine permease which confers sensitivity to canavanine, as canavanine is a structural analogue of arginine. Frameshift or point mutations which render this gene inactive confer resistance to canavanine (Flores-Rozas and Kolodner, 1998). Strains were initially tested by replica plating patches onto lysine omission media and canavanine containing media to give an estimate of the level of mutator phenotype exhibited by the strain. Mutations conferring resistance to canavanine or restoring *LYS2* function will allow colonies to grow on the respective media. Therefore, a modest mutator phenotype is indicated by a low number of papillations (1-10) whereas a strong mutator is indicated by many papillations (>100; Figure 2.3).

Yeast strains to be tested were grown up from frozen stocks on YEPEG, and streaked for single colonies on YEPD. These were grown for 3 days at 30°C. For strains that exhibited a small number of papillations on selective media, 11 singles were cultured overnight in 5 ml liquid YEPD to increase the number of cells, and hence events. Cells were pelleted at 3,600 rpm for 5 minutes and then resuspended in 200 µl sterile distilled water. Eight 1:10 serial dilutions were made and 200 µl of the 10^{-5} to 10^{-8} dilutions was plated on synthetic complete media to determine the total number of cells per culture. 50 µl of the neat solution and 10^{-1} dilution was plated on lysine omission media, and 100 µl of the neat solution and 10^{-1} dilution was plated onto media containing canavanine (Section 2.1.4) to determine the number of mutations occurring in a given population.

For strains displaying a large number of papillations on selective media, single colonies were resuspended in 200 µl sterile distilled water and dilutions made as above. The 10^{-4} to 10^{-7} dilutions were plated on complete media, the neat solution and 10^{-1} dilution were plated on lysine omission media and 10^{-1} and 10^{-2} dilutions were plated on media containing canavanine. The volumes plated were the same as above. These were grown for three days at 30°C and the resulting colonies counted

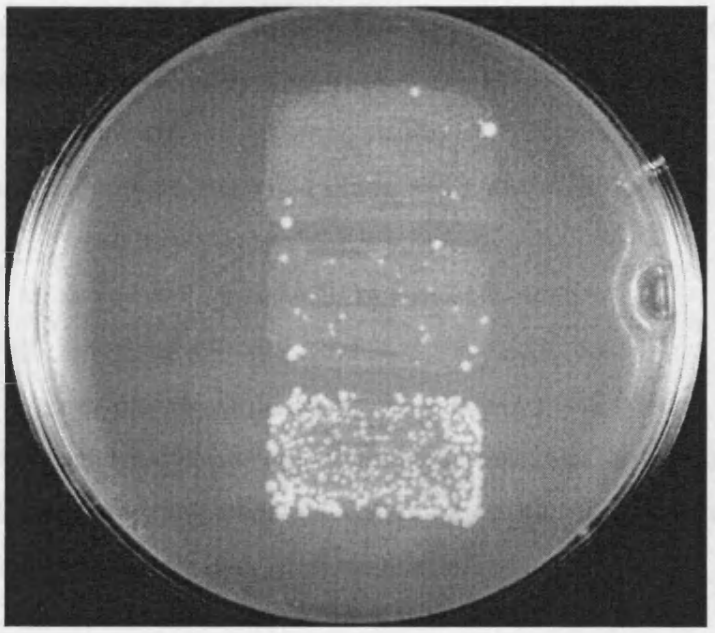


Figure 2.3 Papillation Test
The resulting papillations on lysine omission media are shown to demonstrate the difference between wild-type (top), a weak mutator (middle) and a strong mutator (bottom).

using a colony counter. Three independent experiments were performed for each strain to be tested.

The mutation rate was calculated using the following two formulae:

$$r_0 = M\,(1.24 + \ln M)$$

where r_0 is the median number of colonies on selective media (either lacking lysine or containing canavanine) out of the 11 cultures, and M is the average number of colonies on selective media per culture. Interpolation was used to determine M, and was subsequently used to calculate the mutation rate (r):

$$r = M / N$$

where N is the average number of total cells from the 11 cultures.

2.2.14 Statistical Analysis

Statistical comparisons of crossing over and the distribution of spore classes between strains were made using a G-test of homogeneity (Sokal and Rohlf, 1969). To compare the overall spore viabilities, the z-test was used. The Pearson's chi-squared (χ^2) test for goodness of fit (Sokal and Rohlf, 1969) was used to compare an observed distribution with the expected distribution, whereas a contingency χ^2 test was used to compare two distributions for homogeneity. The contingency χ^2 test and G-test of homogeneity are equivalent. The Yates' correction for continuity was applied to the Pearson's χ^2 test when there was only one degree of freedom (Yates, 1934). The Student's t-test was used to compare means between two populations.

In all statistical analyses where multiple comparisons were made using the same datasets, the Dunn-Sidak correction factor was used for significance rating (Sokal and Rohlf, 1969). With increasing numbers of comparisons to be made, the probability to be considered significant is lowered. This is to prevent the false rejection of the null hypothesis, when it should be accepted (Type I error).

Therefore, a number of different P values were adhered to for significance rating depending on the number of comparisons made.

Chapter 3: The Role of *MLH3* in Meiosis

Chapter 3: The Role of *MLH3* in Meiosis

3.1 Introduction

3.1.1 Structure of MutL and MutL Homologues

The elucidation of the crystal structure of a 40 kDa N-terminal fragment (LN40) of *E. coli* MutL and subsequent functional analyses (Ban and Yang, 1998; Ban *et al.*, 1999) has allowed equivalent studies on the MutL homologues of yeast. The N-terminal ends are conserved amongst the MutL family (Figure 3.1), whereas the C-terminal end is more divergent. MutL functions as a homodimer whereas the yeast MutL homologue Mlh1p functions as a heterodimer with each of the other three homologues, Mlh2p, Mlh3p and Pms1p (Wang *et al.*, 1999). These interactions are mediated through the C-terminus (Ban and Yang, 1998; Pang *et al.*, 1997). In addition it is thought that the N-terminal domain of the proteins also have to interact for the complex to be fully functional (Ban and Yang, 1998). Within this domain, there are four ATPase motifs (Figure 3.1) that are shared with other members of the GHKL ATPase super-family (DNA Gyrase, Hsp90, Histidine Kinase and MutL; Dutta and Inouye, 2000). The N-terminal interaction occurs as a result of ATP binding by both partners, which results in a conformational change of the proteins (Figure 3.2; Ban *et al.*, 1999; Tran and Liskay, 2000). This also allows coordination of downstream events (Tran and Liskay, 2000). Motif III contains the GFRGEAL box, which forms the lid of the ATP-binding pocket, and is also thought to be required for the N-terminal interactions subsequent to ATP binding (Ban and Yang, 1998; Ban *et al.*, 1999).

3.1.2 Functional Analysis of Mlh1p and Pms1p

Based on the functional analogy between MutL and Mlh1p and Pms1p, Tran and Liskay (2000) made mutations to perturb the ATP binding activity of Mlh1p and Pms1p (G98A and G128A respectively) or the ATP hydrolysis activity (E31A and E61A respectively; Ban and Yang, 1998; Ban *et al.*, 1999; Figure 3.1). The functionality of these mutants in mitotic mismatch repair (MMR) was analysed using mutation rate assays (Tran and Liskay, 2000). High mutation rates are exhibited by both Mlh1p and Pms1p ATP binding mutants, although the mutation rates are not equivalent to those of strains in which these genes are deleted. This

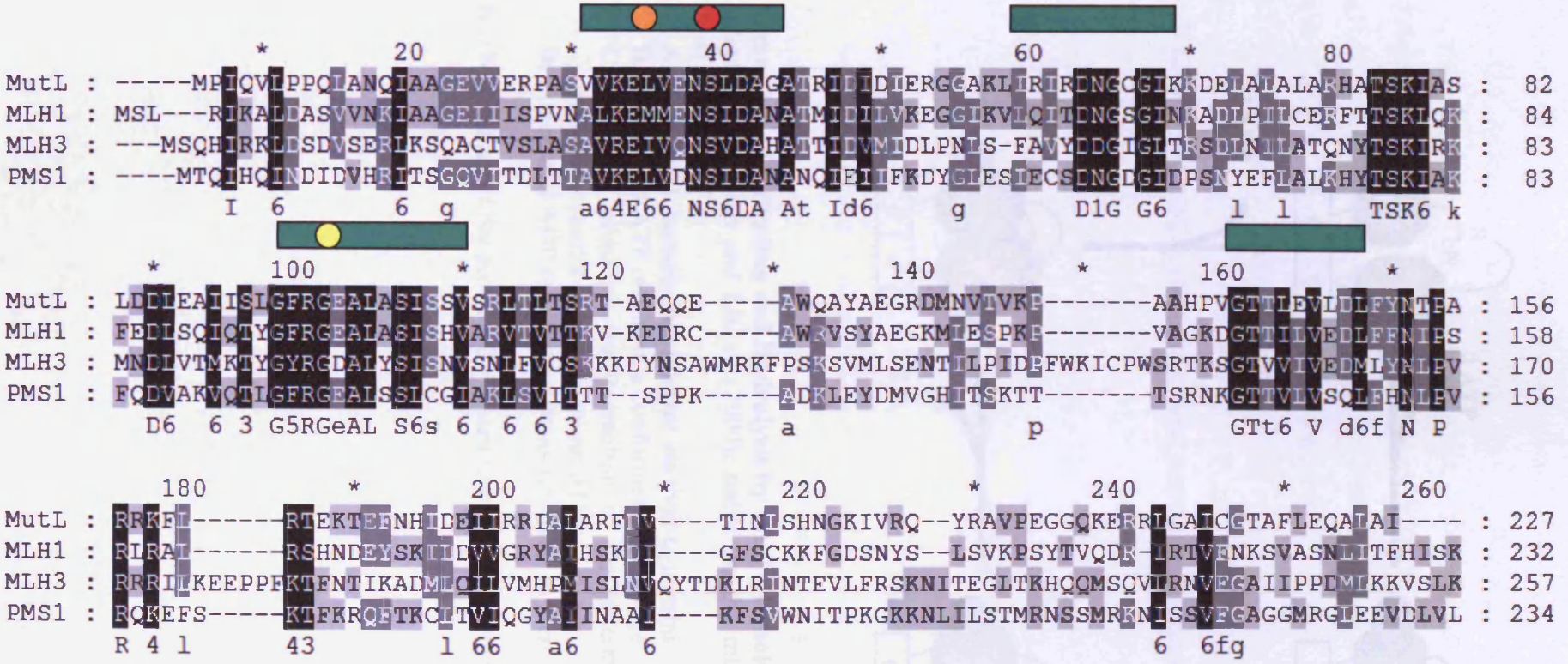


Figure 3.1 Alignment of the N-termini of MutL and MutL Homologues
Alignment of the N-termini of *S.cerevisiae* Mlh1p, Mlh3p, Pms1p and *E. coli* MutL using GeneDoc (Nicholas *et al.*, 1997). The green bars indicate the four ATPase motifs, and the coloured circles indicate the amino acids of interest (positions given with reference to Mlh1p): E31 (orange circle), N35 (red circle) and G98 (yellow circle).

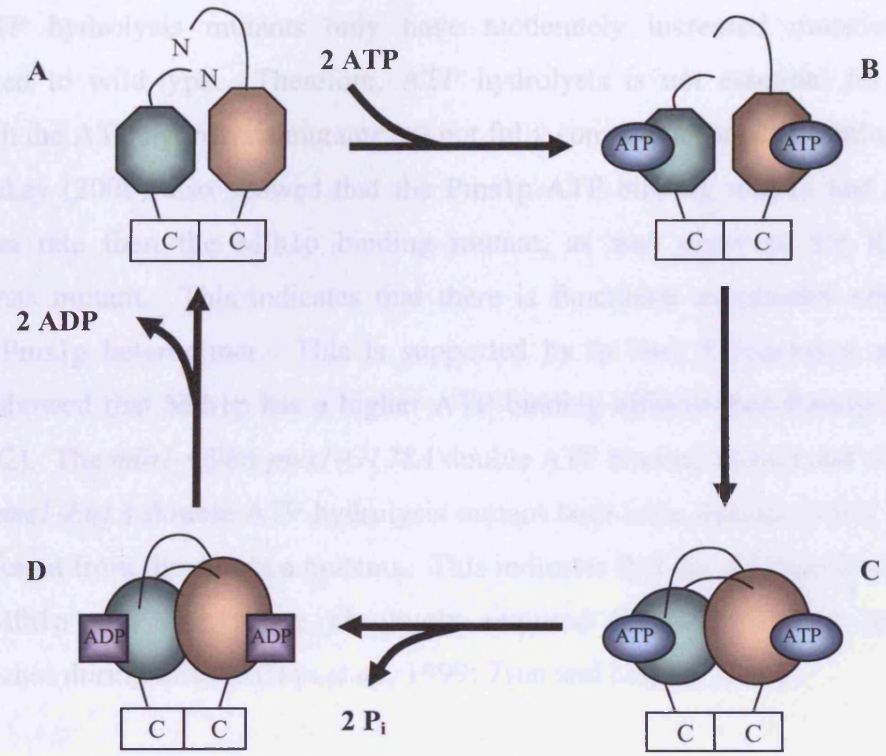


Figure 3.2 ATP Binding and Hydrolysis by MutL Homologues
Adapted from Tran and Liskay (2000), based on the interaction of Mlh1p and Pms1p.

- **A.** Two MutL homologues interact via their C-termini.
- **B.** Binding of ATP results in a conformational change.
- **C.** This also promotes the interaction of the N-termini. Subsequent ATP hydrolysis results in protein bound ADP.
- **D.** Release of ADP results in proteins returning to their original unbound state.

N = N-terminus of the protein; C = C-terminus of the protein.

indicates that ATP binding is necessary for the MMR process to occur. Conversely, the ATP hydrolysis mutants only have moderately increased mutation rates compared to wild-type. Therefore, ATP hydrolysis is not essential for MMR, although the ATP hydrolysis mutants are not fully competent for MMR either. Tran and Liskay (2000) also showed that the Pms1p ATP binding mutant had a lower mutation rate than the Mlh1p binding mutant, as was observed for the ATP hydrolysis mutant. This indicates that there is functional asymmetry within the Mlh1p-Pms1p heterodimer. This is supported by *in vitro* biochemical analysis, which showed that Mlh1p has a higher ATP binding affinity than Pms1p (Hall *et al.*, 2002). The *mlh1-G98A pms1-G128A* double ATP binding mutant and the *mlh1-E31A pms1-E61A* double ATP hydrolysis mutant both have mutation rates that are not different from the deletion mutants. This indicates that the ATPase functions of both Mlh1p and Pms1p are absolutely required for the efficient repair of mismatches during mitosis (Ban *et al.*, 1999; Tran and Liskay, 2000).

The ATP hydrolysis mutant of Mlh1p, *mlh1-E31A*, and a second mutant *mlh1-N35A*, which has been shown to have no ATP binding or hydrolysis activity (Hall *et al.*, 2002), were used to study the requirement of these activities during meiotic recombination (Hoffmann *et al.*, 2003). The *mlh1-N35A* mutant exhibits reduced levels of crossing over and defective repair of heteroduplex DNA (hDNA), whilst *mlh1-E31A* is proficient for both of these functions. These results together indicate that ATP binding alone is sufficient for these functions, and that ATP hydrolysis is not required. In contrast to this, *mlh1-G98A* has differential effects on the meiotic functions. Normal levels of crossing over are observed, whilst the repair of hDNA is reduced (Hoffmann *et al.*, 2003). However, mutating G98 in *MLH1* to valine results in defective crossing over and repair of hDNA (Hoffmann *et al.*, 2003). This mutation also affects the interaction of Mlh1p with Pms1p whereas *mlh1-G98A* does not (Pang *et al.*, 1997). As *mlh1-G98V* is also defective for crossing over, this suggests that the interaction with Mlh3p may also be affected. This is possible as Mlh1p is thought to interact with Mlh3p and Pms1p through the same residues (Kondo *et al.*, 2001; Wang *et al.*, 1999).

3.1.3 The Role of Mlh3p in Mitosis and Meiosis

Mlh1p also interacts with Mlh3p in yeast (Flores-Rozas and Kolodner, 1998; Wang

et al., 1999) and human systems (Kondo *et al.*, 2001). Mlh3p is involved in repair of frameshift mutations in mitosis (as discussed in Chapter 1), however, in the absence of Mlh3p only a weak mutator phenotype is observed due to a partial overlap in function with Pms1p (Flores-Rozas and Kolodner, 1998; Harfe *et al.*, 2000). This is demonstrated by the equivalent mutation rates in *pms1Δmlh3Δ* cells compared with an *mlh1Δ* mutant (Tran and Liskay, 2000).

However, deletion mutants of *MLH1* and *MLH3* in yeast exhibit comparable defects in crossing over, unlike *pms1Δ* mutants which exhibit no crossover defect (Wang *et al.*, 1999). This suggests that Mlh1p and Mlh3p also function together in meiosis. Supporting this, it has been shown in mouse spermatocytes that Mlh1p/Mlh3p colocalise at sites believed to be crossovers (Marcon and Moens, 2003). *MLH3*-deficient mice are infertile and fail to recruit Mlh1p to meiotic chromosomes (Lipkin *et al.*, 2000; reviewed in Svetlanov and Cohen, 2004). This suggests that Mlh3p loads first onto the chromosomes and then recruits Mlh1p, which could explain why foci containing only Mlh3p have been observed (reviewed in Svetlanov and Cohen, 2004). Another possible explanation is that Mlh3p also has a function independent of Mlh1p (reviewed in Svetlanov and Cohen, 2004). Also in mammalian cells, Mlh3p and Msh4p interact, indicating that it is involved in the Msh4p-Msh5p pathway (Santucci-Darmanin *et al.*, 2002) as is Mlh1p (Santucci-Darmanin *et al.*, 2000). Unlike Mlh1p, Mlh3p is not involved in meiotic mismatch repair, as indicated by normal levels of non-Mendelian segregation (NMS) events and the absence of post-meiotic segregation (PMS) events in the *mlh3Δ* mutant (Wang *et al.*, 1999).

3.1.4 Aims

1. To confirm that Mlh1p and Mlh3p function in the same pathway for meiotic recombination, an *mlh1Δmlh3Δ* double mutant will be constructed.

2. To analyse the requirement of ATP binding and/or hydrolysis by Mlh3p for meiotic recombination, ATPase mutants of *MLH3* will be constructed.

3. To mutate the analogous residue of Mlh1p G98, in Mlh3p (G97), to alanine and valine, to determine whether this residue is important for the function of

Mlh3p, and whether mutating the residue to different amino acids has differential effects on the function of Mlh3p, as was shown for Mlh1p.

3.2 Materials and Methods

3.2.1 Strains

All strains used in this chapter were constructed from existing haploid wild-type strains (EY39 and EY1-14B; resulting diploid is ERY68), which have been described previously (Hoffmann *et al.*, 2003). The *mlh1*Δ and *mlh1-E31A* mutant strains used to create the double mutants have also been described previously (Hoffmann *et al.*, 2003). The relevant strains were mated, sporulated and dissected for analysis. The haploid and diploid strains used are listed in Table 3.1 and 3.2 respectively.

3.2.2 Strain Construction

The entire open reading frame of *MLH3* was replaced by *hphMX4* (Chapter 2, 2.12.1), conferring resistance to hygromycin. This was confirmed by PCR (See Appendix I, Table A1.1, for oligonucleotides used) and by crossing to a strain with a known disruption of *MLH3* to confirm segregation. This strain was crossed to an *mlh1*Δ strain, with *MLH1* disrupted with *kanMX4*, and dissected to create the *mlh1*Δ*mlh3*Δ double deletion strain. All point mutants of Mlh3p were constructed using the *delitto perfetto* technique (Chapter 2, 2.12.3). Colonies obtained from transformation with the *mlh3-N35A* mutation were initially screened by digestion as the mutation creates a *Hin*dIII restriction site. Transformants that tested positive for this site were then sequenced. The other mutations did not affect restriction sites and were therefore confirmed by sequencing only. The double mutant *mlh1-E31A mlh3-E31A* was made by crossing. Initially, the *mlh1-E31A* and *mlh3-E31A* strains were crossed to an *mlh1*Δ*mlh3*Δ strain. *MLH3* was disrupted with the *hphMX4* cassette and *MLH1* was disrupted with the *kanMX4* cassette. From these crosses, *mlh1-E31A mlh3*Δ and *mlh3-E31A mlh1*Δ segregants were selected. These segregants were then crossed to bring the two point mutations together in the same strain. Crossing with deletion strains allowed the segregation of the point mutations to be followed without sequencing at every stage.

3.2.3 Fluctuation Tests

Only a mild mutator phenotype is exhibited by *mlh3*Δ strains, and therefore single colonies were propagated overnight in liquid cultures to increase the cell number

Table 3.1 Haploid Strains

Strain Name		Genotype[1]
EY39	Wild-type	*his4-r1 leu2-r1 MATa ade1-1 TRP5 CYH2 MET13 lys2*::insE-A_{14} *CANS ura3-1*
VC29	*mlh3*Δ::*hphMX4*	
VC163	*mlh3-G97A*	
VC136	*mlh3-G97V*	

[1] All strains listed have the same genotype.

Table 3.2 Diploid Strains

Diploid Strain Name	Haploid Parents	Genotype[1]									
ERY68	EY39 EY1-14B	Wild-type	*his4-r1* *HIS4*	*leu2-r1* *LEU2*	*MATa* *MATα*	*ade1-1* *ADE1*	*TRP5* *trp5-1*	*CYH2* *cyh2*	*MET13* *met13-2*	*lys2*::insE-A_{14} *lys2-c*	*CAN1* *CAN1*	*ura3-1* *ura3-Nprob*
VCD8	VC29 VC31	*mlh3Δ::hphMX4* *mlh3Δ::hphMX4*										
VCD6	VC10 VC9	*mlh1Δ::kanMX4 mlh3Δ::hphMX4* *mlh1Δ::kanMX4 mlh3Δ::hphMX4*										
VCD60	VC132 VC31	*mlh3-N35A* *mlh3Δ::hphMX4*										
VCD13	VC35 VC31	*mlh3-E31A* *mlh3Δ::hphMX4*										
VCD29	VC58 VC9	*mlh1-E31A mlh3-E31A* *mlh1Δ::kanMX4 mlh3Δ::hphMX4*										
VCD90	VC163 VC31	*mlh3-G97A* *mlh3Δ::hphMX4*										
VCD62	VC136 VC31	*mlh3-G97V* *mlh3Δ::hphMX4*										

[1] All strains listed have the same genotype.

before testing the mutation rate (Chapter 2, 2.13). The experiments were performed in the same manner for the wild-type, *mlh3*Δ and *mlh3* point mutant strains. Mlh3p is only involved in the removal of frameshift mutations (Flores-Rozas and Kolodner, 1998; Harfe *et al.*, 2000), and therefore the mutants tested were only assayed for reversion to lysine prototrophy, using the *lys2*::InsE-A_{14} allele. The raw data from these experiments can be found in Appendix II (Table A2.1).

3.2.4 Analysis of Meiotic Recombination

Tetrads were analysed for crossing over in the intervals *HIS4-LEU2* and *LEU2-MAT* on chromosome III, and *TRP5-CYH2* and *CYH2-MET13* on chromosome VII (as described in the Materials and Methods (Chapter 2, 2.9.5)). For each strain, 350-450 tetrads were dissected for analysis. In applying the Dunn-Sidak correction for multiple comparisons (Sokal and Rohlf, 1969; Chapter 2, 2.14), a P-value of 0.00568 or less was considered significant (for nine-way comparisons), unless otherwise stated.

3.3 Results

3.3.1 Mlh1p and Mlh3p Function Together in Crossing Over

To assess the relative roles of Mlh1p and Mlh3p in meiotic recombination, *mlh3*Δ and *mlh1*Δ*mlh3*Δ deletion strains were studied, and compared to wild-type and *mlh1*Δ strains (the data for the latter two strains is from Hoffmann *et al.*, 2003; Table 3.3). The *mlh3*Δ mutant exhibited levels of crossing over that were not significantly different from the *mlh1*Δ mutant or the *mlh1*Δ*mlh3*Δ double mutant at all four intervals studied ($P > 0.03$, G-test of homogeneity). All three mutant strains had significantly reduced map distances in comparison to the wild-type strain ($P \leq 2 \times 10^{-04}$, G-test), with one exception. The *mlh1*Δ*mlh3*Δ strain did not exhibit a significantly different level of crossing over in the *HIS4-LEU2* interval compared to the wild-type ($P = 0.0027$, G-test). However, the level of crossing over in this interval was also not significantly different to that observed in either the *mlh1*Δ or *mlh3*Δ mutants ($P > 0.05$, G-test). This indicates that *mlh1*Δ and *mlh3*Δ function in the same pathway for crossing over, in agreement with the observations of Wang *et al.* (1999).

3.3.2 ATP Binding and Hydrolysis by Mlh3p

Crossing over was analysed in strains where Mlh3p was either unable to bind ATP (*mlh3-N35A*) or hydrolyse ATP (*mlh3-E31A*), to determine if these functions are necessary for meiotic recombination (Table 3.3). Comparisons were made to wild-type and *mlh3*Δ strains, and also to the corresponding ATP binding and hydrolysis mutants in *MLH1* (*mlh1-N35A* and *mlh1-E31A* respectively; data from Hoffmann *et al.*, 2003). The *mlh3-N35A* mutant exhibited levels of crossing over that were not different from *mlh1-N35A* ($P > 0.05$, G-test). In addition, neither ATP binding mutant had levels of crossing over which were statistically different to the *mlh3*Δ mutant ($P > 0.05$, G-test). Consistent with this, the levels of crossing over in the *mlh3-N35A* mutant were significantly different to those in the wild-type at all intervals ($P \leq 0.001$, G-test), as was the *mlh3*Δ mutant ($P \leq 0.0002$, G-test). The levels of crossing over in *mlh3-E31A* and *mlh1-E31A* were also not different from one another ($P > 0.05$, G-test). However, in contrast to the ATP binding mutants they were also not significantly different to the wild-type ($P > 0.05$, G-test), but

Table 3.3 Map Distances in Deletion and Separation of Function Mutants

	Interval															
	HIS4-LEU2				*LEU2-MAT*				*MET13-CYH2*				*CYH2-TRP5*			
Genotype	**PD**[1]	**NPD**	**TT**	**cM**	**PD**	**NPD**	**TT**	**cM**	**PD**	**NPD**	**TT**	**cM**	**PD**	**NPD**	**TT**	**cM**
Wild-type[2]	207	3	107	19.7†	191	4	166	26.3†	240	2	104	16.8†	88	26	247	55.8†
*mlh1*Δ[2]	341	2	80	10.9*	318	5	138	18.2*	670	9	112	10.5*	424	14	409	29.1*
*mlh3*Δ	288	0	80	10.9*	299	5	103	16.3*	330	1	55	7.9*	225	7	182	27.1*
*mlh1*Δ*mlh3*Δ	269	1	79	12.2	266	11	95	21.6*	311	1	49	7.6*	185	5	190	28.9*
mlh1-N35A[2]	111	0	24	8.9*	90	1	31	15.2*	105	0	26	9.9*	79	2	58	25.2*
mlh3-N35A	291	1	82	11.8*	304	7	107	17.8*	344	0	60	7.4*	219	17	183	34.0*
mlh1-E31A[2]	124	2	73	21.4†	119	8	94	32.1†	143	1	71	17.9†	65	18	144	55.5†
mlh3-E31A	233	7	150	24.6†	222	12	214	31.9†	293	0	137	15.9†	105	29	322	54.4†
mlh3-G97A	222	3	140	21.6†	200	11	202	32.4†	261	0	142	17.6†	82	40	301	63.9†
mlh3-G97V	307	2	78	11.6*	273	8	136	22.1*	349	0	64	7.7*	199	8	215	31.2*

[1] PD, NPD and TT are parental ditype, non-parental ditype and tetratype respectively, and map distances (cM) were calculated according to Perkins (1949). Comparisons of the distribution of PD, NPD and TT events were made using a G-test of homogeneity, and significant differences are represented as follows:

* Significantly different to corresponding values in wild-type.

† Significantly different to corresponding values in *mlh3*Δ.

[2] Data from Hoffmann *et al.*, 2003.

were different to *mlh1*Δ and *mlh3*Δ ($P \leq 7.22 \times 10^{-05}$, G-test) at all intervals studied.

As crossing over was not defective in either single ATP hydrolysis mutant, it was hypothesised that ATP hydrolysis by either Mlh1p or Mlh3p within the heterodimer was sufficient to maintain normal function. To test this, a double ATP hydrolysis mutant was constructed. As the wild-type, *mlh1-E31A* and *mlh3-E31A* strains had levels of crossing over that were similar the three data sets were compared for homogeneity using a G-test. With a P value of > 0.05, the data sets were considered homogeneous and therefore pooled (referred to as wild-type; Table 3.4). The same rule was applied to the *mlh1*Δ, *mlh3*Δ and *mlh1*Δ*mlh3*Δ strains (referred to as *mlh*Δ). These pooled data groups were used as a comparison for the double ATP hydrolysis mutant (*mlh1-E31A mlh3-E31A*). As only two comparisons were made on this data, a P-value of 0.0253 or less was considered significant (Chapter 2, 2.14).

Unlike the single ATP hydrolysis mutants, the double ATP hydrolysis mutant exhibited levels of crossing over that were significantly lower than those in the wild-type at all intervals ($P \leq 0.004$, G-test; Table 3.4). This strain was also significantly different to *mlh*Δ for crossing over in all intervals ($P \leq 0.018$, G-test) with the exception of *HIS4-LEU2* ($P \geq 0.03$, G-test). At three of the intervals studied, the map distances obtained from the double ATP hydrolysis mutant were not as reduced as those in *mlh*Δ, but at *LEU2-MAT* the map distance was lower than that of the *mlh*Δ grouped data (Table 3.4). This indicates that although a reduction in crossing over was observed compared to wild-type, the double ATP hydrolysis mutant was not as defective for crossing over as the deletion mutants, except in the *LEU2-MAT* interval.

Although the sets of data which were pooled were homogeneous groups, some differences were observed when comparing the *mlh1-E31A mlh3-E31A* double ATP hydrolysis mutant to the pooled "wild-type" group versus the wild-type data alone. While crossing over was significantly different to the pooled wild-type data in the *HIS4-LEU2* and *MET13-CYH2* intervals (Table 3.4), when compared to the wild-type data only (Table 3.3), they were not significantly different ($P \geq 0.06$, G-test).

Table 3.4. Map Distances for Pooled Data

Genotype	Interval															
	HIS4-LEU2				*LEU2-MAT*				*MET13-CYH2*				*CYH2-TRP5*			
	PD[1]	NPD	TT	cM	PD	NPD	TT	cM	PD	NPD	TT	cM	PD	NPD	TT	cM
Wild-type[2]	564	12	330	22.2†	532	24	474	30.0†	676	3	312	16.6†	258	73	713	53.7†
mlhΔ[3]	898	3	239	11.3*	883	21	336	18.6*	1311	11	216	9.2*	834	26	781	28.5*
mlh1-E31A mlh3-E31A	297	3	108	15.4*	317	1	131	15.3*†	322	1	96	12.2*†	186	14	243	36.9*†

[1] PD, NPD and TT are parental ditype, non-parental ditype and tetratype respectively, and map distances (cM) were calculated according to Perkins (1949). Comparisons of the distribution of PD, NPD and TT events were made using a G-test of homogeneity, and significant differences are represented as follows:

* Significantly different to corresponding values in wild-type group.

† Significantly different to corresponding values in the *mlh*Δ group.

[2] Data pooled from the wild-type, *mlh1-E31A* and *mlh3-E31A* strains.

[3] Data pooled from the *mlh1*Δ, *mlh3*Δ and *mlh1*Δ*mlh3*Δ strains.

There were no differences between using the pooled mutant data set or individual mutant data. Despite the differences in statistical comparisons, the map distances obtained were always intermediate between the wild-type and mutant strains, except at *LEU2-MAT*. This was true whether the combined heterogeneous datasets or the individual strain data was used for comparison. The statistical difference observed depending on the dataset used also did not change the fact that the double ATP hydrolysis mutant exhibited reduced crossing over compared to either of the single ATP hydrolysis mutants.

3.3.3 The Meiotic and Mitotic Functions of Mlh3p can be Separated

Two other point mutants of Mlh3p, *mlh3-G97A* and *mlh3-G97V*, were studied as the corresponding mutations in *MLH1* had been observed to have differential effects on the mitotic and meiotic functions of the protein (Hoffmann *et al.*, 2003; Tran and Liskay, 2000). The residue G98 in Mlh1p is situated in the GFRGEAL motif (GYRGDAL in Mlh3p), which forms the lid of the ATP-binding pocket, and is thought to be important for mediating ATP-dependent interactions (Ban and Yang, 1998; Ban *et al.*, 1999).

To determine the effect on meiotic function, crossing over, non-Mendelian segregation and chromosome segregation was analysed (see also sections 3.3.4-3.3.6). Rates of crossing over were not reduced in any of the intervals studied in *mlh3-G97A* compared to wild-type ($P \geq 0.07$, G-test; Table 3.3), and were significantly better than in *mlh3*Δ ($P \leq 4 \times 10^{-07}$, G-test). In contrast, *mlh3-G97V* had rates of crossing over that were reduced to the same level as *mlh3*Δ in all of the intervals studied ($P \geq 0.04$, G-test).

Mutation rate assays were performed to determine the effect of these mutations on the mitotic mismatch repair function of Mlh3p (Table 3.5 and Figure 3.3). For these comparisons, a P-value of 0.0169 or less was considered significant, as only three comparisons were made with this data. The mutation rates for *mlh3*Δ, *mlh3-G97A* and *mlh3-G97V* were all significantly different to the wild-type ($P < 0.001$, contingency χ^2 test). This indicates that these mutants are defective for MMR. In addition, the mutation rates for *mlh3*Δ and *mlh3-G97V* were not different ($P > 0.05$,

Table 3.5 Mutation Rates of Wild-Type and Mutant Strains

Genotype	Mutation Rates x 10^{-07}			Average Mutation Rate x 10^{-07} (Fold Increase)
	1	2	3	
Wild-type	1.82	2.27	0.38	1.49 (1.0)
mlh3Δ	6.96	8.60	7.73	7.76 (5.2)
mlh3-G97A	4.92	6.17	6.40	5.83 (3.9)
mlh3-G97V	7.46	7.49	9.47	8.14 (5.5)

Mutation rates were calculated as described by Reenan and Kolodner (1992; Chapter 2, 2.13), and an average taken from three independent experiments.

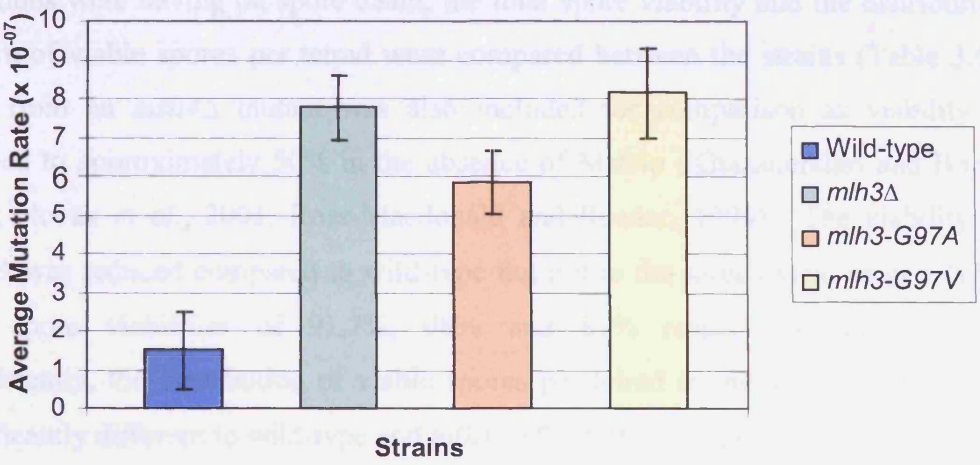


Figure 3.3 Rates of Reversion to Lys$^+$
The mutation rates are a measure of frameshift mutations in the *lys2*::insE-A_{14} allele that restore the reading frame and consequently function of *LYS2*, allowing growth on lysine omission medium. The error bars represent one standard deviation calculated from results of three independent experiments performed on each strain.

contingency χ^2 test), with both exhibiting a 5-fold increase. However, *mlh3-G97A* was significantly different to both *mlh3*Δ and *mlh3-G97V* (P = 0.0014 and 2.95 x 10^{-05} respectively, contingency χ^2 test), with only a 3.9-fold increase. Therefore, although *mlh3-G97A* does exhibit a reduction in the efficiency of repair, it is still partially functional. Together these results suggest that the effects of mutating a single residue can be altered depending on the properties of the resulting amino acid.

3.3.4 Mlh1p and Mlh3p Differentially Affect Spore Viability

Diminished levels of crossing over can cause mis-segregation of chromosomes, resulting in aneuploidy and spore death. To determine the effect the various mutations were having on spore death, the total spore viability and the distribution pattern of viable spores per tetrad were compared between the strains (Table 3.6). Data from an *msh4*Δ mutant was also included for comparison as viability is reduced to approximately 50% in the absence of Msh4p (Khazanehdari and Borts, 2000; Novak *et al.*, 2001; Ross-Macdonald and Roeder, 1994). The viability of *mlh3*Δ was reduced compared to wild-type but not to the same extent as in *mlh1*Δ, with spore viabilities of 91.7%, 98% and 81% respectively (Table 3.6). Consistently, the distribution of viable spores per tetrad in the *mlh3*Δ mutant was significantly different to wild-type and *mlh1*Δ (P < 0.001, G-test). The overall spore viability of *mlh1*Δ*mlh3*Δ was similar to the viability of *mlh1*Δ (79.4% and 81% respectively), but lower than the viability of *mlh3*Δ. The distribution of viable spores per tetrad in the *mlh1*Δ*mlh3*Δ mutant also resembled the distribution in the *mlh1*Δ mutant (P > 0.05, G-test), but not in the *mlh3*Δ mutant (P < 0.001, G-test). This is expected if they function in the same pathway, as *mlh1*Δ has a lower viability than *mlh3*Δ. Therefore, Mlh1p and Mlh3p do not contribute to spore death independently, as this would be indicated by a spore viability that was lower in the *mlh1*Δ*mlh3*Δ strain than in the *mlh1*Δ strain.

The *mlh3-N35A* mutant had a spore viability of 92.8%, which was similar to the viability of the *mlh3*Δ strain, and was lower than the spore viability of the wild-type (Table 3.6). However, the distribution pattern of viable spores per tetrad in the *mlh3-N35A* mutant was significantly different to both wild-type and *mlh3*Δ (P =

Table 3.6 Distribution of Viable Spores per Tetrad and Overall Viability

Genotype	Viable spores per tetrad class					Spore viability	
	4 No. (%)	3 No. (%)	2 No. (%)	1 No. (%)	0 No. (%)	Total tetrads	%[1]
Wild-type[2†]	384 (92.9)	25 (6.1)	4 (1.0)	0 (0.0)	0 (0.0)	413	98.0
*mlh1*Δ[2*†]	1013 (59.8)	279 (16.5)	246 (14.5)	91 (5.4)	66 (3.9)	1695	81.0
*mlh3*Δ[*]	425 (79.9)	64 (12.0)	26 (4.9)	7 (1.3)	10 (1.9)	532	91.7
*mlh1*Δ*mlh3*Δ[*†]	411 (61.0)	114 (16.9)	99 (14.7)	20 (3.0)	30 (4.5)	674	79.4
*msh4*Δ[3*†]	473 (32.5)	117 (8.0)	361 (24.8)	115 (7.9)	391 (26.8)	1457	52.8
mlh3-N35A[*†]	435 (85.0)	31 (6.1)	33 (6.4)	2 (0.4)	11 (2.1)	512	92.8

Table 3.6 Continued

Genotype	Viable spores per tetrad class					Spore Viability	
	4	3	2	1	0		
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	Total Tetrads	%[1]
mlh3-E31A[†]	477 (90.5)	41 (7.8)	5 (0.9)	1 (0.2)	3 (0.6)	527	97.0
mlh3-E31A mlh1-E31A[*]	469 (82.1)	58 (10.2)	33 (5.8)	2 (0.4)	9 (1.6)	571	92.7
mlh3-G97A[*†]	452 (89.0)	38 (7.5)	8 (1.6)	1 (0.2)	9 (1.8)	508	95.4
mlh3-G97V[*]	442 (84.8)	42 (8.1)	23 (4.4)	6 (1.2)	8 (1.5)	521	93.4

[1] Calculated as ((4 x no. of four-spore tetrads + 3 x no. of three-spore tetrads + 2 x no. of two-spore tetrads + no. of one-spore tetrads)/(4 x total no. of tetrads)) x 100. Comparisons of the distribution of viable spores per tetrad class were made using the G-test of homogeneity and significant statistical differences are indicated as follows:

[*] Significantly different to corresponding values in wild-type.

[†] Significantly different to corresponding values in *mlh3*Δ.

[2] Data from Hoffmann *et al.*, 2003.

[3] Data from Hoffmann and Borts, unpublished.

1.66×10^{-07} and 0.004 respectively, G-test). The difference between the distribution patterns in the *mlh3*Δ mutant and *mlh3-N35A* was due to a reduced number of three viable spore tetrads being observed in the latter strain (P = 0.0007, G-test; Table 3.6). Conversely, the *mlh3-E31A* mutant was equally as viable as the wild-type (96.9% compared to 98% respectively), and had improved viability compared to the *mlh3*Δ strain. The distributions of viable-spore tetrads reflected this (P > 0.05 compared to wild-type and $P = 1.39 \times 10^{-06}$ compared to *mlh3*Δ, G-test). This is consistent with the lack of a crossover defect observed. The *mlh3-N35A* and *mlh3-E31A* mutants follow the same trend as observed for the *mlh1* ATP binding and hydrolysis mutants compared to wild-type and *mlh1*Δ (Hoffmann *et al.*, 2003). However, the double ATP hydrolysis mutant (*mlh1-E31A mlh3-E31A*) did have reduced spore viability compared to the wild-type (92.7% compared with 98% respectively). Additionally, the *mlh1-E31A mlh3-E31A* double mutant had a distribution pattern of viable spores that was significantly different to wild-type, *mlh1-E31A* and *mlh3-E31A* strains (P < 0.0001, G-test; Figure 3.5). Instead, the viability most closely resembled that of *mlh3*Δ (P > 0.05, G-test), and was not as severely reduced as the viability of the *mlh1*Δ*mlh3*Δ strain ($P = 6.12 \times 10^{-16}$, G-test). The *mlh3-G97V* mutant also had a spore viability that was similar to *mlh3*Δ (P > 0.05, G-test), but reduced compared to wild-type ($P = 4.66 \times 10^{-06}$, G-test), whereas *mlh3-G97A* has a spore viability that was intermediate between the viabilities of the wild-type and *mlh3*Δ strains (95.4% compared with 98% and 91.7% respectively; $P \leq 8.5 \times 10^{-03}$, G-test).

3.3.5 Chromosome Segregation is Not Severely Impaired in an *mlh3*Δ Mutant

To assess whether there was an increase in meiosis I non-disjunction, the distribution of the two and zero viable spore tetrad classes was analysed (Table 3.6, Figures 3.4 and 3.5). Comparisons were made with an *msh4*Δ strain that exhibits high levels of meiosis I non-disjunction, caused by defects in maintaining the number and distribution of crossovers (Novak *et al.*, 2001; reviewed in Roeder, 1997; Ross-Macdonald and Roeder, 1994). Deletion of *MLH1* causes moderate levels of meiosis I non-disjunction. However, the number of two and zero viable spore tetrads is not an accurate reflection of this as the MMR defect of *mlh1*Δ also causes an increase in the number of tetrads within these classes (Hoffmann *et al.*,

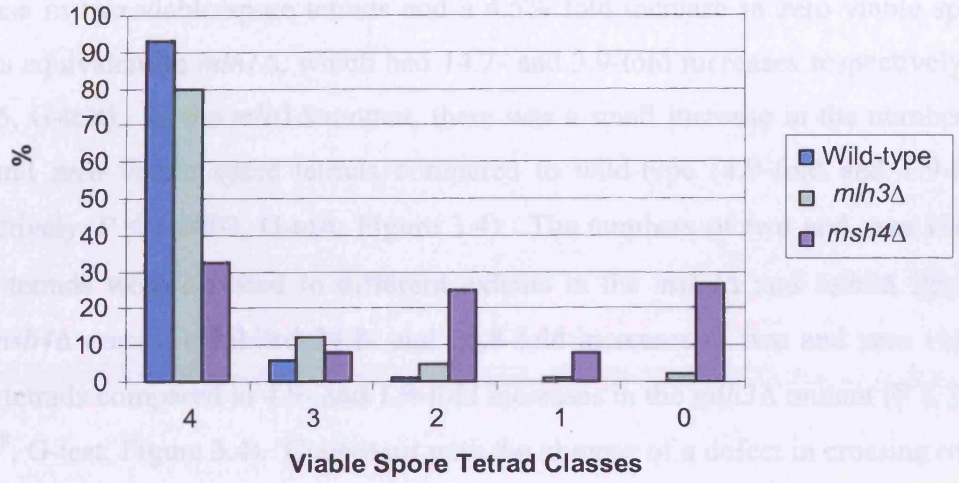


Figure 3.4 Distribution of Viable Spores per Tetrad in Wild-Type and Deletion Strains

The percentages of each class of viable spore tetrads are shown for wild-type, *mlh3*Δ and *msh4*Δ. Data for *msh4*Δ is shown as the distribution of viable spore tetrads is characteristic of meiosis I non-disjunction.

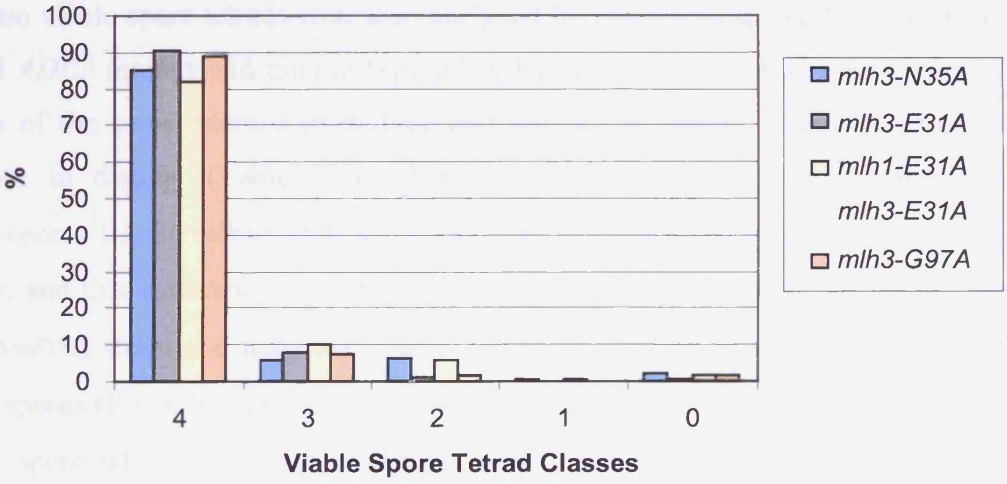


Figure 3.5 Distribution of Viable Spores per Tetrad in Mutant Strains

The percentages of each class of viable spore tetrads are shown for *mlh3-N35A*, *mlh3-E31A*, *mlh1-E31A mlh3-E31A* and *mlh3-G97A*, which exhibit a range of distributions observed from the mutants studied.

2003; Hunter and Borts; 1997). Consistently, *mlh1Δmlh3Δ* exhibited a 14.5% fold increase in two viable spore tetrads and a 4.5% fold increase in zero viable spore tetrads equivalent to *mlh1Δ*, which had 14.7- and 3.9-fold increases respectively ($P > 0.05$, G-test). In the *mlh3Δ* mutant, there was a small increase in the number of two and zero viable spore tetrads compared to wild-type (4.9-fold and 1.9-fold respectively; $P \leq 0.0003$, G-test; Figure 3.4). The numbers of two and zero viable spore tetrads were elevated to different extents in the *msh4Δ* and *mlh3Δ* strains. The *msh4Δ* mutant exhibited 24.8- and 26.8-fold increases in two and zero viable spore tetrads compared to 4.9- and 1.9-fold increases in the *mlh3Δ* mutant ($P \leq 3.54 \times 10^{-08}$, G-test; Figure 3.4). Consistent with the absence of a defect in crossing over, the *mlh3-E31A* mutant did not exhibit an increase in two and zero viable spore tetrads compared to wild-type ($P > 0.05$, G-test; Figure 3.5). The *mlh3-G97A* mutant also did not exhibit an increase in two viable spore tetrads compared to the wild-type ($P > 0.05$, G-test), however, the number of zero viable spore tetrads was significantly higher ($P = 0.001$, G-test). On the other hand, *mlh3-N35A*, *mlh3-G97V* and *mlh1-E31A mlh3-E31A* all exhibited increases equivalent to those observed in *mlh3Δ* ($P > 0.05$, G-test; Figure 3.5).

The two viable spore tetrads were also analysed for disomy by using the centromere linked *ADE1* marker and mating type (Chapter 2, 2.9.5). An increase in pairs of spores of the same adenine phenotype and non-mating spores is indicative of an increase in disomy (Table 3.7). Non-maters specifically identify disomy of chromosome III. In *mlh3Δ* there were more pairs of sister spores compared to non-sisters, and this difference was statistically significant ($P = 0.0017$, χ^2 test). The *mlh1Δmlh3Δ* strain and *mlh3-N35A* also had more pairs of sister spores than non-sister spores ($P = 0.007$ and 0.009 respectively, χ^2 test). The total number of two viable spore tetrads in the *mlh1Δmlh3Δ* strain was considerably higher than the *mlh3Δ* strain due to the spore death attributable to the absence of Mlh1p. The wild-type strain had only four, two viable spore tetrads, with no detectable disomy, showing that there was an increase in the numbers of two viable spore tetrads in the absence of Mlh3p. The *mlh3* ATP hydrolysis mutant (*mlh3-E31A*) also had very few two viable spore tetrads. In the double ATP hydrolysis mutant, the numbers of sisters versus non-sisters were approximately equal (Table 3.7).

Table 3.7 Rates of Non-Disjunction of Chromosomes at Meiosis I

Genotype	No. Sister Spores[1]	No. Non-Sister Spores[1]	Total Tetrads	% Non-Disjunction[2]
mlh3Δ	21	5*	532	3.0
mlh1Δmlh3Δ	63	36*	674	4.0
msh4Δ	99	18*	1457	5.6
mlh3-N35A	24	9*	512	2.9
mlh3-E31A	3	2	527	0.2
mlh1-E31A mlh3-E31A	18	15	571	0.5
mlh3-G97A	3	5	508	0.0
mlh3-G97V	19	4*	520	2.9

[1] Number of two viable spore tetrads with spores of the same adenine phenotype (sisters) or of different adenine phenotypes (non-sisters). * denotes a distribution of sister and non-sister spores which is significantly different to 50:50, as determined using the χ^2 test.

[2] Meiosis I non-disjunction was calculated as:

((number of sister spores – number of non-sister spores) / total tetrads) x 100

The number of non-sister spores was subtracted to give an estimate of the number of sister spores arising due to meiosis I non-disjunction, not accumulation of haplolethal mutations.

Table 3.8 Meiosis I Non-Disjunction of Chromosome III

Genotype	No. Non-Maters[1]	Total Tetrads	% Non-Disjunction[2]
mlh3Δ	5	532	0.9
mlh1Δmlh3Δ	0	674	0.0
msh4Δ	15	1457	1.0
mlh3-N35A	3	512	0.6
mlh3-E31A	0	527	0.0
mlh1-E31A mlh3-E31A	1	571	0.2
mlh3-G97A	0	508	0.0
mlh3-G97V	7	520	1.3

[1] Number of two viable spore tetrads which are non-maters (indicative of meiosis I non-disjunction of chromosome III). These were all sister spores.

[2] Non-disjunction of chromosome III was calculated as:

(No. non-maters / total tetrads) x 100

The non-sister two viable spore tetrads arise from random spore death due to accumulation of mutations in MMR-defective strains during mitotic growth. An equal number of the two viable spore tetrads with sister phenotypes will also arise in this manner. To correct for this when calculating the frequency of meiosis I non-disjunction, the number of non-sister two viable spore tetrads was subtracted from the number of sister two viable spore tetrads (Table 3.7). Additionally, multiple non-disjunction events in a given meiosis will result in the death of all four spores within a tetrad. As a result of this the frequency at which non-disjunction occurs is underestimated, especially in strains with more severe defects in chromosome segregation. Therefore, the overall spore viability and the pattern of distribution of viable spore tetrads, particularly the number of tetrads with zero viable spores, must be considered alongside this data. The frequency of meiosis I non-disjunction was equivalent in the *mlh3*Δ, *mlh3-N35A* and *mlh3-G97V* strains, with 3.0% in the absence of Mlh3p and 2.9% in both of the point mutants ($P > 0.05$, G-test). The frequency of non-disjunction of chromosome III makes up a substantial proportion of the overall frequency of non-disjunction events in these strains (Table 3.8). The *mlh3-E31A*, *mlh1-E31A mlh3-E31A* and *mlh3-G97A* mutants had negligible frequencies of non-disjunction (0.2%, 0.5% and 0% respectively; $P > 0.05$, G-test; Table 3.7). None of the mutants studied here exhibit a large increase in the number of tetrads with zero viable spores and therefore the frequencies of non-disjunction should represent the majority of these events. There may be under-representation of the total amount of non-disjunction events in *msh4*Δ due to the large number of zero viable spores in this mutant.

3.3.6 The Absence of Mlh3p Does Not Affect Non-Mendelian Segregation

The *mlh3*Δ strain does not exhibit an increase in non-Mendelian segregation at *his4-rI* compared to wild-type (10.1% compared to 12.3% respectively; $P > 0.05$, G-test; Table 3.9) nor an increase in post-meiotic segregation events (PMS; 0 events observed). This is in agreement with previous data indicating that Mlh3p, unlike Mlh1p, does not play a role in repair of hDNA during meiosis (Wang *et al.*, 1999). The *mlh1*Δ*mlh3*Δ mutant had an increased number of PMS events like *mlh1*Δ (33.3% and 29.4% respectively; $P > 0.05$, G-test). The *mlh1-E31A mlh3-E31A* mutant also exhibited an increase in PMS events, which was significantly different to wild-type ($P < 0.001$, G-test), but not to *mlh1*Δ ($P = 0.06$, G-test). Interestingly,

Table 3.9 Non-Mendelian Segregation Events at *his4-rI*

Genotype	% NMS[1] (NMS / Total Tetrads)	% PMS[2] (PMS / Total NMS)
Wild-type[3]	12.3 (45 / 366)	0 (0 / 45)
mlh3Δ	10.0 (42 / 418)	0 (0 / 42)
mlh1Δ[3]	8.0* (34 / 427)	29.4* (10 / 34)
mlh1Δmlh3Δ	7.9 (31 / 390)	33.3* (10 / 31)
mlh3-N35A	10.8 (46 / 424)	0 (0 / 46)
mlh3-E31A	13.2 (61 / 463)	0 (0 / 61)
mlh1-E31A mlh3-E31A	9.1 (41 / 452)	12.2* (5 / 41)
mlh3-G97A	11.8 (51 / 432)	0 (0 / 51)
mlh3-G97V	7.4 (32 / 430)	0 (0 / 32)
mlh1-E31A	11.6 (27 / 232)	7.4 (2 / 27)

[1] NMS events include gene conversion and post-meiotic segregation events. % NMS was calculated as the number of NMS events / total number of tetrads x 100. Significant statistical differences (G-test) between mutants and wild-type are denoted by * for both % NMS and % PMS.
[2] % PMS events calculated as the number of PMS events out of total NMS events.
[3] Data from Hoffmann *et al.*, 2003.

the *mlh1-E31A* single ATP hydrolysis mutant also exhibited a small increase in PMS events compared to wild-type (7.4% compared with 0%; Hoffmann *et al.*, 2003), although this was minor compared to that observed in *mlh1*Δ (29.4%). This was contrary to expectation as *mlh1-E31A* does not affect either crossing over or the overall level of non-Mendelian segregation (Hoffmann *et al.*, 2003), and therefore the repair of meiotic heteroduplex DNA was also expected to be normal.

3.4 Discussion

3.4.1 Mlh3p Has a Late Role in Promotion of Crossing Over

In the absence of Mlh3p, crossing over is reduced by 1.6-2.1 fold at the four intervals studied. A concomitant increase in two and zero viable spore tetrads is also observed, indicative of a defect in chromosome segregation at division I of meiosis. The fact that more than 90% of spores are viable may indicate that although crossing over is reduced, the distribution of these events is not greatly affected. This is in contrast to mutants of Msh4p, which reduce crossing over equivalently to *mlh3*Δ mutants, to approximately 40-60% of wild-type levels, but have poor spore viability and also diminish interference (Novak *et al.*, 2001; Ross-Macdonald and Roeder, 1994). This suggests that Mlh3p may only be involved in promoting the resolution of recombination intermediates in a crossover configuration, rather than a role in determining the crossover/non-crossover fate of double-strand breaks (DSBs). This decision is thought to occur prior to or at the time of strand invasion (Borner *et al.*, 2004; Hunter and Kleckner, 2001). Consistent with this hypothesis of a later role for Mlh3p is the observation that MLH3 foci do not appear until early-mid pachytene (Lipkin *et al.*, 2002) around the time when single-end invasion (SEI) intermediates are being stabilised and double Holliday Junctions (dHJ) are formed (reviewed in Zickler and Kleckner, 1999).

3.4.2 Mlh1p and Mlh3p Contribute Equally to Crossing Over in Meiosis

The *mlh3*Δ mutant has equivalent levels of crossing over to *mlh1*Δ, which was also observed by Wang *et al.* (1999). The spore viabilities of the two strains differ due to the additional role of Mlh1p in MMR. Defective MMR results in an accumulation of haplo-lethal mutations during periods of mitotic growth. Crossing over and spore viability in the *mlh1*Δ*mlh3*Δ is not reduced further than in the *mlh1*Δ strain. This suggests that they function in the same pathway for crossing over, which is supported by their known interaction in yeast (Flores-Rozas and Kolodner, 1998; Wang *et al.*, 1999) and colocalisation in mouse spermatocytes, at sites believed to be crossovers (Marcon and Moens, 2003). If they functioned in separate pathways, a double mutant would be more defective for recombination than either single mutant.

The ATP binding and ATP hydrolysis mutants exhibit differential effects on the functions of Mlh3p, as might be expected. According to the model proposed by Tran and Liskay (2000; Figure 3.2), ATP binding by MutL homologues is essential for their interaction and subsequent functions. ATP hydrolysis on the other hand is only necessary for release of the proteins from their interaction, and hence recycling of the components. How severely defects in these ATPase activities affect function, depends on the extent that they are required for the normal functioning of the proteins involved. ATP binding by Mlh3p is essential for its function in meiotic recombination, as the ATP binding mutant (*mlh3-N35A*) exhibits the same defects as a null mutant. This was also observed for the *mlh1-N35A* ATP binding mutant (Hoffmann *et al.*, 2003), indicating that ATP binding and the resultant N-terminal interaction between Mlh1p and Mlh3p is essential for their crossover function. The *mlh3-N35A* and *mlh1-N35A* ATP binding mutants exhibit equivalent defects to one another (Hoffmann *et al.*, 2003 and this study), indicating that Mlh1p and Mlh3p are involved in promotion of crossovers as equal partners.

In contrast to ATP binding, ATP hydrolysis is not essential, as *mlh3-E31A* exhibits wild type levels of function. This also correlates with the observations made for *mlh1-E31A* (Hoffmann *et al.*, 2003). Therefore, when only one of the proteins is defective for ATP hydrolysis, functionality is maintained. However, when neither Mlh1p nor Mlh3p can hydrolyse ATP a defect in crossing over is observed (this study). The double mutant is also not null, indicating that some functionality remains. Tran and Liskay (2000) reported that an N-terminal interaction was detected in the *mlh1* and *pms1* double ATP hydrolysis mutant, but not in the wild-type or the single ATP hydrolysis mutants. Their conclusion was that this mutant prolonged the interaction of the proteins allowing detection, whereas the interaction in the wild-type and single ATP hydrolysis mutants was only transient (Tran and Liskay, 2000). This hypothesis can also be applied to the *mlh1* and *mlh3* ATP hydrolysis mutants. A mutant phenotype was not observed in the single ATP hydrolysis mutants, indicating that the ability of one protein in the heterodimer to hydrolyse ATP is sufficient to release the proteins from the complex. In the double mutant, when neither Mlh1p nor Mlh3p can efficiently hydrolyse ATP, a phenotype is observed presumably due to the inability of the proteins to dissociate from one another. The *mlh1* ATP hydrolysis mutant was shown to exhibit a low level of

ATPase activity (Hall *et al.*, 2002), and therefore it is possible that the *mlh3* ATP hydrolysis mutant also retains some activity, which could explain why a null phenotype is not observed. Alternatively, the mutant complex can perform its function once, but the inability of the proteins to dissociate from one another prevents them from functioning a second time. These are not mutually exclusive, and a combination of the two may result in the observed phenotype. A third possibility is the complex is fully active but destabilised by the presence of two mutant subunits.

There is some evidence for the double ATP hydrolysis mutant impeding the MMR function of Mlh1p. Of the aspects of meiotic recombination tested, Mlh3p is only involved in crossing over. However, Mlh1p is also involved in meiotic mismatch repair independently of Mlh3p. In the double ATP hydrolysis mutant, a number of PMS events are observed, indicative of a failure to repair mismatched DNA. This could be a result of sequestration of Mlh1p-E31A in complex with Mlh3p-E31A, and leaving less free Mlh1p-E31A to carry out its repair function in combination with Pms1p (Wang *et al.*, 1999). That Mlh1p is the protein that is important for meiotic MMR can be seen from the observations made by Hoffmann *et al.* (2003). A small number of PMS events were observed in the *mlh1-E31A* single mutant (compared to no PMS events in the wild-type; Hoffmann *et al.*, 2003), but less than in the double ATP hydrolysis mutant (this study). It is possible that although Mlh3p is functional and capable of dissociating from Mlh1p-E31A, the ATP bound Mlh1p-E31A may not be able to interact with Pms1p to carry out repair. As the *mlh1-E31A* mutant exhibits a low level of ATP hydrolysis activity (Hall *et al.*, 2002), a second possibility is that a reduced ability to dissociate from ATP delays the interaction with Pms1p, also preventing the repair of mismatches. The residual ATPase activity of the single ATP hydrolysis mutants may explain why no other mutant phenotypes are observed in the single ATP hydrolysis mutants. If the defects observed in the double ATP hydrolysis mutant are due to an inability to recycle the proteins or decreased stability of the complex, then over-expression of the mutant forms of *mlh1* and *mlh3* should alleviate this.

3.4.3 Mutating G97 in Mlh3p to Alanine or Valine has Different Effects on Protein Function

The data obtained from the *mlh3-G97A* and *mlh3-G97V* mutants suggest that the structural and/or functional requirements of Mlh3p during mitosis and meiosis are different. Mutating residue G97 to valine disrupts both the mitotic MMR and crossover functions of Mlh3p. In contrast, *mlh3-G97A* functions normally in crossing over, but a small defect was observed in MMR. The spore viability of *mlh3-G97A* was also reduced to an intermediate level between wild-type and *mlh3*Δ. As only a defect in MMR was observed in *mlh3-G97A*, this must be responsible for the reduced viability of the strain, indicating that the contribution of Mlh3p to MMR is important. Consistent with this, *mlh3-E31A*, which has no defect in crossing over (this study) or mismatch repair (preliminary data, not shown) exhibits no reduction in spore viability. Therefore, of the spore death occurring in the *mlh3*Δ strain, at least half can be attributed to MMR defects. However, *mlh3*Δ exhibits a larger increase in mutation rate than *mlh3-G97A* and consequently the resulting proportion of spore death due to this may also be larger. This suggests that the spore death due to reduced crossovers is not substantial, and furthermore, although the number of crossovers is reduced the distribution is unaffected, in agreement with the conclusions drawn above. The majority of chromosome pairs must be receiving at least one crossover otherwise the rates of non-disjunction would be higher, and spore viability would be lower. The mechanism by which this works is unclear. Mlh1p and Mlh3p are recruited to sites designated to become crossovers by Msh4p, and these sites should display interference (Lipkin *et al.*, 2002; Novak *et al.*, 2001; Santucci-Darmanin *et al.*, 2002). Therefore, without Mlh1p or Mlh3p to ensure crossovers occur at these sites, it could be expected that interference would be reduced. Determination of this however, has not been straightforward. Two studies have shown that *mlh1*Δ strains retain interference (Argueso *et al.*, 2002; Hoffmann *et al.*, 2003), whilst a third demonstrated a lack of interference in both *mlh1*Δ and *mlh3*Δ strains (Abdullah *et al.*, 2004). In this study, interference was not detectable in the corresponding wild-type strain and consequently no conclusions can be drawn about *mlh3*Δ.

The results for *mlh3-G97A* and *mlh3-G97V* suggest that not only is the position of

the mutation important, but also the properties of the resulting amino acid. All three amino acids, glycine, alanine and valine are non-polar and aliphatic, with neutral side chains. Glycine is the smallest amino acid with a side chain consisting of a single hydrogen atom. Alanine is also small with a methyl group (CH_3) as a side chain. In contrast, valine has a CH-2CH_3 side chain which is much bulkier. Therefore, whereas the alanine substitution will only disrupt function if that residue is critical for an activity or interaction, substituting glycine for valine may result in physical disruption of interactions. Mutating Asn-35 of Mlh1p to alanine has been shown to disrupt the binding of ATP by Mlh1p (Hall *et al.*, 2002), whereas mutating Gly-98 is predicted to affect ATP binding and/or heterodimerisation of proteins. However, the corresponding mutants in Mlh3p are not equally defective for all functions. Whereas *mlh3-G97A* is competent for crossing over, *mlh3-G97V* and *mlh3-N35A* are not. This is analogous to the observations made for the corresponding mutations in Mlh1p; *mlh1-G98A*, *mlh1-G98V* and *mlh1-N35A* (Hoffmann *et al.*, 2003). Residue G97 is situated in motif III, which forms the 'lid' of the ATP binding pocket. Therefore it is possible that mutating this residue to alanine, which has a small side chain does not largely disrupt the structure of the ATP binding pocket, whereas the bulky side chain of valine does. This would indicate that MMR is more sensitive to any reduced capability of ATP binding compared to crossing over, as MMR is still affected in the *mlh3-G97A* mutant. As *mlh3-N35A* is predicted to be unable to bind ATP, this would directly affect protein interactions which require the ATP-induced conformational change, resulting in the null phenotype observed.

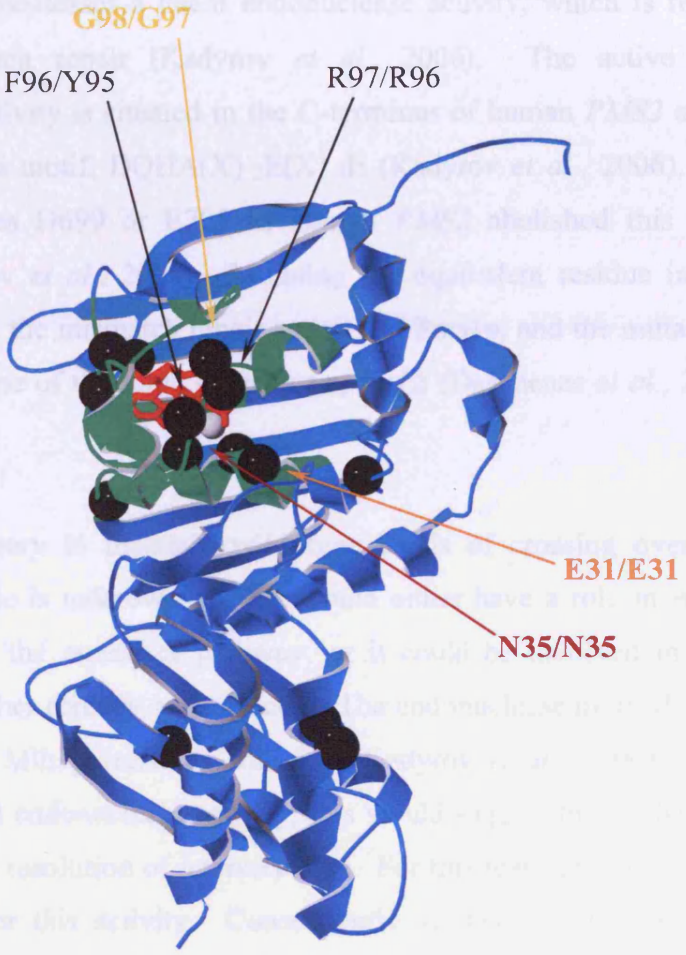


Figure 3.6 Crystal Structure of MutL
The green ribbon indicates the position of the ATP binding site, the red molecule is ATP, the grey ball is Mg^{2+} and the black balls indicate various residues. The positions of the mutations that were made in *MLH3* are shown (coloured text – colours correspond to the residues marked on Figure 3.2), with the equivalent residue in *MLH1* first (*MLH1*/*MLH3*). The residues in black are situated in ATP binding motif III, which comprises the lid of the ATP binding pocket. Figure adapted from Hoffmann *et al.* (2003).

3.5 Future Work

Human MutLα possesses a latent endonuclease activity, which is required for 3′ directed mismatch repair (Kadyrov *et al.*, 2006). The active site for the endonuclease activity is situated in the C-terminus of human *PMS2* and includes a highly conserved motif, DQHA(X) $_2$E(X) $_4$E (Kadyrov *et al.*, 2006). Specifically mutating residues D699 or E705 in human *PMS2* abolished this endonuclease activity (Kadyrov *et al.*, 2006). Mutating the equivalent residue in yeast *PMS1* (E707) disrupted the mismatch repair activity of Pms1p, and the mutant exhibited a mutator phenotype of the same severity as *pms1*Δ (Deschenes *et al.*, 2007; Erdeniz *et al.*, 2007).

Mlh3p is necessary to maintain wild type levels of crossing over, but how it performs this role is unknown. Mlh3p could either have a role in stabilisation of intermediates in the crossover pathway, or it could be involved in resolution of intermediates either directly or indirectly. The endonuclease motif described above is conserved in Mlh3p (residues 523-534; Kadyrov *et al.*, 2006). Therefore, if Mlh3p possessed endonuclease activity, this would suggest that Mlh3p may have a direct role in the resolution of intermediates. For this reason it would be of interest to test Mlh3p for this activity. Consequently, if this activity was confirmed in Mlh3p, mutating the motif to abolish this activity would allow determination of whether it was required for the crossover function of this protein.

Chapter 4: The Role of *EXO1* in Meiotic Recombination

Chapter 4: The Role of *EXO1* in Meiotic Recombination

4.1 Introduction

4.1.1 Exo1p Functions in Mutation Avoidance

Exo1p is a member of the Rad2p nuclease family, which has two highly conserved nuclease domains (N and I) in the N-terminus (Tishkoff *et al.*, 1997a). This family function in a number of different processes, including repair, replication and recombination of DNA. Rad2p, Rad27p and Exo1p all have 5′ exonuclease and flap endonuclease activities, although the extent of these activities and the substrate preference differs amongst them. However, there is some functional overlap between these nucleases (Habraken *et al.*, 1995; Moreau *et al.*, 2001; Sun *et al.*, 2003; Tishkoff *et al.*, 1997b).

Rad27p is involved in base excision repair (BER), mutation avoidance in a pathway distinct from MMR and processing of Okazaki fragments produced during DNA replication. In the absence of Rad27p, over-expression of Exo1p can partially compensate for its function in the removal of Okazaki fragments (Sun *et al.*, 2003). Specifically it is the flap endonuclease activity of Exo1p which is responsible for the partial redundancy observed (Tran *et al.*, 2002). In the absence of *rad27Δ*, strains also exhibit sensitivity to methylmethane sulfonate (MMS), which is thought to be the result of defective base excision repair (Reagan *et al.*, 1995). Over-expression of Exo1p does not rescue this sensitivity, indicating Exo1p does not play a role in BER (Sun *et al.*, 2003).

However, Exo1p does function in the mismatch repair pathway (see Chapter 1). This was first indicated by the interaction of Exo1p with both Msh2p (Schmutte *et al.*, 1998; Tishkoff *et al.*, 1997a) and Mlh1p (Jager *et al.*, 2001; Tran *et al.*, 2001) in yeast and human cells. Interaction between hEXO1 and hMSH3 has also been demonstrated in human cells (Schmutte *et al.*, 2001). In yeast, interaction domains for both Mlh1p and Msh2p within Exo1p have been determined (Figure 4.1; reviewed in Tran *et al.*, 2004). More specifically, a motif has been identified that is required for interaction with Mlh1p (Figure 4.1; Gellon *et al.*, 2002).

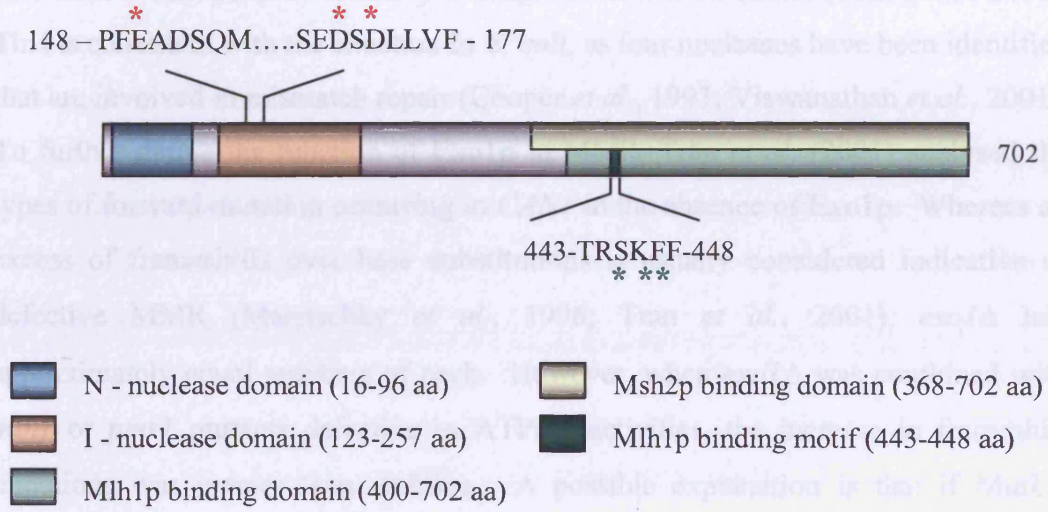


Figure 4.1 Functional Domains of *EXO1*
The functional domains present in *EXO1* are indicated by coloured boxes. The red asterisks mark three residues of interest that lie in a highly conserved nuclease domain; E150, D171 and D173 (further details in 4.1 Introduction). The green asterisks mark residues that when mutated disrupt the interaction between Exo1p and Mlh1p (Gellon *et al.*, 2002).
Adapted from Tran *et al.*, 2004.

In the absence of Exo1p, only a moderate mutator phenotype is observed, indicating that there is functional redundancy amongst nucleases for MMR (Tran *et al.*, 2001). This is consistent with the situation in *E. coli*, as four nucleases have been identified that are involved in mismatch repair (Cooper *et al.*, 1993; Viswanathan *et al.*, 2001). To further define the function of Exo1p in MMR, Tran *et al.* (2001) analysed the types of forward mutation occurring in *CAN1* in the absence of Exo1p. Whereas an excess of frameshifts over base substitutions is usually considered indicative of defective MMR (Marsischky *et al.*, 1996; Tran *et al.*, 2001), *exo1*Δ had approximately equal numbers of each. However, when *exo1*Δ was combined with *mlh1* or *pms1* mutants defective in ATPase activities, the increase in frameshift mutations was greater than additive. A possible explanation is that if MutLα ATPase functions are required to coordinate nucleases redundant with Exo1p, then additional deletion of *EXO1* would be equivalent to inactivating all of the nucleases involved (Tran *et al.*, 2001; Tran *et al.*, 2002). It was also shown that the majority of the mutator phenotype of *exo1*Δ, unlike *mlh1*Δ and *pms1*Δ, is dependent on *REV3* (Tran *et al.*, 2001). *REV3* encodes the catalytic subunit of Polζ (Morrison *et al.*, 1989; Nelson *et al.*, 1996) and is involved in the error-prone branch of the *RAD6* post-replicative repair (PRR) pathway. Specifically *REV3* is involved in the bypass of DNA damage, specifically thymine dimers (Nelson *et al.*, 1996). However, a mutant of *EXO1* that is unable to interact with Mlh1p (*exo1-F447A-F448A*), was defective for MMR but proficient for PRR (Tran *et al.*, 2007). This suggests that the functions of Exo1p in MMR and PRR are separable and therefore are independent.

4.1.2 Mutating the Nuclease Activities of Exo1p

Exo1p has 5′-3′ double-strand DNA exonuclease activity (Fiorentini *et al.*, 1997) and 5′ flap endonuclease activity (Tran *et al.*, 2002). To investigate the requirement of these nuclease activities of Exo1p for MMR, two mutants were made that differentially affected these activities. One mutant, *exo1-D173A*, was defective for both of the nuclease activities whereas *exo1-E150D* was defective for the 5′-3′ exonuclease activity, whilst approximately 20% of flap endonuclease activity was maintained (Tran *et al.*, 2002). Additionally, overexpression of *exo1-D173A* in a *pms1* ATPase mutant conferred a dominant negative mutator phenotype,

indicating that the protein is structurally intact (Tran *et al.*, 2002). The *exo1-D173A* and *exo1-E150D* mutants exhibited mutation rates that were indistinguishable from *exo1*Δ in the *CAN1* forward mutation assay, indicating that the catalytic functions of Exo1p are necessary for mismatch repair (Tran *et al.*, 2002). There is also evidence for Exo1p playing a structural role. A number of strains that were heterozygous for recessive *mlh1* and *pms1* alleles in an otherwise wild-type background were not defective for MMR. However, when *EXO1* was deleted in these strains, a strong mutator phenotype was observed (Amin *et al.*, 2001). The observed synergy is either due to functional redundancy or because the proteins involved need to interact to perform their function. Diploids that are heterozygous for recessive mutations in two proteins which form a complex, sometimes still exhibit a mutant phenotype. This is due to a reduced number of fully functional complexes being formed. Introduction of wild-type Exo1p suppressed this phenotype, suggesting that the *mlh1* and *pms1* mutations disrupted complex formation in the absence of Exo1p. This implies that Exo1p is necessary to stabilise MMR complexes for them to be fully functional (Amin *et al.*, 2001). Further support for this hypothesis comes from experiments performed by Tran *et al.* (2007). Due to the synergy observed when *exo1*Δ was combined with a *pms1* ATPase mutant, *pms1-E61A* (Tran *et al.*, 2001; Tran *et al.*, 2002), point mutants of *exo1* were combined with *pms1-E61A* to analyse their contribution to MMR (Tran *et al.*, 2007). Using the *hom3-10* allele, an increase in mutation rate was observed in the double *exo1-D173A pms1-E61A* mutant, but this was not equivalent to the mutation rate observed in the *exo1*Δ *pms1-E61A* double mutant. However, when the *exo1-D173A-FF447AA* mutant (deficient for both nuclease activities and for the interaction with Mlh1p) was combined with *pms1-E61A*, the mutation rate was equivalent to that observed in the *exo1*Δ *pms1-E61A* double mutant. Therefore Exo1p has both catalytic and structural functions in MMR (Tran *et al.*, 2002; Tran *et al.*, 2007).

A third mutation of interest is *exo1-D171A*, which is situated in the I nuclease domain, in close proximity to residue D173. From its location, it is predicted that the D171A mutation would have the same effect on function as mutating D173A. Additionally, the residues D78, D173 and D225 in hEXO1 were thought to be important for substrate binding and/or cleavage (Lee Bi *et al.*, 2002), based on the

functional analysis of hFEN1 (Shen *et al.*, 1997). This was confirmed by mutating these residues, as this resulted in the loss of the exonuclease and endonuclease functions of hEXO1 (Lee Bi *et al.*, 2002). The nuclease functions of the D171A mutation have not been tested biochemically, however, the fact that other aspartic acid residues are critical for functionality suggests that this residue may also be important for the functions of Exo1p.

4.1.3 Exo1p and Meiosis

Exo1p also functions in meiosis, and expression of *EXO1* is increased substantially during this process (Chu *et al.*, 1998; Tsubouchi and Ogawa, 2000). A number of observations have shown that Exo1p is important for meiotic recombination. In the absence of Exo1p, the levels of crossing over are reduced by approximately 50% at all intervals, and an associated increase in mis-segregation of chromosomes is observed (Khazanehdari and Borts, 2000; Kirkpatrick *et al.*, 2000; Tsubouchi and Ogawa, 2000). The overall spore viability is also decreased compared to wild-type indicating these defects lead to spore death (Khazanehdari and Borts, 2000; Kirkpatrick *et al.*, 2000; Tsubouchi and Ogawa, 2000).

Exo1p and Msh4p function in the same pathway, as a double mutant is not more severely affected for crossing over than either single mutant (Khazanehdari and Borts, 2000; Tsubouchi and Ogawa, 2000). However, this is not reflected by the spore viability, which was significantly worse than that observed in either single mutant. Additionally, the spore viability of the *exo1Δmsh4Δ* double mutant was lower than expected than if each single mutant caused spore death independently from the other. This indicates that they can partially compensate for each other in a particular function, which results in spore death when defective (Khazanehdari and Borts, 2000). The fact that *exo1Δ* has better viability than *msh4Δ*, although crossing over is affected to the same extent, could indicate that Exo1p is involved in the production of crossovers but not their distribution, whereas Msh4p has roles in both (Tsubouchi and Ogawa, 2000). As discussed in the previous chapter, Mlh1p is also involved in the promotion of crossovers in conjunction with Mlh3p (Hunter and Borts, 1997; Wang *et al.*, 1999). These proteins also form part of the Msh4p/Msh5p pathway, as demonstrated by an *msh4Δmlh1Δ* double mutant, which was not

significantly worse for crossing over than the *msh4*Δ single mutant (Hunter and Borts, 1997).

In the absence of Exo1p, the production of double strand breaks (DSBs) occurs with normal timing, but the processing is delayed by 1-2 hours compared to wild-type (Tsubouchi and Ogawa, 2000). Levels of gene conversion are also reduced in *exo1*Δ strains, but only at some loci. Events at both the *HIS4* (Khazanehdari and Borts, 2000) and *ARG4* (Kirkpatrick *et al.*, 2000) double-strand break hotspots are affected, whilst *LEU2* and *MET13* appear unaffected (Khazanehdari and Borts, 2000). The non-Mendelian segregation (NMS) events at *HIS4* will be discussed further in Chapters 5 and 6. A reduction in gene conversion could be caused by reduced resection of the 5′ ends of DSBs, a role that Exo1p has been implicated in (Khazanehdari and Borts, 2000; Kirkpatrick *et al.*, 2000; Tsubouchi and Ogawa, 2000). However, reduced resection could either be due to resection of fewer molecules or shorter resection of every molecule (Khazanehdari and Borts, 2000). Therefore, Exo1p may have an early role in recombination in processing DSB ends, and/or a later role in formation of crossovers. Alternatively, the reduction in gene conversion and crossing over could both be a result of reduced resection.

4.1.4 Aims

1. To determine if Exo1p has a catalytic and/or structural role in meiotic recombination, defined mutants *exo1-E150D* and *exo1-D173A* will be constructed.

2. To establish if a second conserved aspartic acid is important for meiotic functions, the residue D171 will be mutated.

3. To determine if the interaction between Mlh1p and Exo1p is important for mitotic mismatch repair and/or meiotic recombination, a mutant that abolishes this interaction, *exo1-S445A-F447A-F448A*, will be constructed.

4.2 Materials and Methods

4.2.1 Strains

All diploid strains used in this chapter were derived from the wild-type haploids EY97 and EY128 (resulting diploid is ERY103; Table 4.2). EY39, ERY103 and ERY112 have been described previously (Hoffmann *et al.*, 2003). The haploid *exo1*Δ strains EY142 and EY143 were constructed and analysed previously (Meadows, Hoffmann and Borts, unpublished). All strains used were isogenic, with the exception of the introduction of the *lys2*::InsE-A_{14} allele into the *exo1* mutants. The haploid and diploid strains used for analysis are listed in Tables 4.1 and 4.2 respectively.

4.2.2 Strain Construction

To determine the ability of *exo1* mutants to repair frameshift mutations, the *lys2-d* allele of EY128 was converted to the *lys2*::InsE-A_{14} allele. EY128 was initially transformed with the *LYS2* gene amplified from genomic DNA (Appendix I, Table A1.1), to make the strain Lys^+ (VC73). Plasmid p93 (Table 2.1) bearing the *lys2*::InsE-A_{14} allele and the *URA3* selectable marker, was digested using *Hpa*I, to linearise it. This product was transformed into VC73, and transformants were selected on uracil omission media (Chapter 2, 2.1.4 Media). Transformants were purified and replicated to 5′-FOA to select for "pop-out" events (loss of the plasmid). These were screened for the inability to grow on lysine and uracil omission medium. All *exo1* point mutations were introduced into the resulting strain, VC77, and mated to EY143 for analysis (Table 4.2).

To introduce the mutations E150D, D171A and D173A into genomic *EXO1*, a PCR-based allele replacement method was used (Chapter 2, 2.12.2; Erdeniz *et al.*, 1997). Insertion of the mutations was confirmed by restriction enzyme digestion where possible, and all were sequenced. Introduction of D173A destroys a *Drd*I enzyme restriction site, whereas the E150D and D171A changes are not detectable by restriction enzyme digestion. The mutations were amplified from plasmids pJM3, pEAM69 and pEAM71 respectively (Table 2.1; Appendix I, Table A1.1). The triple mutation S445A-F447A-F448A was created using *Dpn*I mediated

Table 4.1 Haploid Strains

Strain Name		Genotype
EY39	Wild-type	*his4:r*I *leu2:r*I *MATa ade1-1 TRP5 CYH2 MET13 lys2*::insE-A_{14} *CAN1 ura3-1*
VC178	*exo1Δ::kanMX4*	*his4:r*I *LEU2 MATα ade1-1 trp5-1 cyh2 MET13 lys2*::insE-A_{14} *CAN1 ura3-1*
VC310	*exo1-S445A-F447A-F448A*	*HIS4-Hha*I *leu2-r*I *MATa ADE1 TRP5 CYH2 met13-2 lys2*::insE-A_{14} *CAN1 ura3-1*

Table 4.2 Diploid Strains

Diploid Strain Name	Haploid Parents	Genotype[1]
ERY103	EY128 EY97	Wild-type *HIS4-HhaI BIK1-939 leu2-rI MATa ADE1 TRP5 CYH2 met13-2 lys2-d* *RRP7::NAT his4-ATC FUS1::HYG LEU2 MATα ade1-1 trp5-1 cyh2 MET13 lys2-c*
ERY112	EY131 EY130	*mlh1Δ::kanMX4* *mlh1Δ::kanMX4*
ERY160	EY142 EY143	*exo1Δ::kanMX4* *exo1Δ::kanMX4*
VCD71	VC121 VC142	*mlh1Δ::kanMX4 exo1Δ::kanMX4* *mlh1Δ::kanMX4 exo1Δ::kanMX4*
VCD76	VC144 EY143	*exo1-D171A* *HIS4-HhaI BIK1-939 leu2-rI MATa ADE1 TRP5 CYH2 met13-2 lys2::insE-A$_{14}$* *exo1Δ::kanMX4* *RRP7::NAT his4-ATC FUS1::HYG LEU2 MATα ade1-1 trp5-1 cyh2 MET13 lys2-c*
VCD77	VC146 EY143	*exo1-E150D* *exo1Δ::kanMX4*
VCD84	VC154 EY143	*exo1-D173A* *exo1Δ::kanMX4*
VCD166	VC310 EY143	*exo1-S445A F447A F448A* *exo1Δ::kanMX4*

[1] Strains grouped together have the same genotype. All of the strains are also *ura3-1 / ura3-1* and *CAN1 / CAN1*.

mutagenesis (Chapter 2, 2.12.4; Fisher and Pei, 1997). A pre-existing plasmid with a clone of the *EXO1* ORF present (pEAM67; Table 2.1) was used as a template for PCR (Appendix I, Table A1.1). The resulting plasmid, pVC8 (Table 2.1), was digested with *Drd*I and *Ava*I to give an 1170 bp fragment containing the mutations. The *delitto perfetto* technique (Chapter 2, 2.12.3; Storici *et al.*, 2001) was used to insert the fragment into the genome. Insertion of the mutations was confirmed initially by restriction enzyme digestion. F447A creates an *Alu*I recognition site, and S445A and F448A together create a *Mwo*I recognition site. Correct transformants were also sequenced to ensure no additional changes had been introduced. The resulting strain, VC310 (Tables 4.1 and 4.2), was used for analysis. To create the *mlh1*Δ*exo1*Δ double mutant, an *mlh1*Δ::*kanMX4* strain was crossed to an *exo1*Δ strain, also disrupted with *kanMX4*, and the necessary segregants were obtained by dissection. VC178 was obtained through crossing (Table 4.1).

4.2.3 Fluctuation Tests

Fluctuation tests were used to determine the mutation rates of *exo1* mutants. Due to the minor mutator phenotype of wild-type and *exo1*Δ strains (Tishkoff *et al.*, 1997a; Tran *et al.*, 2002), single colonies were propagated overnight in liquid cultures to increase the cell number before testing the mutation rate (Chapter 2, 2.13). The raw data from these experiments can be found in Appendix II (Table A2.2).

4.2.4 Genetic Analysis

To determine the effects of *exo1* mutants on meiotic recombination, crossing over was assessed in the intervals *NAT-HYG*, *HYG-LEU2* and *LEU2-MAT* on chromosome III, and *TRP5-CYH2* and *CYH2-MET13* on chromosome VII (as described in Chapter 2, 2.9.5). The additional markers *NAT* and *HYG* that flank *HIS4* in EY97 and its derivatives (Table 4.2), allowed more detailed analysis of the events occurring at the DSB hotspot (Figure 4.2; Hoffmann *et al.*, 2005). For consistency, all *exo1* mutants were crossed with the same haploid *exo1*Δ strain, EY143. The level of repair of the *his4-ATC* and *met13-2* alleles during meiosis was also determined, by comparing the level of gene conversion and post-meiotic segregation events which reflect repair and non-repair of mismatches respectively. At least 400 tetrads were dissected for each strain studied. Data from an isogenic *mlh1*Δ strain (Hoffmann and Borts, unpublished) was included for comparison with

the *mlh1Δexo1Δ* double mutant and the *exo1* mutant that is unable to interact with Mlh1p (*exo1-S445A-F447A-F448A*).

4.2.5 Statistics

In applying the Dunn-Sidak correction for multiple comparisons (Sokal and Rohlf, 1969; Chapter 2, 2.14), a P-value of 0.0073 or less was considered significant (for seven-way comparisons of all mutant strains with the wild-type and comparisons amongst the datasets as required), unless otherwise stated.

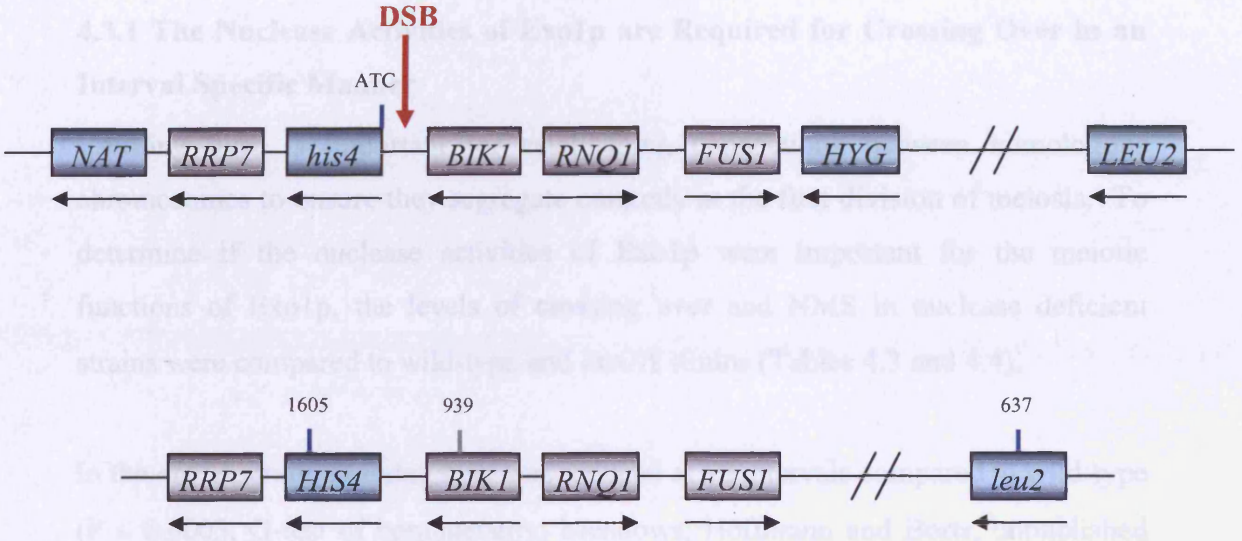


Figure 4.2 The *HIS4* Region on Chromosome III.
The *HIS4* locus in haploid strains EY97 (top) and EY128 (bottom) are shown. The modifications to this region have been described previously (Hoffmann *et al.*, 2005). The cassettes *natMX4* (*NAT*) and *hphMX4* (*HYG*) conferring resistance to nourseothricin and hygromycin B respectively, were inserted into EY97 to add additional intervals over which to monitor crossing over. These flank *HIS4*, which contains a G-C mutation in the start codon. *HIS4* in EY128 contains a silent mutation which ablates a *Hha*I restriction site (C to G change at position 1605). *BIK1* contains a silent mutation which ablates a *Pvu*II restriction site (G to A change at position 939). *LEU2* in EY128 also contains a 4 bp insertion which disrupts an *Eco*RI restriction site (at position 637), and function of the gene. Heterozygosity at the *LEU2* locus (and others; Chapter 4, 2.4), allows assessment of crossing over between *LEU2* and adjacent intervals. The red arrow marks the position of the *HIS4* DSB, approximately 300 bp upstream of the start of the ORF (Hoffmann *et al.*, 2005). The blue lines mark the positions of mutations and the black arrows indicate the direction of transcription. The genes used for monitoring meiotic recombination are highlighted in blue.
Adapted from Hoffmann *et al.*, 2005, not drawn to scale.

4.3 Results

4.3.1 The Nuclease Activities of Exo1p are Required for Crossing Over in an Interval Specific Manner

Crossing over is important for establishing connections between homologous chromosomes to ensure they segregate correctly at the first division of meiosis. To determine if the nuclease activities of Exo1p were important for the meiotic functions of Exo1p, the levels of crossing over and NMS in nuclease deficient strains were compared to wild-type and *exo1*Δ strains (Tables 4.3 and 4.4).

In the *exo1*Δ strain, crossing over was reduced at all intervals compared to wild-type ($P \leq 0.0003$, G-test of homogeneity; Meadows, Hoffmann and Borts, unpublished and Hoffmann and Borts unpublished, respectively). The mutant *exo1-D173A*, which is defective for both the 5′-3′ exonuclease and flap endonuclease activities, exhibited levels of crossing over that were equivalent to those exhibited by *exo1*Δ in all of the intervals studied ($P > 0.05$, G-test). Consistent with this, the levels of crossing over were significantly lower than those in the wild-type ($P \leq 6.0 \times 10^{-05}$, G-test) except at *HYG-LEU2* ($P > 0.05$, G-test). The *exo1-E150D* mutant, that is defective for the 5′-3′ exonuclease activity but retains some flap endonuclease activity, exhibited interval specific effects. The levels of crossing over were significantly higher than those in the *exo1*Δ strain in all intervals tested ($P \leq 0.001$, G-test), with the exception of *NAT-HYG* ($P > 0.05$, G-test). Consistent with this, the map distance at *NAT-HYG* was also lower than the corresponding map distance in the wild-type ($P = 0.0002$, G-test). However, crossing over in *exo1-E150D* was also worse than in the wild-type at *LEU2-MAT* and *CYH2-TRP5* ($P \leq 7.0 \times 10^{-05}$, G-test) indicating that there is an intermediate effect in these larger intervals. There was no decrease in crossing over in the smaller intervals, *HYG-LEU2* and *MET13-CYH2*, compared to wild-type ($P > 0.05$, G-test). Together these data suggests that the two nuclease activities of Exo1p are required to different extents for crossing over.

4.3.2 How Functional is *exo1-D171A*?

To determine the functionality of *exo1-D171A*, comparisons were made with the wild-type and *exo1*Δ strains, and also the functionally defined mutants, *exo1-E150D*

Table 4.3 Map Distances for Intervals on Chromosome III

	Interval											
	NAT-HYG				*HYG-LEU2*				*LEU2-MAT*			
Genotype	**PD**[1]	**NPD**	**TT**	**cM**	**PD**	**NPD**	**TT**	**cM**	**PD**	**NPD**	**TT**	**cM**
Wild-type[2]	1347	7	336	11.2†	1435	7	245	8.5†‡	828	66	799	35.3†‡
*exo1*Δ[3]	372	2	43	6.6*‡	377	0	33	4.0*	305	6	100	16.5*
*mlh1*Δ[2]	463	1	103	9.6†	522	1	40	4.1*	375	14	175	23.0*
*mlh1*Δ*exo1*Δ	351	0	42	5.3*‡	354	1	33	5.0*	276	7	106	19.0*
exo1-E150D	394	0	57	6.3*	377	0	73	8.1†‡	282	3	164	20.3*†
exo1-D171A	366	0	61	7.1*	380	0	50	5.8	252	3	173	22.3*†‡
exo1-D173A	341	0	44	5.7*‡	337	1	43	6.4	282	2	98	14.4*‡
exo1-S445A-F447A-F448A	332	0	72	8.9†	342	2	61	9.0†‡	188	11	206	33.6†‡

[1] PD, NPD and TT are parental ditype, non-parental ditype and tetratype respectively, and map distances (cM) were calculated according to Perkins (1949). Comparisons of the distribution of PD, NPD and TT events were made using a G-test of homogeneity, and significant statistical differences are represented as follows:

* Significantly different to corresponding values in wild-type.

† Significantly different to corresponding values in *exo1*Δ.

‡ Significantly different to corresponding values in *mlh1*Δ.

[2] Data from Hoffmann and Borts, unpublished.

[3] Data from Meadows, Hoffmann and Borts, unpublished.

Table 4.4 Map Distances for Intervals on Chromosome VII

	Interval							
	MET13-CYH2				*CYH2-TRP5*			
Genotype	**PD**[1]	**NPD**	**TT**	**cM**	**PD**	**NPD**	**TT**	**cM**
Wild-type[2]	1160	8	474	15.9†‡	314	174	1216	66.3†‡
*exo1*Δ[3]	352	0	52	6.4*	223	6	182	26.5*
*mlh1*Δ[2]	458	6	81	10.7*	299	9	261	27.7*
*mlh1*Δ*exo1*Δ	306	0	65	8.8*	209	11	170	30.3*
exo1-E150D	310	1	122	14.8†‡	126	37	287	56.6*†‡
exo1-D171A	294	0	122	14.7†‡	133	30	267	52.0*†‡
exo1-D173A	318	0	51	6.9*	201	9	174	29.7*
exo1-S445A-F447A-F448A	266	2	128	17.7†‡	70	27	314	57.9†‡

[1] PD, NPD and TT are parental ditype, non-parental ditype and tetratype respectively, and map distances (cM) were calculated according to Perkins (1949). Comparisons of the distribution of PD, NPD and TT events were made using a G-test of homogeneity, and significant statistical differences are represented as follows:

* Significantly different to corresponding values in wild-type.

† Significantly different to corresponding values in *exo1*Δ.

‡ Significantly different to corresponding values in *mlh1*Δ.

[2] Data from Hoffmann and Borts, unpublished.

[3] Data from Meadows, Hoffmann and Borts, unpublished.

and *exo1-D173A* (Tables 4.3 and 4.4). Although the nuclease activities of *exo1-D171A* have not been determined, it is thought that D171 may be one of the residues important for substrate binding and or/cleavage. Therefore it might be predicted to have similar effects to *exo1-D173A*. Like *exo1-D173A*, the *exo1-D171A* mutant had an equivalent map distance to *exo1*Δ at *NAT- HYG* ($P = 0.05$, G-test). However, at the other intervals studied differences were observed. In contrast to *exo1-D173A*, *exo1-D171A* was comparable to the wild-type at *MET13-CYH2* ($P > 0.05$, G-test) and had intermediate levels of crossing over between wild-type and *exo1*Δ in the intervals *LEU2-MAT* and *CYH2-TRP5* ($P \leq 4.0 \times 10^{-06}$ for both comparisons, G-test). In addition, although the distribution of parental ditype, non-parental ditype and tetratype (PD, NPD and TT respectively) events were not different to wild-type or *exo1*Δ, the cM value for the *HYG-LEU2* interval was intermediate (5.8 cM compared with 4 cM for wild-type and 8.5 cM for *exo1*Δ; Table 4.3). This may be partly due to the small size of the interval. Overall, the phenotype of *exo1-D171A* in terms of crossing over was not as severe as that exhibited by *exo1-D173A*.

In contrast, the *exo1-D171A* mutant exhibited equivalent levels of crossing over to *exo1-E150D*, and they were not statistically different to one another at any of the intervals studied ($P > 0.05$, G-test; Tables 4.3 and 4.4). Both *exo1-E150D* and *exo1-D171A* had larger map distances than *exo1-D173A* in the *LEU2-MAT*, *MET13-CYH2* and *CYH2-TRP5* intervals ($P \leq 0.003$, G-test). All three mutants exhibited levels of crossing over equivalent to *exo1*Δ in the *NAT-HYG* interval ($P \geq 0.05$, G-test). At *HYG-LEU2*, *exo1-E150D* exhibited wild-type levels of crossing over ($P > 0.05$, G-test), whereas the other two mutants displayed intermediate levels of crossing over. However, neither *exo1-D171A* nor *exo1-D173A* was statistically different to the wild-type or *exo1*Δ strains ($P \geq 0.06$ for all comparisons, G-test). Therefore the crossover phenotype of *exo1-D171A* most closely resembles the phenotype of *exo1-E150D*, not *exo1-D173A*.

4.3.3 Mlh1p and Exo1p Function in the Same Crossover Pathway

Mlh1p and Exo1p are known to interact (Jager *et al.*, 2001; Tran *et al.*, 2001), and as both proteins are known to contribute to the production of crossovers (Hunter and Borts, 1997; Khazanehdari and Borts, 2000; Kirkpatrick *et al.*, 2000; Tsubouchi and

Ogawa, 2000; Wang *et al.*, 1999), it is possible that they function together in this process. To test this hypothesis two mutants were made; an *mlh1Δexo1Δ* double mutant and an *exo1* single mutant that was unable to interact with Mlh1p (S445A-F447A-F448A; Figure 4.1). To determine the relationship between Exo1p and Mlh1p, *mlh1Δ* and *exo1Δ* strains were compared to wild-type and *mlh1Δexo1Δ* (Tables 4.3 and 4.4). The *mlh1Δ* strain had levels of crossing over that were statistically lower than those in the wild-type in all of the intervals ($P \leq 5.2 \times 10^{-06}$, G-test) except *NAT-HYG* ($P > 0.05$, G-test; Tables 4.3 and 4.4; Hoffmann and Borts, unpublished data). Crossing over in the *mlh1Δ* and *exo1Δ* strains was only different in the *NAT-HYG* interval ($P = 0.0017$, G-test; Table 4.3). The levels of crossing over in the *mlh1Δexo1Δ* mutant were not different from the *exo1Δ* mutant in any of the intervals studied ($P > 0.05$, G-test). Consistent with the above data, crossing over in the *mlh1Δexo1Δ* double mutant was different to *mlh1Δ* at *NAT-HYG* ($P = 0.003$, G-test).

To determine if Mlh1p and Exo1p act at the same stage in meiosis, or if the interaction between them is required for their meiotic functions, the Mlh1p interaction mutant, *exo1-S445A-F447A-F448A*, was compared to wild-type, *exo1Δ* and *mlh1Δexo1Δ*. The *exo1-S445A-F447A-F448A* mutant was proficient for crossing over and was not different to the wild-type in any of the intervals studied ($P \geq 0.04$; G-test). Additionally the mutant exhibited significantly higher levels of crossing over than *exo1Δ* in all intervals ($P < 0.0022$, G-test), and than *mlh1Δexo1Δ* in three intervals, *LEU2-MAT*, *MET13-CYH2* and *CYH2-TRP5* ($P \leq 2.6 \times 10^{-06}$, G-test). However, the levels of crossing over in *exo1-S445A-F447A-F448A* and *mlh1Δexo1Δ* were not different in the *NAT-HYG* or *HYG-LEU2* intervals ($P \geq 0.013$, G-test). This suggests that the interaction between Mlh1p and Exo1p is not required for them to perform their functions during recombination.

4.3.4 The 5′-3′ Exonuclease Activity of Exo1p is Required for Gene Conversion but not Meiotic Mismatch Repair at *HIS4*

If the prediction that Exo1p is involved in resection is correct, then the frequency of total NMS should decrease but the frequency of PMS events should remain the same. To determine the effects of the different *exo1* mutants on NMS and hDNA

repair, the type of events at two alleles, *his4-ATC* and *met13-2*, were measured (Table 4.5). The data obtained was compared to wild-type and *exo1*Δ data using the G-test of homogeneity. In the absence of Exo1p, a 2-fold reduction in NMS was observed at the *his4-ATC* allele compared to wild-type ($P = 5.1 \times 10^{-05}$, G-test; Meadows, Hoffmann and Borts, unpublished) as has been seen previously for other alleles of *HIS4* (Khazanehdari and Borts, 2000). The *exo1-E150D*, *exo1-D171A* and *exo1-D173A* mutants all reduced NMS at *his4-ATC* to the same extent as the *exo1*Δ mutant ($P > 0.05$, G-test). None of the mutants studied exhibited a level of NMS at *met13-2* that was different to the wild-type level ($P \geq 0.04$, G-test). The *exo1-E150D* mutant, that is defective for the 5′-3′ exonuclease activity but proficient for flap endonuclease activity, exhibited a frequency of NMS of the *his4-ATC* allele that was not different to *exo1*Δ. This suggests that it is the 5′-3′ exonuclease activity, but not the flap endonuclease activity, that is required for generating these events. This is consistent with a role in the resection of the *HIS4* DSB.

To determine if the interaction of Mlh1p and Exo1p was important for producing the frequency of NMS observed in the wild-type, the level of NMS in the *exo1-S445A-F447A-F448A* mutant was compared to wild-type, *exo1*Δ and *mlh1*Δ. The *exo1-S445A-F447A-F448A* mutant did not affect NMS at *his4-ATC* or *met13-2* compared to the wild-type ($P > 0.05$ for both comparisons, G-test), indicating that the interaction with Mlh1p is also not important in this function. The *mlh1*Δ strain had a higher percentage of NMS events at *his4-ATC* than the wild-type strain (19.8% compared with 14.0% respectively; $P = 0.001$, G-test; Table 4.5; Hoffmann *et al.*, 2005). In contrast, in the *mlh1*Δ*exo1*Δ double mutant, NMS at *his4-ATC* was reduced approximately 2-fold compared to the *mlh1*Δ single mutant ($P = 6.2 \times 10^{-05}$, G-test). This suggests that the increased events observed in the absence of Mlh1p are dependent upon the presence of Exo1p.

The level of repair of hDNA formed during meiosis was also analysed using the *his4-ATC* and *met13-2* alleles (Chapter 2, 2.9.5). The ratio of gene conversion events (repair) to post-meiotic segregation events (PMS; non-repair) is reflective of this (Holliday, 1964). Comparisons were made using the G-test of homogeneity. In

Table 4.5 Non-Mendelian Segregation at Two Alleles

Genotype	% Non-Mendelian Segregation[1] (NMS / Total Tetrads) *his4-ATC*[2]	*met13-2*[2]
Wild-type[3]	14.0†‡ (243 / 1731)	4.5 (78 / 1731)
exo1Δ[4]	7.1*‡ (30 / 421)	3.3 (14 / 421)
mlh1Δ[3]	19.8*† (116 / 585)	5.5 (32 / 585)
mlh1Δexo1Δ	10.8‡ (43 / 400)	6.3 (25 / 400)
exo1-E150D	6.2*‡ (28 / 453)	4.2 (19 / 453)
exo1-D171A	8.8*‡ (38 / 434)	3.9 (17 / 434)
exo1-D173A	10.1‡ (39 / 387)	4.4 (17 / 387)
exo1-S445A-F447A-F448A	14.3† (59 / 414)	4.1 (17 / 414)

[1] Non-Mendelian segregation includes gene conversion and post-meiotic segregation events. % NMS was calculated as the number of NMS events / total number of tetrads x 100. Comparisons were made using the G-test, and significant statistical differences are represented as follows:
* Significantly different to corresponding values in wild-type.
† Significantly different to corresponding values in *exo1Δ*.
‡ Significantly different to corresponding values in *mlh1Δ*.

[2] The *his4-ATC* allele makes a C-C mispair in the ATG start codon of *HIS4* and *met13-2* contains the mutation C278A.

[3] Data from Hoffmann *et al.*, 2005.

[4] Data from Meadows, Hoffmann and Borts, unpublished.

Table 4.6 Frequency of Repair of DNA Mismatches

Genotype	% Repair (No. of Repair Events / Total NMS Events)[1] *his4-ATC*[2]	*met13-2*[2]
Wild-type[3]	86.8‡ (211 / 243)	100‡ (78 / 78)
*exo1*Δ[4]	96.7‡ (29 / 30)	100‡ (7 / 7)
*mlh1*Δ[3]	10.3*† (12 / 116)	28.1*† (9 / 32)
*mlh1*Δ *exo1*Δ	34.9*†‡ (15 / 43)	40.0*† (10 / 25)
exo1-E150D	85.7‡ (24 / 28)	94.7‡ (18 / 19)
exo1-D171A	84.2‡ (32 / 38)	82.4*‡ (14 / 17)
exo1-D173A	66.7*†‡ (26 / 39)	100‡ (17 / 17)
exo1-S445A-F447A-F448A	79.7‡ (47 / 59)	100‡ (17 / 17)

[1] Repair events are manifested as gene conversions. Total NMS events include gene conversion and post-meiotic segregation events. Comparisons were made using the G-test, and significant statistical differences are represented as follows:

* Significantly different to corresponding values in wild-type.

† Significantly different to corresponding values in *exo1*Δ.

‡ Significantly different to corresponding values in *mlh1*Δ.

[2] The *his4-ATC* allele makes a C-C mispair in the ATG start codon of *HIS4* and *met13-2* contains the mutation C278A.

[3] Data from Hoffmann and Borts, unpublished.

[4] Data from Meadows, Hoffmann and Borts, unpublished.

most cases *met13-2* was repaired between 90 - 100% (Table 4.6). The *exo1Δ*, *exo1-E150D* and *exo1-D171A* mutants all exhibited wild-type levels of repair at *his4-ATC* ($P > 0.05$, G-test). However, *exo1-D173A* reduced the level of repair of the *his4-ATC* allele significantly compared to wild-type (66.7% compared with 86.8%; $P = 0.0034$, G-test) and *exo1Δ* (66.7% compared with 96.7%; $P = 0.0008$, G-test). Unlike the other point mutants, *exo1-D171A* also reduced the repair of the *met13-2* allele significantly compared to wild-type ($P = 0.001$, G-test).

As Exo1p is not involved in meiotic repair, it was not expected that the interaction between Mlh1p and Exo1p would be required for this function. To confirm this, the levels of repair were analysed in the *exo1-S445A-F447A-F448A* interaction mutant, *exo1Δ* and *mlh1Δ*. The level of repair at *his4-ATC* in the e*xo1-S445A-F447A-F448A* mutant was not different to the wild-type ($P > 0.05$, G-test) as was observed for *exo1Δ* ($P > 0.05$, G-test). Repair of both *his4-ATC* and *met13-2* was severely reduced in the absence of Mlh1p to 10.3% and 28.1% respectively (compared to 86.8% and 100% respectively for wild-type; $P \leq 5.26 \times 10^{-18}$, G-test). A double *mlh1Δexo1Δ* mutant was also analysed, and surprisingly mismatches produced at the *his4-ATC* allele were repaired better than in the *mlh1Δ* mutant (34.9% compared with 10.3%; $P = 0.0005$, G-test) although not as well in the *exo1Δ* mutant (96.7%; $P = 6.4 \times 10^{-09}$, G-test). This was not reflected in the repair of the *met13-2* allele, where there was no difference between the *mlh1Δ* and *mlh1Δexo1Δ* mutants (28.1% compared with 40.0% respectively; $P > 0.05$, G-test). The *mlh1Δexo1Δ* double mutant exhibited significantly lower levels of repair than the *exo1-S445A-F447A-F448A* interaction mutant at both alleles tested ($P \leq 4 \times 10^{-06}$, G-test). This supports the fact that Exo1p is not involved in the repair of mismatches during meiosis.

To test the hypothesis that Exo1p may play a role in the restoration of G-G mismatches, the patterns of non-Mendelian segregation of the *his4-ATC* allele were analysed (Table 4.7). Given that DSBs are initiated on both parental strands of DNA with equal frequency, equal numbers of 6:2 and 2:6 or 5:3 and 3:5 NMS events should be detected. It has been shown previously that in the absence of Mlh1p more 3:5 PMS events than 5:3 were obtained, although equal numbers of 6:2 and 2:6 gene conversions were observed (Hoffmann *et al.*, 2005; results reproduced

Table 4.7 Patterns of Non-Mendelian Segregation of *his4-ATC*

Genotype	NMS Class										Total NMS	Total Tetrads	NMS (%)
	6:2	2:6	5:3	3:5	8:0	0:8	7:1	1:7	Ab4:4	Others[1]			
Wild-type[2]	96	111	14	16	1	3	1	1	0	0	243	1731	14.0
*exo1*Δ[3]	13	16	0	1	0	0	0	0	0	0	30	421	7.1
*mlh1*Δ[2]	5	7	35	64	0	0	0	0	2	3	116	585	19.8
*mlh1*Δ*exo1*Δ	5	3	9	18	6	1	0	0	1	0	43	400	10.8

[1] This group consisted mainly of aberrant 6:2 and 2:6 segregation events.
[2] Data from Hoffmann *et al.* (2005).
[3] Data from Meadows, Hoffmann and Borts, unpublished.

in Table 4.7). In the *exo1*Δ mutant, equivalent numbers of 6:2 and 2:6 gene conversion events were observed ($P > 0.05$, χ^2 test), but as Exo1p is not involved in meiotic repair (Khazanehdari and Borts, 2000) only one PMS event was detected. Interestingly, in the *mlh1*Δ*exo1*Δ double mutant a disparity between the 5:3 and 3:5 PMS events was still observed, although not significant from the "expected" equal distribution (9 and 18 events of each type respectively; $P = 0.08$, χ^2 test). Additionally, the disparity in *mlh1*Δ*exo1*Δ was not different to that observed in *mlh1*Δ ($P > 0.05$, G-test) when comparing the distribution of 5:3 and 3:5 PMS events. This suggests that the origin of the disparity is not dependent on Exo1p.

4.3.5 The Association Between NMS and Crossing Over is Not Altered in the Absence of Exo1p

As both crossovers and gene conversion events arise from the repair of DSBs, the association of these events arising from the *HIS4* DSB was analysed. The proportions of gene conversion events associated with a crossover or a non-crossover were compared using the G-test of homogeneity in the wild-type and *exo1* mutants (Table 4.8). In the wild-type, 58.9% of the NMS events that occur at *HIS4* were associated with a crossover between *NAT-HYG*. This was not altered in *exo1*Δ, where 56.7% of the NMS events were associated with a crossover ($P > 0.05$, G-test), nor in any of the four *exo1* point mutants ($P > 0.05$ compared to wild-type and *exo1*Δ, G-test). In the absence of Mlh1p, a small reduction in the association between NMS and crossing over was observed compared to wild-type ($P = 0.0019$, G-test). This may be a result of the increase in NMS observed in *mlh1*Δ without a concomitant increase in crossing over. This was also true for *mlh1*Δ*exo1*Δ, which was significantly different to wild-type (33.3% compared with 58.9% respectively; $P = 0.0021$, G-test), but not to either *mlh1*Δ or *exo1*Δ ($P \geq 0.05$, G-test). Therefore, when there is a concomitant decrease in NMS and crossing over, as in the *exo1*Δ strain, the association between the events remains the same. This suggests that the same defect leads to the decrease in both NMS and crossover events at *HIS4*.

4.3.6 In the Absence of Exo1p Chromosome Segregation is Affected

Decreased crossing over can cause non-disjunction of chromosomes, which leads to a specific pattern of spore death. The patterns of tetrads with four, three, two, one and zero viable spores were compared using the G-test (Table 4.9). The distribution

Table 4.8 The Association Between Non-Mendelian Segregation and Crossing Over at *HIS4*

Genotype	NMS[1]	CO[2]	% NMS with a CO[3] (NMS with a CO/Total NMS)	CO / NCO Ratio[6]
Wild-type[4]	243	343	58.9 (143 / 243)	1.43
exo1Δ[5]	30	45	56.7 (17 / 30)	1.31
mlh1Δ[4]	116	104	41.4* (48 / 116)	0.71
mlh1Δ exo1Δ	42	42	33.3* (14 / 42)	0.50
exo1-E150D	28	57	75.0 (21 / 28)	3.00
exo1-D171A	38	61	68.4 (26 / 38)	2.17
exo1-D173A	39	44	46.2 (18 / 39)	0.86
exo1-S445A-F447A-F448A	59	72	52.5 (31 / 59)	1.11

[1] Number of tetrads with a non-Mendelian segregation event at *HIS4*. NMS includes gene conversion and post-meiotic segregation events.
[2] Number of tetrads with a crossover in the *NAT-HYG* interval which spans *HIS4*.
[3] The percentage of tetrads which have a NMS event and an associated crossover. Calculated as the number of tetrads with an NMS event with a crossover / number of tetrads with an NMS event. Comparisons were made using the G-test, and * denotes values which were significantly different to wild-type.
[4] Data from Hoffmann and Borts, unpublished.
[5] Data from Meadows, Hoffmann and Borts, unpublished.
[6] Calculated by:
% NMS with a CO / % NMS without a CO

of the viable spore tetrad classes can give an indication of the type of errors occurring to cause spore death. The pattern of viable spore tetrads observed in the absence of Msh4p (Hoffmann and Borts, unpublished; data reproduced in Table 4.9) provides an example of the effects a reduction in frequency and distribution of crossovers has on chromosome segregation (Khazanehdari and Borts, 2000; Novak *et al.*, 2001; Ross-Macdonald and Roeder, 1994). The overall spore viability of *exo1*Δ was reduced 1.26-fold compared to wild-type (75.2% and 95.1% respectively) and as can be seen in Table 4.9 and Figure 4.3, the strains had a significantly different distribution pattern of viable spores ($P = 2.4 \times 10^{-104}$, G-test; Meadows, Hoffmann and Borts, unpublished). Particularly there was an increase in two and zero viable spore tetrads in *exo1*Δ compared to wild-type (4.4-fold and 8.0-fold respectively), which is indicative of meiosis I non-disjunction. The increases in these viable spore tetrad classes were not equivalent to those observed in *msh4*Δ ($P < 1.87 \times 10^{-11}$ for both comparisons, G-test). The *exo1-E150D*, *exo1-D171A* and *exo1-D173A* mutants all had significantly better viability than the *exo1*Δ mutant (ranging from 80.9 to 85.5%), but did not have equivalent viability to the wild-type (Table 4.9). Increased numbers of two viable spore tetrads were also observed in the *exo1-E150D, exo1-D171A* and *exo1-D173A* strains, equivalent to those observed in *exo1*Δ ($P > 0.05$, G-test). In contrast, the number of zero viable spore tetrads in these mutants was not increased to the same extent. This difference was only significant when comparing the *exo1-E150D* and *exo1-D171A* mutants to *exo1*Δ ($P < 9.5 \times 10^{-10}$ for both comparisons, G-test). The overall distribution of viable spore tetrad classes in these mutants was different to the distribution in the *exo1*Δ ($P \leq 0.0008$, G-test) and wild-type strains ($P \leq 1.83 \times 10^{-53}$, G-test). Compared to wild-type, a 3-fold increase in three viable spore tetrads was also observed in the absence of *exo1*Δ (Table 4.9; 8.4×10^{-32}, G-test). An increase of equal magnitude was also observed in the *exo1-E150D*, *exo1-D171A* and *exo1-D173A* mutants (2.6-4 fold; $P > 0.01$ compared to *exo1*Δ, G-test). The *exo1-E150D* mutant exhibited an increase in three viable spore tetrads that was larger than observed in *exo1*Δ, however, fewer one and zero viable spore tetrads were obtained indicating a shift in the number of spores per tetrad being affected by defects in the *exo1-E150D* mutant.

Table 4.9 Distribution of Viable Spores per Tetrad and Overall Viability

Genotype	Viable spores per tetrad class					Spore Viability	
	4	3	2	1	0		
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	Total Tetrads	%[1]
Wild-type[2†]	1746 (87.9)	148 (7.4)	59 (3.0)	14 (0.7)	20 (1.0)	1987	95.1
*msh4*Δ[2*†]	473 (32.5)	117 (8.0)	361 (24.8)	115 (7.9)	391 (26.8)	1457	52.8
*exo1*Δ[3*]	428 (49.1)	208 (23.9)	117 (13.4)	48 (5.5)	70 (8.0)	871	75.1
*mlh1*Δ*exo1*Δ[*†]	418 (43.1)	178 (18.4)	203 (20.9)	76 (7.8)	94 (9.7)	969	69.3
exo1-E150D[*†]	472 (53.2)	261 (29.4)	116 (13.1)	22 (2.5)	17 (1.9)	888	82.3
exo1-D171A[*†]	447 (59.0)	194 (25.6)	106 (14.0)	8 (1.1)	2 (0.3)	757	85.5
exo1-D173A[*†]	400 (59.1)	129 (19.1)	87 (12.9)	29 (4.3)	32 (4.7)	677	80.9
exo1-S445A F447A F448A[*†]	430 (90.0)	39 (8.2)	6 (1.3)	0 (0.0)	3 (0.6)	478	96.7

[1] Calculated as ((4 x no. of 4-spore tetrads + 3 x no. of 3-spore tetrads + 2 x no. of 2-spore tetrads + no. of 1-spore tetrads) / (4 x total no. of tetrads)) x 100. Comparisons of overall spore viability were made using the z-test. All mutants were significantly different to wild-type and *exo1*Δ. The distributions of viable spore tetrads were compared using the G-test. Significant differences in overall spore viability are indicated as follows:

[*] Significantly different to corresponding values in wild-type.

[†] Significantly different to corresponding values in *exo1*Δ.

[2] Data from Hoffmann and Borts, unpublished.

[3] Data from Meadows, Hoffmann and Borts, unpublished.

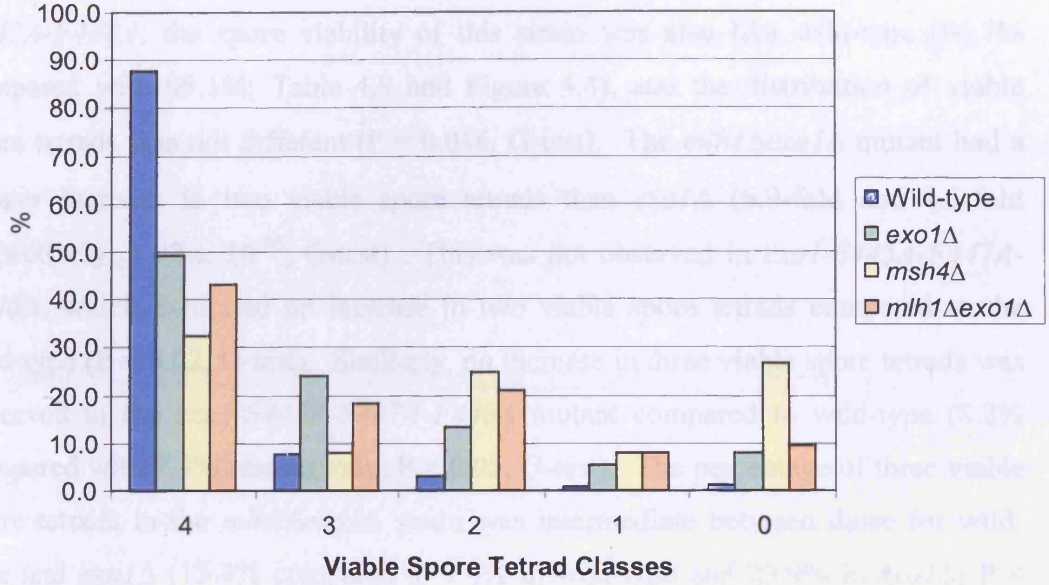


Figure 4.3 Distribution of Viable Spores per Tetrad in Wild-Type and Deletion Strains
The percentages of each class of viable spores per tetrad are shown for wild-type, *exo1*Δ, *mlh1*Δ*exo1*Δ and *msh4*Δ. Data for *msh4*Δ is shown as the distribution of viable spores per tetrads is characteristic of meiosis I non-disjunction.

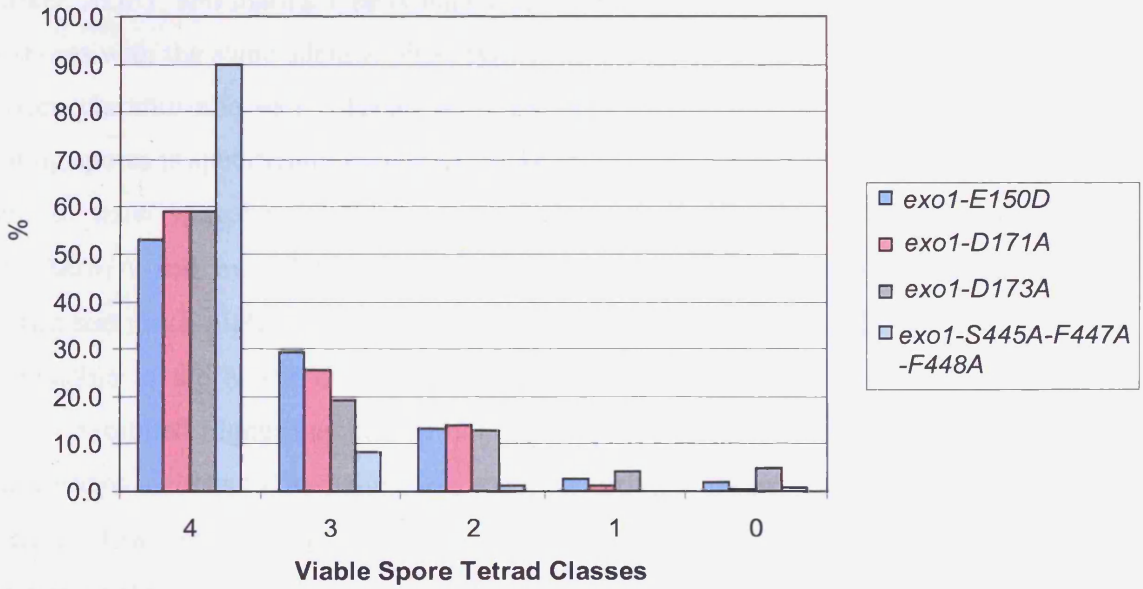


Figure 4.4 Distribution of Viable Spores per Tetrad in Mutant Strains
The percentages of each class of viable spores per tetrad are shown for *exo1-E150D*, *exo1-D171A*, *exo1-D173A* and *exo1-S445A-F447A-F448A*.

Consistent with the wild-type levels of crossing over observed in *exo1-S445A-F447A-F448A*, the spore viability of this strain was also like wild-type (96.7% compared with 95.1%; Table 4.9 and Figure 4.4), and the distribution of viable spore tetrads was not different (P = 0.016, G-test). The *mlh1Δexo1Δ* mutant had a greater increase in two viable spore tetrads than *exo1Δ* (6.9-fold and 4.5-fold respectively; 1.92 x 10^{-05}, G-test). This was not observed in *exo1-S445A-F447A-F448A*, which exhibited no increase in two viable spore tetrads compared to the wild-type (P = 0.02, G-test). Similarly, no increase in three viable spore tetrads was observed in the *exo1-S445A-F447A-F448A* mutant compared to wild-type (8.2% compared with 7.4% respectively; P > 0.05, G-test). The percentage of three viable spore tetrads in the *mlh1Δexo1Δ* strain was intermediate between those for wild-type and *exo1Δ* (15.4% compared to 7.4% in wild-type and 23.9% in *exo1Δ*; P < 0.004, G-test). Therefore, the additional deletion of Mlh1p in an *exo1Δ* background either alleviates some of the death of single spores caused by the absence of Exo1p, or causes a shift in the pattern of spore death.

To determine if meiosis I non-disjunction was contributing to spore death, the two-viable spore tetrads were analysed for disomy by using the centromere linked marker, *ADE1*, and mating type (Chapter 2, 2.9.5). The presence of increased pairs of spores with the same adenine phenotype is indicative of disomy of any one of the sixteen chromosome pairs (Tables 4.10 and 4.11). An increase in pairs of non-mating spores is specifically indicative of non-disjunction of chromosome III. More pairs of sister spores were obtained compared to non-sister spores in the *exo1Δ*, *mlh1Δexo1Δ* and *exo1-D173A* mutants (P < 0.007, χ^2 test). The *mlh1Δexo1Δ* mutant had much higher numbers of two viable spore tetrads due to the spore death attributable to the MMR defect of *mlh1Δ*. Surprisingly, *exo1-E150D* and *exo1-D171A* exhibited higher numbers of non-sister spores than sister spores, suggesting that perhaps meiosis I non-disjunction is not occurring in these mutants (P < 0.0002, χ^2 test). However, some pairs of non-mating spores were obtained in these mutants, suggesting that chromosome III is undergoing some non-disjunction (Table 4.11). The *exo1-S445A-F447A-F448A* mutant only had six two viable spore tetrads, consistent with the wild-type level of spore viability of the strain.

Table 4.10 Rates of Chromosome Non-Disjunction at Meiosis I

Genotype	No. Sister Spores[1]	No. Non-Sister Spores[1]	Total Tetrads	% Non-Disjunction[2]
exo1Δ	73	44*	871	3.3
mlh1Δexo1Δ	140	63*	969	7.9
msh4Δ	99	18*	1457	5.6
exo1-E150D	29	87*	888	0.0
exo1-D171A	34	72*	757	0.0
exo1-D173A	69	18*	677	7.5
exo1-S445A-F447A-F448A	5	1	478	0.8

[1] Number of two-viable spore tetrads with spores of the same adenine phenotype (sisters) or of different adenine phenotypes (non-sisters). * denotes a distribution of sister and non-sister spores which is significantly different to 50:50, determined using the χ^2 test.

[2] Meiosis I non-disjunction was calculated as:

((number of sister spores – number of non-sister spores) / total tetrads) x 100

The number of non-sister spores was subtracted to give an estimate of the number of sister spores arising due to meiosis I non-disjunction, not accumulation of haplolethal mutations.

Table 4.11 Meiosis I Non-Disjunction of Chromosome III

Genotype	No. Non-Maters[1]	Total Tetrads	% Non-Disjunction[2]
exo1Δ	0	871	0.0
mlh1Δexo1Δ	6	969	0.6
msh4Δ	15	1457	1.0
exo1-E150D	3	888	0.3
exo1-D171A	5	757	0.7
exo1-D173A	14	677	2.1
exo1-S445A-F447A-F448A	0	478	0.0

[1] Number of two-viable spore tetrads which are non-maters (indicative of meiosis I non-disjunction of chromosome III). These were all sister spores.

[2] Non-disjunction of chromosome III was calculated as:

(No. non-maters / total tetrads) x 100

The frequency of meiosis I non-disjunction was also compared amongst strains using the G-test (Tables 4.10 and 4.11), and P-values of 0.0085 or less were considered significant (for six way comparisons). The overall rate of non-disjunction in *exo1*Δ was not significantly different to that observed in *msh4*Δ (P = 0.012, χ^2 test). However, rates of non-disjunction can be under-estimated, as multiple non-disjunction events in a given meiosis will result in the death of all four spores within a tetrad. The *msh4*Δ mutant had 26.8% zero viable spore tetrads compared to 8.0% in *exo1*Δ (P = 3.23 x 10^{-31}, G-test), suggesting that more non-disjunction is occurring in *msh4*Δ than *exo1*Δ. Although the viability of *exo1-D173A* was better than the viability of *exo1*Δ, *exo1-D173A* exhibited a higher rate of meiosis I non-disjunction (P = 0.0002, G-test). It was surprising that non-disjunction of chromosome III was not observed in the absence of Exo1p. However, the *mlh1*Δ*exo1*Δ mutant only exhibited 0.6% non-disjunction of chromosome III, although the overall rate of non-disjunction in this mutant was 7.9%. If every pair of chromosomes has an equal probability of non-disjoining, then the expected rate of non-disjunction for chromosome III in the *mlh1*Δ*exo1*Δ mutant would be 0.49% (7.9% / 16 chromosomes), and this is equivalent to the rate observed. Therefore in the *exo1*Δ strain, only 0.2% non-disjunction of chromosome III would be expected. However, as smaller chromosomes have a higher rate of non-disjunction, this may be an under-representation. This suggests that the reduction in crossing over in the absence of Exo1p also affects chromosome segregation.

4.3.7 The Interaction Between Mlh1p and Exo1p is not Required for MMR

Exo1p has been shown to interact with MutLα, which is involved in MMR (Tran *et al.*, 2001). Therefore, the interaction of Mlh1p and Exo1p may be necessary for this function. To test this hypothesis, the mutator phenotype of the *exo1-S445A-F447A-F448A* mutant, which is unable to interact with Mlh1p, was analysed. Initially, a patch test was performed for wild-type, *exo1*Δ, *mlh1*Δ and *exo1-S445A-F447A-F448A* strains on lysine omission media and canavanine containing media (Chapter 2, 1.4). The *exo1-S445A-F447A-F448A* strain had slightly more papillations on the lysine omission media than the wild-type strain, although less than the *exo1*Δ strain (Figure 4.5). Therefore, fluctuation tests were performed to determine if there was a true quantitative difference. The strains were assayed for

reversion to lysine prototrophy and forward mutation to canavanine resistance (Tables 4.12 and 4.13, and Figures 4.6 and 4.7).

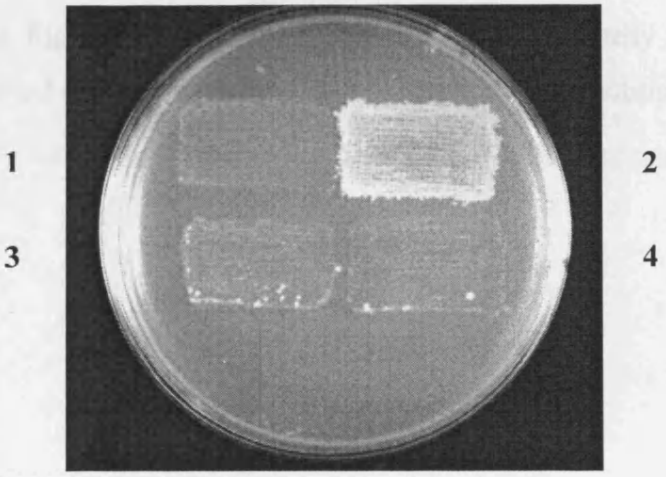


Figure 4.5 Papillations on Lysine Omission Media.
The visible colonies have reverted to lysine prototrophy.
1. Wild-type
2. *mlh1*Δ
3. *exo1*Δ
4. *exo1-S445A-F447A-F448A*

In comparing the wild-type, *exo1*Δ and *exo1-S445A-F447A-F448A* strains, a P-value of 0.0253 or less was considered significant for two-way comparisons. The *exo1*Δ mutant had higher mutation rates than the wild-type in both assays ($P = 1.6 \times 10^{-09}$ for both assays, contingency χ^2 test; Figures 4.6 and 4.7). This was also true for *exo1-S445A-F447A-F448A*, although only the mutation rate obtained in the forward mutation assay was significantly higher than the mutation rate in the wild-type ($P = 0.016$, contingency χ^2 test). The *exo1*Δ mutant exhibited a 5.7-fold increase in reversion to Lys^+ (Table 4.12 and Figure 4.6) and a 6.1-fold increase in mutation of the *CAN1* gene (Table 4.13 and Figure 4.7). However, the mutation rates of *exo1*Δ were also different to those exhibited by *exo1-S445A-F447A-F448A*. In the lysine reversion assay, the *exo1-S445A-F447A-F448A* mutant exhibited a 1.8-

fold increase in mutation rate which was significantly less than the increase observed in the *exo1*Δ strain ($P = 5.7 \times 10^{-08}$, contingency χ^2 test; Table 4.12 and Figure 4.6). In the forward mutation assay, the fold increase in mutation rate for the *exo1-S445A-F447A-F448A* mutant was not as great as that observed in the absence of *exo1*Δ (1.5-fold and 6.1-fold respectively; $P = 1.6 \times 10^{-09}$, contingency χ^2 test; Table 4.13 and Figure 4.7). This suggests that the majority of the mutator phenotype observed in *exo1*Δ is not due to a defect in the interaction between Exo1p and Mlh1p.

Table 4.12 Rates of Reversion to Lys$^+$

Genotype	Mutation Rates x 10^{-07}			Average Mutation Rate x 10^{-07} (Fold Increase)
	1	2	3	
Wild-type	1.82	2.27	0.38	1.49 (1.0)
exo1Δ	8.08	8.99	-	8.54 (5.7)
exo1-S445A-F447A-F448A	3.19	2.13	-	2.66 (1.8)

Mutation rates were calculated as described by Reenan and Kolodner (1992; Chapter 2, 2.13), and an average taken from two or three independent experiments.

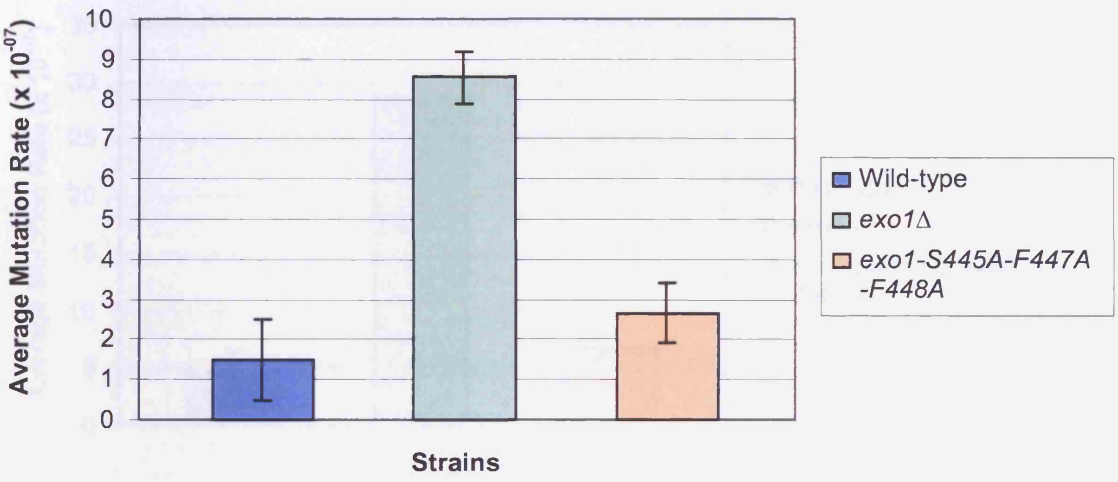


Figure 4.6 Reversion Rates in Wild-Type and Mutant Strains
The mutation rates are a measure of frameshift mutations in the *lys2*::insE-A_{14} allele that restore the reading frame and consequently function of *LYS2*, allowing growth on lysine omission medium. The error bars represent one standard deviation calculated from results of two or three independent experiments performed on each strain.

Table 4.13 Rates of Forward Mutation to Canavanine Resistance

Genotype	Mutation Rates x 10^{-07}			Average Mutation Rate x 10^{-07} (Fold Increase)
	1	2	3	
Wild-type	2.64	5.75	5.79	4.73 (1.0)
*exo1*Δ	29.2	28.2	-	28.7 (6.1)
exo1-S445A-F447A-F448A	7.59	6.28	-	6.94 (1.5)

Mutation rates were calculated as described by Reenan and Kolodner (1992; Chapter 2, 2.13), and an average taken from two or three independent experiments. The wild-type data was obtained from M. Almeida (unpublished).

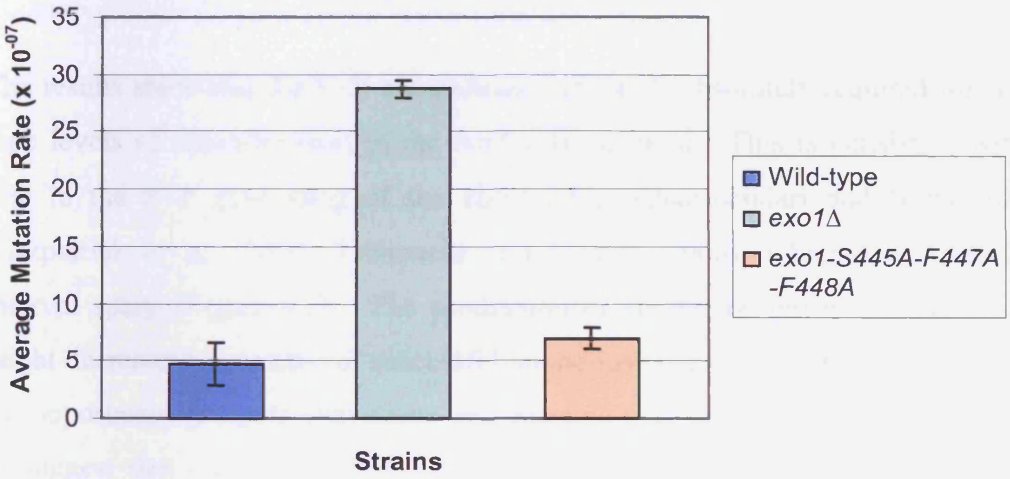


Figure 4.7 Forward Mutation Rates in Wild-Type and Mutant Strains
The mutation rates are a measure of frameshift and point mutations, which inactivate the *CAN1* gene and confer resistance to canavanine. The wild-type data was obtained from M. Almeida (unpublished). The error bars represent one standard deviation calculated from results of two or three independent experiments performed on each strain.

4.4 Discussion

4.4.1 The 5′-3′ Exonuclease and Flap Endonuclease Activities of Exo1p are Required for Crossing Over

The precise roles of Exo1p in meiotic recombination are unknown. However, the phenotypes exhibited in the absence of Exo1p suggest roles in the processing of DSB ends, and crossover formation (Khazanehdari and Borts, 2000; Kirkpatrick *et al.*, 2000; Tsubouchi and Ogawa, 2000). The mutant phenotypes observed could arise from the same basic defect in function or be a result of two independent functions of Exo1p. To increase the understanding of the roles of Exo1p, a number of specific nuclease and interaction mutants were studied (summarised in Table 7.1).

The results show that the 5′-3′ exonuclease activity is absolutely required for wild-type levels of recombination in the *NAT-HYG* interval. This is consistent with a role in the 5′-3′ processing of the *HIS4* DSB (Khazanehdari and Borts, 2000; Kirkpatrick *et al.*, 2000; Tsubouchi and Ogawa, 2000), which the *NAT-HYG* interval spans (Figure 4.2). The production of shorter or fewer 3′ ssDNA tails might decrease the number of successful strand invasions, and consequently reduce the opportunity for gene conversion and crossing over to occur. There is evidence to suggest that crossover events are more often associated with longer tracts of repair synthesis than non-crossovers are (Terasawa *et al.*, 2007). Less resection of a DSB would concomitantly reduce the amount of hDNA between homologues (and the amount of repair synthesis), and therefore may be more likely to be associated with non-crossovers. As gene conversion and crossing over is only reduced to approximately half of that observed in the wild-type, this suggests that there are other exonucleases that are required for DSB resection in addition to Exo1p (Khazanehdari and Borts, 2000; Kirkpatrick *et al.*, 2000; Tsubouchi and Ogawa, 2000). Additionally, not all DSBs are resected by Exo1p, illustrated by the fact that a reduction in crossing over is not observed in all of the intervals studied in the *exo1* mutant that is defective for the 5′-3′ exonuclease activity.

Differential effects on crossing over are displayed in the other genetic intervals

studied, which roughly correlate with the size of the interval. This may be due to the fact that the larger the interval is, the higher the probability is that it encompasses a DSB which is processed by Exo1p. The mutant lacking both nuclease activities is *exo1*Δ like for crossing over in all intervals studied, whilst retaining the flap endonuclease activity is sufficient to maintain crossing over in some intervals. This suggests that the flap endonuclease activity of Exo1p is required for crossing over independently of the requirement for the 5′-3′ exonuclease activity. One possibility is that this activity is required for the resolution of intermediates in a crossover configuration. Abdullah *et al.* (2004) have hypothesised that Exo1p is involved in the removal of DNA flaps that are produced when DNA synthesis primed by the 3′ invading end of the DSB is not terminated correctly (Abdullah *et al.*, 2004). Therefore it is possible that flap production occurs frequently at some loci, and these are dependent on Exo1p for removal (See Chapter 7). These results collectively indicate that both of the exonuclease activities are required to maintain wild-type levels of crossing over, either directly or indirectly.

In some intervals, crossing over is more severely reduced in the absence of Exo1p than it is in the absence of Mlh1p (Meadows, Hoffmann and Borts, unpublished). This suggests that although Exo1p and Mlh1p function in the same pathway (this study), Exo1p has additional functions to Mlh1p. Although Mlh1p and Exo1p may function together to promote crossovers, the role of Exo1p in processing of DSBs is likely to be independent of Mlh1p. Additionally this suggests that Exo1p initially functions earlier than Mlh1p, although has a later role also, alongside Mlh1p.

4.4.2 The Presence of Mutant Exo1p Protein Improves Viability Compared to the Complete Absence of the Protein

Spore viability is reduced significantly in the absence of Exo1p compared to the wild-type (Meadows, Hoffmann and Borts, unpublished data and Hoffmann and Borts, unpublished data), as has been observed previously (Khazanehdari and Borts, 2000; Kirkpatrick *et al.*, 2000; Tsubouchi and Ogawa, 2000). The *exo1* point mutants, including *exo1-D173A* that lacks both nuclease activities, have better viability than in the complete absence of Exo1p. This suggests that having the protein, although defective, is better than having no Exo1p. In support of this it has

been shown that the hFEN1 D181A mutant, which is equivalent to yeast *exo1-D173A*, retains the ability to bind to DNA (Shen *et al.*, 1997) and an interaction has been demonstrated between yeast *exo1-D173A* and Msh2p (Sokolsky and Alani, 2000). The interaction domains for Mlh1p and Msh2p within Exo1p overlap (reviewed in Tran *et al.*, 2004), and as the interaction with Msh2p is unaffected, it is possible that the interaction with Mlh1p is also not directly affected although this has not been tested. However, if the mutation alters the conformation of the protein this may also disrupt the interaction. Therefore, it may be that the mere presence of the protein, although non-functional, stabilises complex formation, as was shown for MMR (Amin *et al.*, 2001), and/or can still recruit Mlh1p or Msh2p to the DNA via their interaction, enabling some functionality to be rescued. The *mlh1Δexo1Δ* double mutant has a viability worse than expected for them functioning in the same pathway, but not as severe as expected for completely independent roles. This indicates that Exo1p and Mlh1p function in the same pathway, but additionally one either has a role independent of the other or they can partially compensate for one another in a given function. This is consistent with the crossover data. As discussed earlier, an Mlh1p-independent role of Exo1p is probably in the resection of DSBs, whereas more spore death will occur in the absence of Mlh1p due to a defect in mitotic MMR.

4.4.3 Increased Death of Two, Three and Four Spores in the Absence of Exo1p

In the absence of Exo1p, an increase in two and zero viable spore tetrads was observed, which is indicative of meiosis I non-disjunction (Khazanehdri and Borts, 2000; Meadows, Hoffmann and Borts, unpublished). This is expected due to the decrease in crossovers, which will reduce interactions between homologous chromosomes. In contrast, the *exo1* point mutants cause an increase in the two viable spore tetrad class but not the zero viable spore tetrad class. One explanation for this is that the mutants cause some non-disjunction but it is not widespread enough to result in the presence of multiple disomes per tetrad. This correlates with *exo1-E150D* and *exo1-D171A* not reducing crossovers in all of the intervals.

Specifically, a higher frequency of sister spores than non-sister spores in the two viable spore tetrad class, as determined by using a centromere marker, is indicative of meiosis I non-disjunction. Interestingly, whereas *exo1Δ* and *exo1-D173A* did

produce more sister spores than non-sisters spores, the opposite was observed in the *exo1-E150D* and *exo1-D171A* mutants. This suggests that there was a defect in the separation of non-sister chromatids, and this may be due to the formation of an abnormal recombination intermediate that cannot be resolved in these mutants.

An increase in three viable spore tetrads was also observed in *exo1*Δ (Meadows, Hoffmann and Borts, unpublished). Two chromosome segregation defects result in the death of a single spore. At the first division of meiosis, precocious separation of sister chromatids (PSSC) can occur, and at meiosis II the sister chromatids can fail to segregate (meiosis II non-disjunction). No evidence for PSSC has been found in the absence of Exo1p (Khazanehdari and Borts, 2000). Instead a third possibility was proposed, whereby death of a single spore occurs due to the presence of an unrepaired DSB (Khazanehdari and Borts, 2000). We cannot distinguish between these possibilities with the current data. However, given the evidence that suggests Exo1p is involved in resection of some DSBs, we favour the unrepaired DSB hypothesis. Additionally, in the *exo1-E150D* mutant that is defective in 5′-3′ processing, death of a single spore is also elevated (this study). This supports the above hypothesis, as reduced processing of DSBs could lead to less efficient repair as a result of instability in DNA interactions.

4.4.4 Reduced Resection of DSBs Affects Non-Mendelian Segregation

It has been shown previously that the absence of Exo1p reduces NMS at *HIS4*, but does not increase PMS events, indicating that Exo1p does not function in the repair of mismatches arising during meiosis (Khazanehdari and Borts, 2000; Meadows, Hoffman and Borts, unpublished). This was also observed in the mutant defective for the 5′-3′ exonuclease activity (E150D), indicating that this activity, and not the flap endonuclease activity, is necessary to generate these events. Less resection would reduce the frequency with which the *his4-ATC* mismatch would become incorporated into hDNA and as a result there would be less opportunity for gene conversion to occur. This phenomenon was observed at *his4-ATC* but not *met13-2*, consistent with previous studies in suggesting that Exo1p is only involved in processing a subset of DSBs (Khazanehdari and Borts, 2000; Kirkpatrick *et al.*, 2000). However, the *met13-2* allele is a C to A change at position 278 in *MET13* (Abdullah *et al.*, 2004). Therefore, given that gene conversion gradients are

observed at a number of loci (Alani *et al.*, 1994; White *et al.*, 1992), another possibility is that the mismatch in this allele is too far away from the initiating DSB. The frequency of NMS observed at this allele in the wild-type is small (4.5%; Hoffmann *et al.*, 2005). Therefore even if Exo1p does reduce the frequency, the numbers may be too small to detect a significant difference. It has been demonstrated previously that Mlh1p is involved in repair of mismatches during meiosis (Hoffmann *et al.*, 2003; Hunter and Borts, 1997; Prolla *et al.*, 1994; Reenan and Kolodner, 1992), and at *HIS4* total NMS events actually increase (Hoffmann *et al.*, 2005) in these mutants. These increases occur due to the uncovering of events that would normally be restored to a 2:2 segregation in MMR-proficient cells (Hoffmann *et al.*, 2005). The additional events observed in *mlh1*Δ are dependent on the presence of Exo1p, as this increase was not observed in the *mlh1*Δ*exo1*Δ double mutant. In the absence of Exo1p, there are fewer mismatches present to be repaired by Mlh1p due to the reduced processing of the *HIS4* DSB. Therefore, in the double mutants less of a defect is observed. The disparity observed between 3:5 and 5:3 PMS events in the absence of Mlh1p was proposed to be due to restoration of G-G mismatches through an alternative pathway (Hoffmann *et al.*, 2005). In the combined *mlh1*Δ*exo1*Δ mutant this disparity remains, providing further support that Exo1p does not function in either of the meiotic mismatch repair pathways.

The association of NMS events at *his4-ATC* with crossovers in the *NAT-HYG* interval was reduced in the *mlh1*Δ mutant, possibly due to the increase in NMS events observed in the absence of Mlh1p. The extra NMS events detected are from events that would normally undergo restoration in the wild-type. This suggests that mismatches associated with non-crossover events arising through the synthesis-dependent strand annealing (SDSA) pathway may preferentially be repaired as a restoration rather than a conversion. The association of NMS events at *his4-ATC* with crossovers in the *NAT-HYG* interval is not affected in the absence of Exo1p. This suggests that Exo1p is not involved in making the crossover/non-crossover decision. As mentioned earlier, it has been shown that crossovers are more likely to be associated with long tracts of hDNA (Terasawa *et al.*, 2007). Intermediates produced whereby resection reaches and/or extends further than the *his4-ATC* mismatch, which will consequently give rise to an NMS event, may have substantial amounts of hDNA present. Even in the absence of Exo1p, Exo1p-independent DSB

resection can lead to the production of long tracts of hDNA and consequently stable intermediates, resulting in crossover formation. Therefore, the observations made here are consistent with those of Terasawa *et al.* (2007). The association of crossovers with hDNA tracts of differing lengths will be tested further in Chapter 6. Although Exo1p does not function in the repair of mismatches during meiosis, the mutant *exo1-D173A* does reduce the efficiency of repair of the *his4-ATC* allele. As mentioned earlier, it is possible that *exo1-D173A* is still interacting with DNA, potentially for a prolonged period of time as it can't perform its normal function. If it binds in close proximity to any mismatches present, it could interfere with the normal repair process by blocking access to the mismatches. Exo1p has been shown to interact with Msh2p (Tishkoff *et al.*, 1997a), which is known to function in meiotic repair, as *msh2*Δ strains exhibit levels of PMS events similar to those observed in *pms1*Δ and *mlh1*Δ strains (Hoffmann *et al.*, 2003; Reenan and Kolodner, 1992). The *exo1-D173A* mutant retains the ability to interact with Msh2p (Sokolsky and Alani, 2000) and therefore another possibility is that *exo1-D173A* is sequestering away Msh2p from forming an active repair complex with functional proteins. However, it is unclear why Exo1p and Msh2p would be interacting during meiosis. Consistent with this hypothesis, deleting *EXO1* in an *mlh1*Δ background improves repair, suggesting that the presence of Exo1p normally inhibits this process. Potentially in the absence of Exo1p, a MMR-independent process can repair these mismatches, which would normally be obscured from detection. These effects are all specific to *HIS4*, as the same effects were not observed for the *met13-2* allele.

4.4.5 The *exo1-D171A* Mutant Does Not Mimic *exo1-D173A*

The mutations D171A and D173A are in close proximity in a region of highly conserved residues and therefore they might be expected to disrupt similar functions. However, this is not reflected in their phenotypes. Instead, in terms of proficiency for crossing over, *exo1-D171A* resembles *exo1-E150D* more closely than *exo1-D173A*. This suggests that *exo1-D171A* possesses some flap endonuclease activity like *exo1-E150D*, whilst still being defective for the 5′-3′ exonuclease activity. Any slight differences in phenotypes between *exo1-E150D* and *exo1-D171A* may be due to differences in their respective levels of nuclease activity. As expected, the production of non-Mendelian segregation events, which

requires the 5′-3′ exonuclease activity of Exo1p, was affected to the same extent in all three point mutants. Together these data indicates that residue D171 is less critical for the flap endonuclease activity of Exo1p than for the 5′-3′ exonuclease activity, unlike residue D173 which is necessary for both of these activities.

4.4.6 Disruption of the Interaction Between Mlh1p and Exo1p

Mutating the residues that are required for the interaction of Mlh1p with Exo1p does not have an effect on the levels of crossing over, meiotic repair or spore viability. Therefore, either these proteins do not interact during meiotic recombination, or their interaction is not critical for these functions. It is possible that Exo1p and Mlh1p interact only during MMR (Tran *et al.*, 2001), and although they appear to function in the same pathway for meiotic recombination, there may be no direct link between their roles. A third possibility is that Mlh1p and Exo1p do form a complex in conjunction with other proteins, and these other components are sufficient to stabilise the complex and keep Mlh1p and Exo1p together. However, this would suggest that the interaction between Mlh1p and Exo1p is only required for recruitment purposes and/or stabilisation of complexes, rather than for a specific function. A number of mutations in *hMLH1* (T117M, Q426X and 1813insA) identified in HNPCC patients were also shown to abolish the interaction between hMLH1 and hEXO1 (Jager *et al.*, 2001). The equivalent residue to T117M in yeast *MLH1* was mutated and shown to have no effect on crossing over (Hoffmann *et al.*, 2003). Together these results suggest that the interaction between Mlh1p and Exo1p is not essential for meiotic recombination.

In addition, mutating the Mlh1p interaction domain of Exo1p did not cause any major defects in MMR. A modest increase in mutation rate compared to wild-type was only observed in the forward mutation assay. This was not surprising as the majority of the mutator phenotype of *exo1*Δ is from the PRR pathway, not MMR (Tran *et al.*, 2001; Tran *et al.*, 2007), and the interaction of Exo1p with Mlh1p is only likely to be important for MMR. This is in agreement with the results of a recent study by Tran *et al.* (2007), which used an *exo1-FF447AA* mutant that was defective for interaction with Mlh1p as demonstrated by yeast-two hybrid (Gellon *et al.*, 2002; Tran *et al.*, 2007). This mutant did not display a mutator phenotype, and was able to complement the phenotype of an *exo1*Δ strain in a forward mutation

assay (Tran *et al.*, 2007). This suggests that either this interaction is not required for the proteins to perform their normal function, or despite this interaction being disrupted, the complex involved still forms normally. Therefore it is possible that in this situation Exo1p plays a structural role only. That Exo1p plays a structural role has been postulated previously, as some mutations of *MLH1* and *PMS1* show an *exo1*-dependent mutator phenotype (Amin *et al.*, 2001). Furthermore, other proteins form complexes with MutLα to carry out MMR, for example MutSα (Msh2p and Msh6p) and MutSβ (Msh2p and Msh3p), which also interact with Exo1p (Tishkoff *et al.*, 1997a). Therefore if this mutant is still capable of binding to Msh2p, then this interaction may be sufficient to recruit Mlh1p to the complex. Additionally, overexpression of *EXO1* has been shown to partially suppress the temperature sensitive mutator phenotype of an *msh2* mutant, *msh2-L560S* (Sokolsky and Alani, 2000). The *msh2-L560S* also co-immunoprecipitated with Exo1p, suggesting that their interaction is unaffected (Sokolsky and Alani, 2000). Therefore, the interaction between Exo1p and Msh2p may be the important interaction for stabilisation of complexes. This may explain why disrupting the interaction of Mlh1p and Exo1p does not have a major detrimental effect on MMR. Additionally, Tran *et al.* (2007) also demonstrated that combining *exo1-FF447AA* with *mlh1-E31A* (an ATPase mutant) resulted in a synergy with respect to mutator phenotype. This is consistent with the hypothesis that the interaction of Exo1p with MutLα is necessary to provide structural support for complexes, as suggested previously (Amin *et al.*, 2001; Tran *et al.*, 2002; Tran *et al.*, 2007), as introducing additional instability by means of mutation has a detrimental effect. This suggests that the inability to interact with Mlh1p may be destabilising complexes but does not directly disrupt functions.

4.5 Future Work

It is possible that Mlh1p and Exo1p are present in the same complex during meiosis, and it is through the actions of other components of this complex that function is not disturbed in the *exo1* mutant that is unable to interact with Mlh1p. In MMR, combining nuclease deficient mutants of Exo1p with an ATPase mutant of Mlh1p, *mlh1-E31A*, resulted in a synergistic effect on mutator phenotype (Tran *et al.*, 2002), and this was also observed when using an Mlh1p interaction mutant of Exo1p (Tran *et al.*, 2007). This shows that combining two mutant proteins that may be present in a complex together can result in further destabilisation of that complex. The *mlh1-E31A* ATPase mutant does not exhibit a defect in crossing over or hDNA repair during meiosis (Hoffmann *et al.*, 2003), as was observed for the *exo1-S445A-F447A-F448A* interaction mutant in this study. However, combining the Mlh1p-interaction deficient *exo1* and *mlh1-E31A* mutants to study recombination may reveal more about the nature of the relationship between Mlh1p and Exo1p during meiosis.

Additionally, the *mlh1*Δ*exo1*Δ mutant exhibits better repair of mismatches than *mlh1*Δ alone. We propose that Exo1p normally blocks MMR-independent repair in the absence of Mlh1p, such that upon the additional deletion of Exo1p, this repair can occur. To determine if this is true, the *exo1-D173A* mutant, which is defective for the 5′-3′ exonuclease and the flap endonuclease activities, could be combined with *mlh1*Δ. The *exo1-D173A* mutant is still structurally intact, and therefore would still be capable of blocking repair, if this is indeed what is happening. Consequently, the levels of repair should resemble those in the single *mlh1*Δ mutant. This hypothesis is supported by the fact that *exo1-D173A* already displays some reduction in repair compared to *exo1*Δ.

We would also like to measure the length of resection that occurs at the *HIS4* DSB. This would be possible using the *HIS4-Hha*I allele, which is positioned at 1605 bp into *HIS4* (Figure 4.2). The frequency of gene conversion events occurring at this downstream allele would indicate how frequently this marker is included in hDNA. Determining how frequently this marker is converted in an *exo1*Δ mutant would provide information on whether resection of some, or all DSBs are affected.

Chapter 5: Modulation of Non-Mendelian Segregation Events at *HIS4*

Chapter 5: Modulation of Non-Mendelian Segregation Events at *HIS4*

5.1 Introduction

5.1.1 Exo1p Functions in Resection of Some Double-Strand Breaks

Exo1p is involved in the 5′-3′ resection of the *HIS4* double-strand break (DSB), as discussed in Chapter 4. However, in the absence of Exo1p, some processing still occurs, albeit delayed (Tsubouchi and Ogawa, 2000) suggesting that there are additional exonucleases that can perform this function. Furthermore, preliminary data suggests that Exo1p may only process a subset of DSBs at *HIS4*, as non-Mendelian segregation (NMS) is only reduced by approximately 50% in the absence of Exo1p (Khazanehdari and Borts, 2000; Meadows, Hoffmann and Borts, unpublished). We were interested in studying the contribution Exo1p makes to the resection of DSBs, however, given that only 14% NMS of the *his4-ATC* allele is observed in the wild-type, the number of Exo1p-dependent events available to study is limited. Consequently it was of interest to try and use phenotypic modifications to increase the initiation of events occurring at *HIS4* (Abdullah and Borts, 2001), to enable the easier study of the functions of Exo1p at this locus.

5.1.2 Double-Strand Break Hotspot Activity in Meiosis is Affected by Transcription Factors

Chromosomal regions exist that display particularly high or low levels of meiotic recombination (hotspots and coldspots respectively; reviewed in Petes, 2001). Hotspots are associated with DNA DSBs, which are the initiators of recombination (Wu and Lichten, 1994). However, hotspots are not all defined by the same characteristics, but rather are grouped into three types; α, β and γ hotspots. A common feature to all hotspots is the requirement for an open chromatin structure to allow recombination factors access to the DNA, but this is achieved in different ways (reviewed in Petes, 2001). α-hotspots require the presence of transcription factors for activation, although they do not require transcription *per se*. Deletion of the TATA box upstream of the *HIS4* α-hotspot produced a 20-fold reduction in expression levels but hotspot activity was unaffected (White *et al.*, 1992). To

enable binding of transcription factors, the DNA must be unwound from nucleosomes, and the consequent transcription factor binding maintains an open state. Maintaining an open chromatin state should allow an increase in the frequency of DSB formation in that region, consequently increasing recombination events associated with that DSB. It is also possible that the transcription factors are directly involved in the recruitment of recombination factors (reviewed in Petes, 2001). β-hotspots occur at sites with tandem repeats of a specific motif, 5′-CCGNN, which has been shown to prevent the establishment of nucleosomes (Kirkpatrick *et al.*, 1999; Wang and Griffith, 1996). There is also evidence that poly(A) and poly(T) sequences can block nucleosome formation (Struhl, 1985). The third type of hotspot, γ hotspots, occur at regions with a high content of G and C bases (Gerton *et al.*, 2000), although it is unknown how this creates an 'open' configuration.

Meiotic hotspots that are dependent on transcription factors for their activity have also been identified in *Schizosaccharomyces pombe.* The *ade6-M26* mutation was shown to increase gene conversion and meiotic crossing over several fold over other mutations in *ade6* (Gutz, 1971; Schuchert and Kohli, 1988). The mutation identified in *ade6* was a G to T change (Ponticelli *et al.*, 1988), and this produces a sequence motif, 5′-ATGACGT-3′ (Schuchert *et al.*, 1991), that creates a meiotic recombination hotspot (Ponticelli *et al.*, 1988; Schuchert and Kohli, 1988). The transcription factors, Atf1 and Pcr1 bind to this sequence motif (Wahls and Smith, 1994), and are absolutely required for hotspot activity (Kon *et al.*, 1997). It has also been demonstrated that upon entry to meiosis, the chromatin configuration around the *ade6-M26* hotspot is altered (Mizuno *et al.*, 1997), becoming more open to allow proteins access to the DNA, as in *S. cerevisiae* (Wu and Lichten, 1995). Hotspot activity of *ade6-M26* has also been shown to require Rec10, a component of linear elements in *Sz. pombe*, indicating that as in *S. cerevisiae* there is interaction between elements of recombination and chromosome structure (Pryce *et al.*, 2005).

5.1.3 Transcriptional Activation of *HIS4*

HIS4 exhibits high levels of meiotic recombination and NMS (Detloff *et al.*, 1992; White *et al.*, 1992; White *et al.*, 1993), and this hotspot activity is controlled by

transcription factors, specifically Bas1p, Bas2p (**Bas**al), Rap1p (**R**epressor **A**ctivator **P**rotein) and Gcn4p (**G**eneral **C**ontrol **N**on-derepressible; Arndt and Fink, 1986; Devlin *et al.*, 1991; Tice-Baldwin *et al.*, 1989; White *et al.*, 1993). However, there are two independent control systems involved in the regulation of *HIS4*; general control and basal control. Gcn4p is involved in the general control and up-regulates expression of genes involved in several amino acid biosynthesis pathways in response to amino acid starvation (reviewed in Hinnebusch, 2005). Bas1p and Bas2p are responsible for the basal expression level of *HIS4* and in addition, they further stimulate transcription of *HIS4* under conditions of starvation for either adenine or phosphate (Arndt *et al.*, 1987; Tice-Baldwin *et al.*, 1989). Cells lacking Gcn4p, Bas1p and Bas2p are auxotrophic for histidine, demonstrating the necessity of their presence for histidine biosynthesis (Arndt *et al.*, 1987). Additionally, mutation of the binding sites of Rap1p or of Bas1p/2p results in the loss of a detectable DSB at *HIS4* (Fan *et al.*, 1995). Rap1p binding is not sufficient to activate *HIS4* alone, but is required by both Gcn4p and Bas1p/2p, to open up the chromatin structure and allow access to their respective binding sites (Devlin *et al.*, 1991). Bas1p and Gcn4p have the same consensus binding site, TGACTC (Arndt and Fink, 1986; Daignan-Fornier and Fink, 1992; Mieczkowski *et al.*, 2006) and this sequence appears five times in the promoter region of *HIS4* (Figure 5.1). These sites are designated A-E (Arndt and Fink, 1986). Bas1p only binds two of these sites, A and B (Tice-Baldwin *et al.*, 1989), indicating that other factors besides sequence define a Bas1p binding site. Gcn4p binds all five sites, but has greater affinity for site C (Arndt and Fink, 1986). Site C also overlaps the Rap1p binding site (Arndt and Fink, 1986; Devlin *et al.*, 1991) and these two factors bind competitively. This site is usually occupied by the protein that is being expressed at the highest level (Arndt and Fink, 1986). Mutating binding site C to affect binding of Gcn4p but not Rap1p, in a *bas1-2 bas2-2* double mutant resulted in an inability to synthesise histidine (Devlin *et al.*, 1991). This demonstrates that this binding site is crucial for the transcriptional activation of *HIS4* by Gcn4p.

5.1.4 The Absence of Adenine Promotes the Interaction of Bas1p with Bas2p

Bas1p is a myb-related protein that interacts with DNA via its N-terminal myb-domain (Hovring *et al.*, 1994), and Bas2p is a homeodomain protein (Burglin, 1988). All ten steps of the adenine biosynthesis pathway are regulated by Bas1p/2p

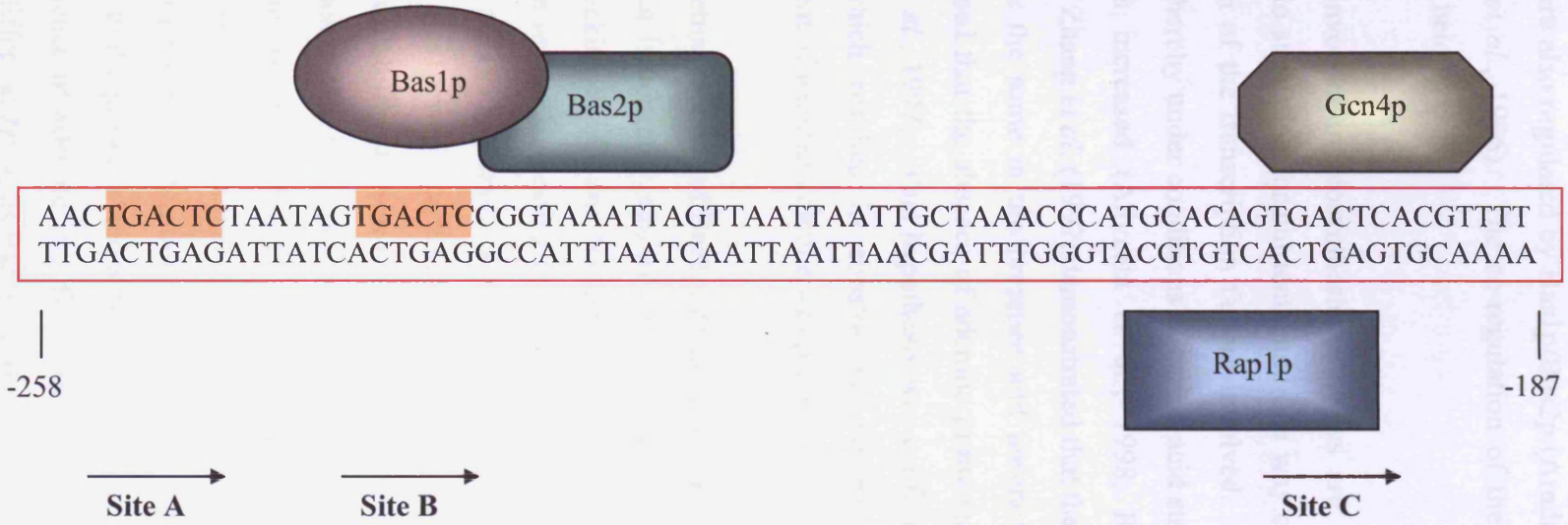


Figure 5.1 Transcription Factor Binding Sites in the *HIS4* Promoter
Binding sites A, B and C are shown. Sites D and E are situated more proximal to *HIS4*, and are sites to which only Gcn4p binds. The sequence of sites D and E also differs from the consensus by a single base (Arndt and Fink, 1986). Bas1p and Gcn4p are shown at the sites for which they have the highest affinity. However, Gcn4p does bind to all five sites (A-E), whereas Bas1p binds to sites A and B. The Rap1p binding site overlaps with site C. The numbers are the positions relative to the ATG start codon of *HIS4*.
Adapted from Devlin *et al.* (1991).

in response to adenine availability (Daignan-Fornier and Fink, 1992; Denis *et al.*, 1998; Figure 5.3). In addition, *HIS1*, *HIS4* and *HIS7* in the histidine biosynthesis pathway are also regulated by Bas1p/Bas2p (Arndt *et al.*, 1987; Denis *et al.*, 1998; Springer *et al.*, 1996). The co-regulation of these pathways and others will be discussed below.

Proteins involved in biosynthesis pathways are upregulated and/or activated in response to starvation for nutrients, and one way of achieving this is to control the expression of the transcription factors involved. This has been demonstrated for Gcn4p, whereby under conditions of amino acid starvation, translation of the *GCN4* mRNA is increased (Albrecht *et al.*, 1998; Rolfes and Hinnebusch, 1993). However, Zhang *et al.* (1997) demonstrated that the level of expression of *BAS1* and *BAS2* was the same in the presence and absence of adenine. Therefore it was hypothesised that the absence of adenine promoted the interaction of these factors (Zhang *et al.*, 1997). This hypothesis was confirmed using a fusion of Bas1p and Bas2p, which resulted in constitutive expression of an *ADE1-lacZ* reporter, independent of whether adenine was present or absent (Pinson *et al.*, 2000).

Using deletion mutants of Bas1p, Zhang *et al.* (1997) identified a domain within the protein that is normally masked by the C- and N-termini of Bas1p. Furthermore, mutants lacking the C- and N- terminal domains resulted in activation independent of adenine levels, suggesting that this unmasking is the step regulated by adenine (Zhang *et al.*, 1997). Residues 630-664 (**Bas1p Interaction and Regulatory Domain**; BIRD) in Bas1p were identified as being critical to this regulation in response to adenine availability (Pinson *et al.*, 2000; Figure 5.2). In the presence of adenine, Bas1p binds to the *ADE1* promoter and BIRD represses the transactivation domain of Bas1p to prevent activation. In the absence of adenine, Bas2p is recruited to the promoter and complex formation with Bas1p results in a conformational change and exposure of the transactivation domain. Although Bas1p binds to the *ADE* gene promoters in the presence and absence of adenine, binding is increased substantially in the absence of adenine in a Bas2p-dependent manner (Som *et al.*, 2005). The situation differs at *HIS4*, as Bas1p needs to interact with Bas2p to be able to bind to the *HIS4* promoter at all (Pinson *et al.*, 2000). Therefore, even in the presence of adenine some interaction must occur between Bas1p and Bas2p to allow promoter

binding and basal expression of *HIS4*. However, in the absence of adenine, the transactivation domain of Bas1p is exposed allowing a fuller interaction between Bas1p and Bas2p, and BIRD behaves as an activator. Therefore, promoter specific regulation occurs, where BIRD mediates interactions (either through repression or activation) in response to adenine levels.

In the absence of adenine, derepression of the *ADE* genes occurs through activation of the Bas1p-Bas2p complex as discussed above. This pathway is negatively regulated by the end product, in that the presence of ADP and ATP inhibits the activity of the first enzyme in the pathway, Ade4p (Figure 5.3; Guetsova *et al.*, 1997). The interaction between Bas1p and Bas2p is promoted by the presence of the pathway intermediates 5-amino-1-(5′-phosphoribosyl)-imidazole-4-carboxyamide (AICAR) and 1-(5′-phosphoribosyl)-4-(*N*-succinocarboxyamido)-5-aminoimidazole (SAICAR), not adenine itself (Rebora *et al.*, 2001; Rebora *et al.*, 2005). This was determined by measuring the ability of various *ade* mutants to activate expression of an *ADE1-lacZ* fusion construct present on a plasmid (Rebora *et al.*, 2001). Mutating the first seven genes in the adenine pathway (*ADE4* to *ADE1*; Figure 5.3) resulted in very low expression of the reporter whereas mutating *ADE13* or *ADE17* resulted in constitutive expression. This indicated that the first seven steps were involved in the production of an intermediate necessary for the activation of this pathway, whereas mutating the later steps resulted in accumulation of the required product(s), due to the prevention of further processing (Rebora *et al.*, 2001). When AICAR and SAICAR are only present at low levels, the *ADE* genes are expressed at a basal level (reviewed in Rolfes, 2006). It is unknown if these intermediates are involved directly, or whether further processing occurs to form another intermediate, or that another as yet unidentified protein is activated to promote the interaction between Bas1p and Bas2p (Rebora *et al.*, 2001; Rebora *et al.*, 2005).

5.1.5 Co-Regulation of the Adenine and Histidine Biosynthesis Pathways

In addition to the regulation of the *ADE* genes in response to purine limitation (Daignan-Fornier and Fink, 1992; Denis *et al.*, 1998), the expression of a number of other genes are co-regulated by Bas1p/2p. In addition to *HIS4* (Arndt *et al.*, 1987; Tice-Baldwin *et al.*, 1989), *HIS1* (Denis *et al.*, 1998) and *HIS7* (Springer *et al.*,

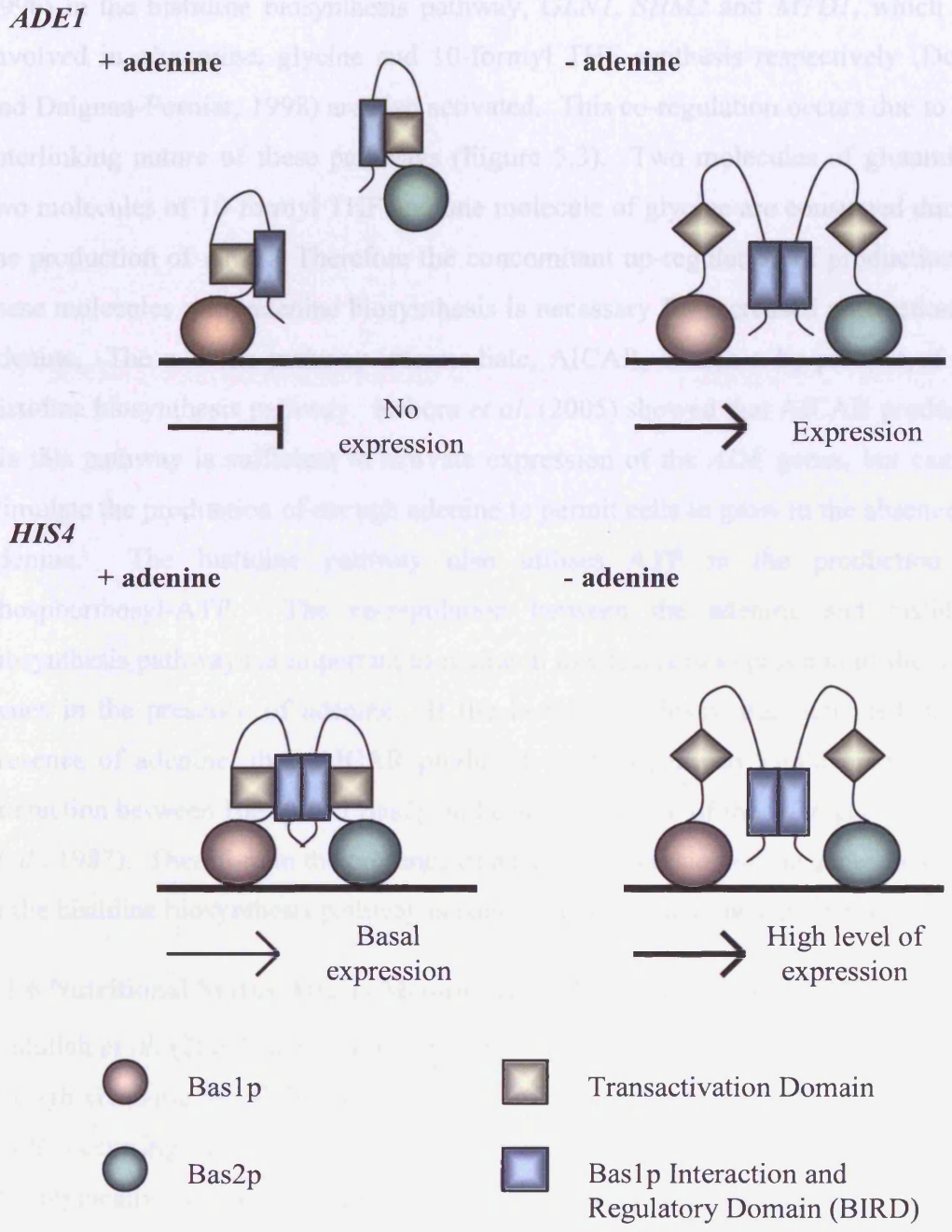


Figure 5.2 The Adenine Regulated Interaction of Bas1p and Bas2p.
ADE1: in the presence of adenine, Bas1p is bound to the promoter but Bas2p is not recruited. The transactivation domain is masked and in this case represses activity. In the absence of adenine, interaction of Bas1p and Bas2p causes the proteins to undergo a conformational change, resulting in exposure of the transactivation domain, allowing full activation.
HIS4: in the presence of adenine, low levels of Bas1/Bas2p bind to the promoter, and although the activation domain is still repressed there is a low (basal) level of expression. In the absence of adenine, more Bas1p/2p complexes are recruited and further activation occurs through unmasking of the activation domain, mediated by BIRD.
Adapted from Pinson *et* al., 2000 and Som *et al.*, 2005.

1996) in the histidine biosynthesis pathway, *GLN1*, *SHM2* and *MTD1*, which are involved in glutamine, glycine and 10-formyl THF synthesis respectively (Denis and Daignan-Fornier, 1998) are also activated. This co-regulation occurs due to the interlinking nature of these pathways (Figure 5.3). Two molecules of glutamine, two molecules of 10-formyl THF and one molecule of glycine are consumed during the production of AMP. Therefore the concomitant up-regulation of production of these molecules with adenine biosynthesis is necessary for increased production of adenine. The adenine pathway intermediate, AICAR, is also a by-product of the histidine biosynthesis pathway. Rebora *et al.* (2005) showed that AICAR produced via this pathway is sufficient to activate expression of the *ADE* genes, but cannot stimulate the production of enough adenine to permit cells to grow in the absence of adenine. The histidine pathway also utilises ATP in the production of phosphoribosyl-ATP. The co-regulation between the adenine and histidine biosynthesis pathways is important to maintain low levels of expression of the *ADE* genes in the presence of adenine. If the histidine pathway was activated in the presence of adenine, then AICAR produced via this pathway could promote the interaction between Bas1p and Bas2p and cause activation of the *ADE* genes (Arndt *et al.*, 1987). Therefore in the presence of adenine, expression of the genes involved in the histidine biosynthesis pathway is concomitantly maintained at a basal level.

5.1.6 Nutritional Status Affects Meiotic Recombination Events at *HIS4*

Abdullah *et al.* (2001) demonstrated that by altering the metabolic state of the cell, through changing the ability to synthesise leucine, lysine and adenine, the level of NMS occurring at *HIS4* could be increased. Specifically, cells that were phenotypically Leu^+ Lys^- exhibited the highest levels of NMS at *HIS4*, and Leu^- Lys^+ cells exhibited the lowest (Abdullah and Borts, 2001). NMS increased even further to 37-40% in cells that were auxotrophic for adenine, and this was independent of leucine and lysine phenotype. In Ade^+ cells, deletion of *GCN4*, which is upregulated in response to amino acid starvation (reviewed in Hinnebusch, 2005), resulted in a decrease in NMS to approximately the same level as was observed in the Leu^- Lys^+ strain. This suggests that this is the basal level of NMS obtained in the absence of Gcn4p activation. However, even in the absence of Gcn4p, Ade^- cells still exhibited an increase in NMS compared with Ade^+ cells. Therefore, the number of NMS events at *HIS4* can be modulated genetically by

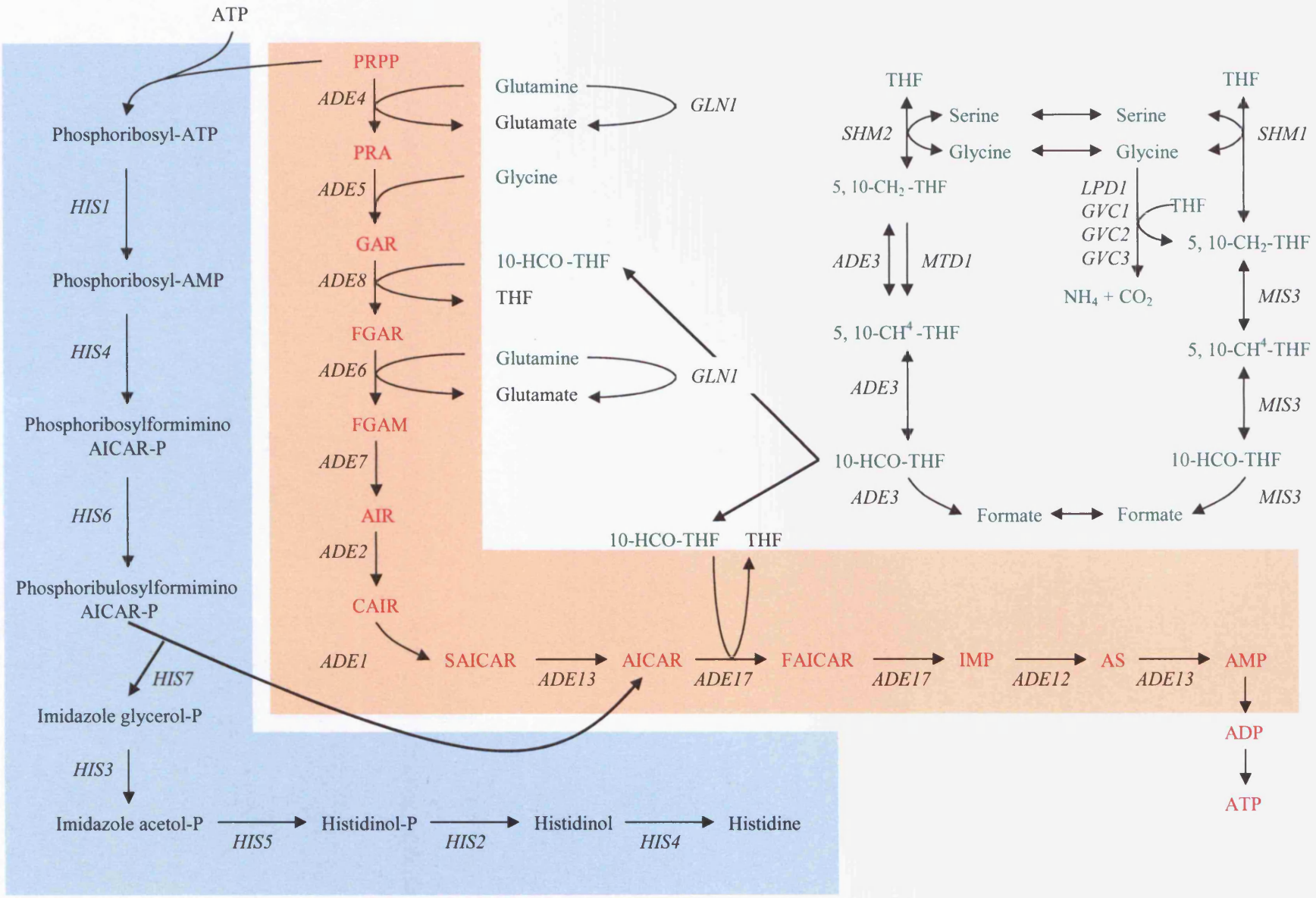

ATP
PRPP
ADE4
PRA
ADE5
GAR
ADE8
FGAR
ADE6
FGAM
ADE7
AIR
ADE2
CAIR
ADE1
SAICAR
ADE13
AICAR
ADE17
FAICAR
ADE17
IMP
ADE12
AS
ADE13
AMP
ADP
ATP
Glutamine
Glutamate
GLN1
Glycine
10-HCO-THF
THF
Glutamine
Glutamate
GLN1
10-HCO-THF THF
THF
SHM2
Serine
Glycine
5, 10-CH2-THF
ADE3
MTD1
5, 10-CH+-THF
ADE3
10-HCO-THF
ADE3
Formate
Serine
Glycine
LPD1
GVC1
GVC2
GVC3
THF
NH4 + CO2
THF
SHM1
5, 10-CH2-THF
MIS3
5, 10-CH+-THF
MIS3
10-HCO-THF
MIS3
Formate
Phosphoribosyl-ATP
HIS1
Phosphoribosyl-AMP
HIS4
Phosphoribosylformimino AICAR-P
HIS6
Phosphoribulosylformimino AICAR-P
HIS7
Imidazole glycerol-P
HIS3
Imidazole acetol-P
HIS5
Histidinol-P
HIS2
Histidinol
HIS4
Histidine

Figure 5.3 Interlinking Biosynthesis Pathways of Adenine, Histidine and One Carbon Metabolism
The adenine biosynthesis pathway is shown in the pink box, with intermediates of this pathway highlighted in red. The histidine biosynthesis pathway is shown in the blue box, with intermediates highlighted in dark blue. A number of products from the one-carbon metabolism pathway, shown in green, also feed into the adenine biosynthesis pathway. Two molecules of 10-HCO-THF, two molecules of glutamine and one molecule of glycine are consumed in the production of one molecule of AMP.
Combined from Mieczkowski *et al.*, 2006 and Denis and Daignan-Fornier, 1998.

Abbreviations are as follows: PRPP, 5-phosphoribosyl diphosphate; PRA, 5-phosphorybosylamine; GAR, 5′-phosphoribosylglycinamide; FGAR, 5′-phosphoribosyl-*N*′-formylglycinamide; FGAM, 5′-phosphoribosyl-*N*′-formylglycinamidine; AIR, 1-(5′-phosphoribosyl)-5-aminoimidazole; CAIR, 1-(5′-phosphoribosyl)-5-aminoimidazole-4-carboxylate; SAICAR, 1-(5′-phosphoribosyl)-4-(*N*-succinocarboxyamido)-5-aminoimidazole; AICAR, 5-amino-1-(5′-phosphoribosyl)-imidazole-4-carboxyamide; FAICAR, 5-formamido-1-(5′-phosphoribosyl)-imidazole-4-carboxyamide; IMP, inosine 5′-monophosphate; THF, tetrahydrofolate; 10-HCO-THF, 10-formyl tetrahydrofolate.

varying the phenotype of cells.

At the time this work was performed, it was thought that adenine directly prevented the interaction between Bas1p and Bas2p (Zhang *et al.*, 1997). Therefore, the increase in NMS events at *HIS4* in Ade$^-$ cells was attributed to activation of Bas1p/Bas2p complex formation and the consequent increase in DSB production. However, the particular mutant used to block adenine synthesis is a mutation of the *ADE1* gene. Consequently, the subsequent discovery that the intermediates AICAR and SAICAR promote the interaction between Bas1p and Bas2p (Rebora *et al.*, 2001; Rebora *et al.*, 2005) means that this hypothesis was incorrect. In the adenine biosynthesis pathway, *ADE1* converts 1-(5′-phosphoribosyl)-5-aminoimidazole-4-carboxylate (CAIR) into SAICAR (Figure 5.3). Therefore the production of the intermediates AICAR and SAICAR is blocked by the *ade1-1* mutation. Consequently, Bas1p/2p should not be activated and increased DSB production at *HIS4* should not occur. Reasons why this up-regulation may still occur will be discussed later (see Section 5.4.1).

5.1.7 Aims

1. To determine if Exo1p-dependent events at *HIS4* can also be modulated by genetically altering the adenine phenotype of cells.

2. To determine the effects of varying the nutritional environment on NMS and crossing over at *HIS4*.

5.2 Materials and Methods

5.2.1 Strains

All strains used are listed in Table 5.1. The diploid strains ERY103 and ERY160 were described in Chapter 4.

5.2.2 Strain Construction

To create Ade$^-$ *exo1*Δ haploid strains that were isogenic to VC155 and VC156, these strains were transformed with a *kanMX4* disruption cassette targeted to *EXO1* (Chapter 2, 2.12.1), conferring resistance to geneticin. Potential transformants were confirmed by PCR (See Appendix I for oligonucleotides used). Independent transformants obtained were used to create the diploids VCD98 and VCD132.

5.2.3 Sporulation

All diploid strains used were sporulated on either complete potassium acetate (KAc) media (Chapter 2, 1.4) or minimal KAc supplemented with the necessary nutrients (uracil and lysine for all strains used, and also adenine for VCD97, VCD98 and VCD132). Prior to sporulation on supplemented minimal KAc, selection for diploids was also performed (Chapter 2, 2.9.3).

5.2.4 Statistics

To compare levels of NMS and meiotic recombination, the G-test of homogeneity was used. In applying the Dunn-Sidak correction for multiple comparisons (Sokal and Rohlf, 1969; Chapter 2, 2.14), a P-value of 0.0169 or less was considered significant (for three-way comparisons), unless otherwise stated. The z-test was used to compare fold differences between data comparisons.

Table 5.1 Strains

Diploid Strain Name	Haploid Parents		Genotype[1]
ERY103	EY97 EY128	Wild-type	*HIS4-HhaI* *leu2-rI* *MATa* *ADE1* *TRP5* *CYH2* *met13-2* *lys2-d* *ura3-1* *RRP7::NAT* *his4-ATC* *FUS1::HYG* *LEU2* *MATα* *ade1-1* *trp5-1* *cyh2* *MET13* *lys2-c* *ura3-1*
ERY160	EY142 EY143	*exo1*Δ::*kanMX4*	
VCD97	VC155 VC156	Wild-type	*HIS4* *leu2* *MATa* *ade1-1* *TRP5* *CYH2* *met13-4* *lys2* *ura3* *RRP7::NAT* *his4-ATC* *FUS1::HYG* *LEU2* *MATα* *ade1-1* *trp5-1* *cyh2* *MET13* *lys2-c* *ura3*
VCD98	VC159 VC161	*exo1*Δ::*kanMX4*	
VCD132	VC160 VC162	*exo1*Δ::*kanMX4*	

[1] Strains grouped together have the same genotype. All strains were also all *CAN1 / CAN1*.

5.3 Results

5.3.1 Nutritional Starvation Increases Non-Mendelian Segregation at *HIS4*

Strains either auxotrophic or prototrophic for adenine were sporulated on complete or minimal sporulation media to determine the effect of nutritional status on non-Mendelian segregation (Table 5.2). However, diploids were selected prior to sporulation on minimal KAc and in addition it was necessary to supplement the media with any nutrients the cells could not synthesise themselves. For the Ade$^-$ cells, this meant supplementing with adenine and therefore under these conditions the cells were not completely starved for adenine.

Strains auxotrophic for adenine exhibited 26.7% NMS (Table 5.2). This was a 1.9-fold increase compared to the 14% NMS observed in the prototrophic strain ($P = 2.03 \times 10^{-09}$, G-test of homogeneity). Sporulation on minimal media also generated an increase in NMS. The Ade$^+$ strain exhibited 22.6% NMS when sporulated on minimal KAc. This was a 1.6-fold increase compared to the Ade$^+$ strain sporulated on complete KAc media ($P = 0.002$, G-test). A similar fold increase was observed in the Ade$^-$ strains between the different types of KAc media (26.7% to 48.2% giving a 1.8-fold increase; $P = 7.4 \times 10^{-08}$, G-test). The Ade$^-$ wild-type strain sporulated on minimal KAc exhibited a significantly higher frequency of NMS than the corresponding Ade$^+$ strain sporulated under the same conditions (48.2% compared to 22.6%; $P = 0.014$, G-test).

5.3.2 Non-Mendelian Segregation at *HIS4* in the Absence of Exo1p

Experiments modulating adenine phenotype and sporulation media were also performed in an *exo1*Δ background to determine if similar effects on the levels of NMS were observed (Table 5.2). As discussed in Chapter 4, in the *exo1*Δ strain only 7.1% NMS was observed at *his4-ATC* in Ade$^+$ cells (Meadows, Hoffmann and Borts, unpublished). This is a highly significant 2-fold reduction in NMS compared to wild-type ($P = 5.0 \times 10^{-05}$, G-test). This rate can also be influenced by complete starvation as a significantly higher rate of NMS was seen when sporulation occurred on minimal KAc compared to on complete KAc (15.9% compared to 7.1% in the Ade$^+$ strain respectively; $P = 5 \times 10^{-05}$, G-test). The rate was also influenced by auxotrophy as was observed for the wild-type. The *exo1*Δ Ade$^-$ strain sporulated on

Table 5.2 Non-Mendelian Segregation at *HIS4* under Varying Conditions

Phenotype	Sporulation Media[1]	Non-Mendelian Segregation (%)[2] (NMS / Total Tetrads) Wild-Type	*exo1*Δ
Ade$^+$	Complete	14.0[3] (243 / 1731)	7.1[4] (30 / 421)
Ade$^-$	Complete	26.7* (113 / 423)	9.6[5] (60 / 626)
Ade$^+$	Minimal (+ Ura and Lys)	22.6† (45 / 199)	15.9† (34 / 214)
Ade$^-$	Minimal (+ Ura, Lys and Ade)	48.2*† (105 / 218)	29.6*† (67 / 226)

[1] Sporulation media was either supplemented with all nutrients (complete) or only with the nutrients required by the diploid strain (minimal).

[2] Non-Mendelian segregation includes gene conversion and post-meiotic segregation events. % NMS was calculated as the number of NMS events / total number of tetrads x 100. Comparisons were made using the G-test, and significant statistical differences are represented as follows:

* Significant difference between the corresponding Ade$^+$ and Ade$^-$ strains on the same sporulation media.

† Significant difference between the same strains on a different sporulation medium.

[3] Data from Hoffmann and Borts, unpublished.

[4] Data from Meadows, Hoffmann and Borts, unpublished.

[5] Data from VCD98-1 and VCD132 (Table 5.3) was pooled (see text).

minimal KAc had a significantly higher frequency of NMS than the *exo1*Δ Ade$^+$ strain sporulated on minimal KAc (29.6% compared to 15.9% respectively; P = 0.001, G-test).

However, for the *exo1*Δ Ade$^-$ strain sporulated on complete KAc, variable results were obtained (Table 5.3). The initial result of dissecting VCD98 yielded 9.6% NMS of *his4-ATC*. However, after a second independent mating and sporulation of VCD98 (VCD98-2), a higher level of NMS was observed, which was significantly different to the first value obtained (22.4%; P = 0.0008, G-test). Therefore this cross was remade and dissected a third time, and another control was performed using a second isogenic diploid, VCD132 (Table 5.1 and Section 5.2.2). The frequency of NMS observed in VCD98-3 was consistent with the frequency of NMS obtained from VCD98-2 (23.3% compared with 22.4% respectively (P > 0.05, G-test). Dissection of the second diploid, VCD132, gave a result of 9.6% NMS at *HIS4*, concurring with the initial result obtained from VCD98-1 (P > 0.05, G-test). Clearly these four datasets do not constitute a homogeneous group (P = 1.3 x 10^{-05}, G-test); instead the data falls into two distinct groups. VCD98-1 and VCD132 did not show a substantial difference in levels of NMS at *his4-ATC* compared to the *exo1*Δ Ade$^+$ strain. A 1.4-fold increase in NMS was observed from 7.1% in the *exo1*Δ Ade$^+$ strain to 9.6% in the *exo1*Δ Ade$^-$ strains VCD98-1 and VCD132, and these values were not significantly different (P > 0.05 for both comparisons, G-test). However, the remaining two datasets from the Ade$^-$ *exo1*Δ strain, VCD98-2 and VCD98-3, exhibited a 3-fold increase in NMS. This was significantly higher than that observed in the Ade$^+$ *exo1*Δ strain (7.1% compared with 22.4% and 23.3%; P < 0.001 for both comparisons, G-test). The levels of NMS observed in VCD98-2 and VCD98-3 were also significantly higher than those obtained from VCD98-1 and VCD132 (P < 0.007 for all comparisons, G-test). Therefore, this gives contrasting views on the effect of adenine auxotrophy in the absence of Exo1p. In an attempt to further understand this, *exo1*Δ Ade$^+$ and Ade$^-$ strains were dissected from a different strain background, with a different *his4* allele (*his4::Xho*I at 96 bp from the start of the *HIS4* open reading frame). This experiment yielded similar results as from VCD98-1 and VCD132; from the Ade$^+$ strain 7.0% (14 / 201) NMS was obtained at *his4::Xho*I, whereas in the Ade$^-$ strain 11.2% (24 / 214) NMS was observed (P > 0.05, G-test). The lower values of NMS obtained are more consistent with an

Table 5.3 Non-Mendelian Segregation in *exo1*Δ Strains Auxotrophic for Adenine Sporulated on Complete KAc

Strain[1]	Non-Mendelian Segregation (%)[2] (NMS / Total Tetrads)
VCD98 – 1	9.6 (22 / 229)
VCD98 – 2	22.4* (32 / 143)
VCD98 – 3	23.3* (27 / 116)
VCD132	9.6 (38 / 397)

[1] VCD98 was dissected three times, and VCD132 was dissected as a further control.

[2] Comparisons were made using the G-test of homogeneity, and values statistically different to those in dataset 1 of VCD98 are represented by *.

*exo1*Δ phenotype, given that the absence of Exo1p reduces NMS events at *HIS4* (Abdullah *et al.*, 2004; Khazanehdari and Borts, 2000). Additionally, the levels of NMS greater than 20% (observed in VCD98-2 and VCD98-3) were equivalent to the levels observed in the Ade$^-$ wild-type strain ($P > 0.05$, G-test; see also Section 5.4.3). Therefore all four datasets will be presented, but only the data obtained from VCD98-1 and VCD132 will be discussed. The data from VCD98-1 and VCD132 was homogeneous (for NMS $P > 0.05$, G-test) and therefore pooled for all subsequent comparisons (Table 5.2).

The frequencies of NMS at *his4-ATC* obtained in the *exo1*Δ strains were also compared to those obtained from the wild-type strains. From sporulation on complete KAc, the 9.6% NMS observed at *HIS4* in the *exo1*Δ Ade$^-$ strain was significantly lower than the 26.7% NMS observed in the wild-type Ade$^-$ strain ($P \leq 4.3 \times 10^{-06}$, G-test). Although the level of NMS at *HIS4* observed in the *exo1*Δ Ade$^+$ strain sporulated on minimal KAc was lower than the wild-type (15.9% compared with 22.6% respectively), these values were not significantly different ($P = 0.08$, G-test). However, the *exo1*Δ and wild-type Ade$^-$ strains sporulated on minimal KAc did exhibit significantly different levels of NMS (29.6% compared with 48.2% respectively; $P = 5.9 \times 10^{-05}$, G-test). Therefore the increases in NMS observed in the wild-type and *exo1*Δ strains differed depending on the media used for sporulation.

Another way of evaluating the differences between these strains is to compare the fold differences observed in NMS between the different adenine phenotypes and environmental conditions studied (Table 5.4). A 1.9-fold increase in NMS was observed in the wild-type Ade$^-$ strain compared to the Ade$^+$ strain when they were sporulated on complete KAc. This was significantly different to the 1.4-fold increase observed in the *exo1*Δ Ade$^-$ strain compared to the Ade$^+$ strain also sporulated on complete KAc ($P = 0.01$, z-test). In contrast, the 2.1-fold increase in NMS at *HIS4* observed in the wild-type Ade$^-$ strain compared to the wild-type Ade$^+$ strain when they were sporulated on minimal KAc was not significantly different to the 1.9-fold increase observed between the Ade$^+$ and Ade$^-$ *exo1*Δ strains sporulated on minimal KAc ($P = 0.05$, z-test). The 2-fold difference in the frequency of NMS

Table 5.4 Fold Differences Between NMS for Wild-Type and *exo1*Δ Strains under Varying Conditions

		Fold Differences[1] (% NMS / % NMS)				
		Wild-Type : *exo1*Δ	Ade⁻ : Ade⁺		Minimal : Complete	
Phenotype	**Sporulation Media**		**Wild-Type**	***exo1*Δ**	**Wild-Type**	***exo1*Δ**
Ade⁺	Complete	2.0* (14.0 / 7.1)				
		|				
Ade⁻	Complete	2.8 (26.7 / 9.6)	1.9 (26.7 / 14.0)	— 1.4* (9.6 / 7.1)		
Ade⁺	Minimal (+ Ura and Lys)	1.4 (22.6 / 15.9)			1.6 (22.6 / 14.0)	2.2 (15.9 / 7.1)
		|			|	|
Ade⁻	Minimal (+ Ura, Lys and Ade)	1.6 (48.2 / 29.6)	2.1 (48.2 / 22.6)	— 1.9 (29.6 / 15.9)	1.8 (48.2 / 26.7)	3.1* (29.6 / 9.6)

[1] Values in brackets are NMS (%) from Table 5.2 used to calculate the fold differences represented. Comparisons were made between corresponding fold differences, indicated by the lines. Significant differences were denoted by *.

observed in the wild-type Ade$^+$ strain compared to the *exo1*Δ Ade$^+$ strain was significantly different to the 2.8-fold difference observed between the corresponding Ade$^-$ strains (P = 0.01, z-test) when the strains were sporulated on complete KAc. However, when the strains were sporulated on minimal KAc, the fold differences between the wild-type and *exo1*Δ Ade$^+$ strains, and the wild-type and *exo1*Δ Ade$^-$ strains were not different (1.4-fold compared with 1.6-fold respectively; P = 0.05, z-test). Additionally, the fold increase in NMS observed in the wild-type strains sporulated on minimal KAc compared to sporulation on complete KAc was equivalent in the Ade$^+$ and Ade$^-$ strains (1.6-fold compared to 1.8-fold respectively, P > 0.1, z-test). This was not true for the *exo1*Δ strains, where the fold increase observed in the Ade$^-$ strains when sporulated on minimal KAc compared to sporulation on complete KAc was significantly different to the fold increase observed in the *exo1*Δ Ade$^+$ strains (P = 0.01, z-test). Therefore, in the absence of Exo1p auxotrophy for adenine does not elevate NMS to the same extent as in the wild-type. However, starvation for amino acids in addition to auxotrophy for adenine stimulates an equivalent response in wild-type and *exo1*Δ strains.

5.3.3 Crossing Over in the *NAT-HYG* Interval Increases with NMS at *HIS4*

At *HIS4* it is thought that the increase in recombination events at transcription factor regulated hotspots arise through the increased production of DSBs (Fan *et al.*, 1995). As both crossovers and gene conversion events arise from the repair of DSBs, it was possible that crossover events would also be increased by modulating the phenotype of the cells and the nutritional environment. To determine if this was true, crossing over in the *NAT-HYG* interval that spans *HIS4* was also analysed (Table 5.5). As NMS increased at *HIS4*, there was a concomitant increase in crossing over between *NAT-HYG*. When sporulating on complete KAc, the Ade$^-$ wild-type strain exhibited a 1.8-fold increase in crossing over compared to the Ade$^+$ strain (P = 3.6 x 10^{-07}, G-test). The Ade$^-$ strain sporulated on minimal KAc, which showed the highest level of NMS at *HIS4*, also had the highest level of recombination between *NAT-HYG*, being significantly higher than for the other conditions tested (P < 0.002, G-test). Therefore, a direct correlation between the level of NMS and crossing over was observed (Figure 5.4A).

The same effects were observed for the *exo1*Δ strains. With increasing NMS at

HIS4, crossing over also increased in the *NAT-HYG* interval (Table 5.5). The *exo1*Δ Ade$^-$ strain sporulated on minimal KAc exhibited the highest level of NMS out of the *exo1*Δ strains analysed, and also the highest level of crossing over between *NAT-HYG*. A map distance of 33.5 cM was observed in the *exo1*Δ Ade$^-$ strain sporulated on minimal KAc, whereas the corresponding Ade$^+$ strain only exhibited a map distance of 11.7 cM ($P = 2.19 \times 10^{-08}$, G-test). Sporulation of the *exo1*Δ Ade$^+$ strain on complete KAc produced a small map distance of 6.6 cM in the *NAT-HYG* interval which was significantly different to the map distances obtained from sporulation on minimal KAc ($P \leq 0.0024$, G-test). The crossover data obtained from the *exo1*Δ Ade$^-$ strains VCD98 (1-3) and VCD132 are shown in Tables 5.7 and 5.8. The data from VCD98-1 and VCD132 was combined and used to make comparisons as they formed a homogeneous group ($P > 0.05$ for all comparisons, G-test; Tables 5.5 and 5.6). The Ade$^-$ *exo1*Δ strain did not exhibit an increase in crossing over in the *NAT-HYG* interval compared to the Ade$^+$ *exo1*Δ strain ($P > 0.05$, G-test). This was significantly different to the increase in crossing over in the *NAT-HYG* interval observed in the Ade$^-$ strain sporulated on minimal KAc (5.3 cM compared with 33.5 cM respectively; 4.57×10^{-29}, G-test). This reflects the observations made for the frequencies of NMS at *HIS4*. Although the Ade$^-$ *exo1*Δ strain did not exhibit significant increases in either NMS at *HIS4* or crossing over in the *NAT-HYG* interval, a positive correlation was still observed between these events, as was observed in the wild-type strains (Figure 5.4B).

The wild-type and *exo1*Δ strains were also compared for crossing over. In the *NAT-HYG* interval, crossing over was reduced in the Ade$^+$ *exo1*Δ strain compared to the Ade$^+$ wild-type (6.6 cM compared with 11.2 cM respectively; $P = 9.74 \times 10^{-06}$, G-test; Meadows, Hoffmann and Borts, unpublished) as was shown in Chapter 4. On minimal KAc however, the *exo1*Δ Ade$^+$ strain exhibited a level of crossing over in the *NAT-HYG* interval that was not different to the level in the wild-type Ade$^+$ strain (11.7 cM compared with 18.5 cM respectively; $P > 0.05$, G-test) whereas the Ade$^-$ strains were statistically different (33.5 cM compared with 37.8 cM respectively; $P = 0.0041$, G-test). In the *exo1*Δ Ade$^-$ strain sporulated on complete KAc, the level of crossing over in the *NAT-HYG* interval was significantly lower than observed for the wild-type Ade$^-$ strain ($P = 1.32 \times 10^{-18}$, G-test).

Table 5.5 Map Distances for Intervals on Chromosome III

Genotype	Phenotype	Sporulation Media[1]	Interval											
			NAT-HYG				*HYG-LEU2*				*LEU2-MAT*			
			PD[2]	NPD	TT	cM	PD	NPD	TT	cM	PD	NPD	TT	cM
Wild-type[3]	Ade^+	Complete	1347	7	336	11.2	1435	7	245	8.5	828	66	799	35.3
Wild-type	Ade^-	Complete	273	5	130	19.6*	362	0	45	5.5	186	9	208	32.5
Wild-type	Ade^+	Minimal (+ Ura and Lys)	136	3	53	18.5†	165	0	29	7.5	95	4	98	31.0
Wild-type	Ade^-	Minimal (+ Ura, Lys and Ade)	82	6	125	37.8*†	204	0	12	2.8*	107	4	106	30.0
*exo1*Δ[4]	Ade^+	Complete	372	2	43	6.6	377	0	33	4.0	305	6	100	16.5
*exo1*Δ[5]	Ade^-	Complete	552	0	65	5.3	564	0	47	3.8	475	3	133	12.4
*exo1*Δ	Ade^+	Minimal (+ Ura and Lys)	168	1	44	11.7†	202	0	10	2.4	158	1	53	13.9
*exo1*Δ	Ade^-	Minimal (+ Ura, Lys and Ade)	119	9	96	33.5*†	218	0	6	1.3	173	2	50	13.8

[1] Sporulation media was either supplemented with all nutrients (complete) or only with the nutrients required by the diploid strain (minimal).

[2] PD, NPD and TT are parental ditype, non-parental ditype and tetratype respectively, and map distances (cM) were calculated according to Perkins (1949). Comparisons of the distribution of PD, NPD and TT events were made using a G-test of homogeneity, and significant statistical differences are represented as follows:

* Significant difference between corresponding values for Ade^+ and Ade^- strains sporulated on the same media.

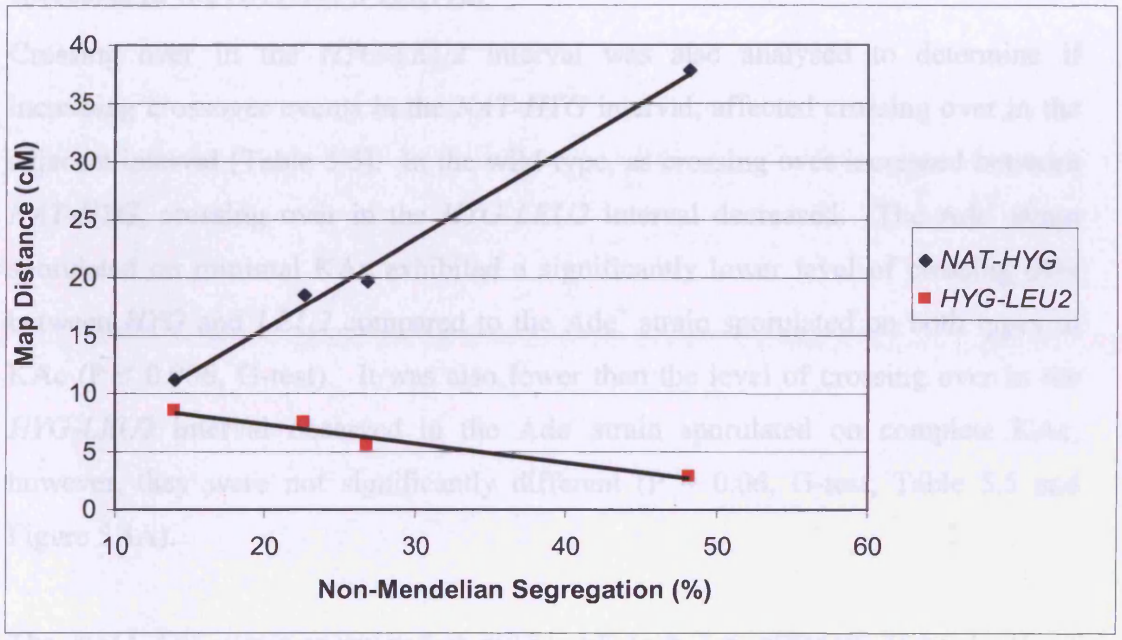
† Significant difference between corresponding values for Ade^+ and Ade^- strains sporulated on different media.

[3] Data from Hoffmann and Borts, unpublished.

[4] Data from Meadows, Hoffmann and Borts, unpublished.

[5] Data from VCD98-1 and VCD132 (Table 5.7) was pooled.

A. Wild-type Strains



B. *exo1*Δ Strains

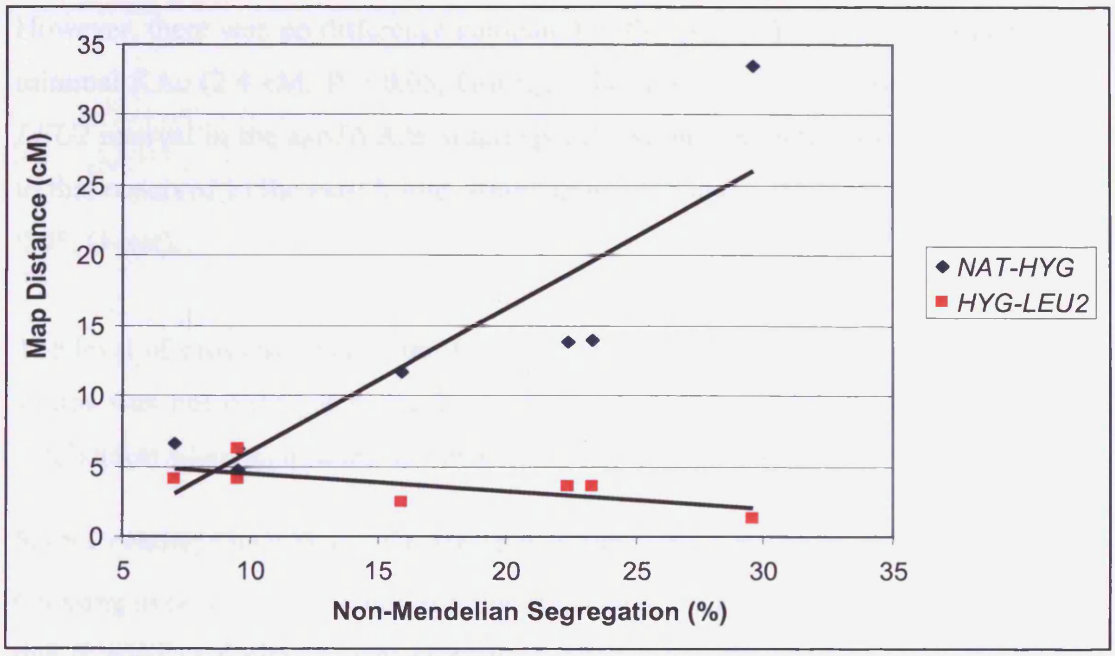


Figure 5.4 Correlations Between Crossing Over in the *NAT-HYG* and *HYG-LEU2* Intervals with NMS at *HIS4*
The data from each wild-type or *exo1*Δ strain analysed was plotted. For each strain, the map distances for the *NAT-HYG* and *HYG-LEU2* intervals were both plotted against the NMS at *HIS4*.

5.3.4 Crossing Over in the *HYG-LEU2* Interval Decreases as Crossing Over Increases in the *NAT-HYG* Interval

Crossing over in the *HYG-LEU2* interval was also analysed to determine if increasing crossover events in the *NAT-HYG* interval, affected crossing over in the adjacent interval (Table 5.5). In the wild-type, as crossing over increased between *NAT-HYG*, crossing over in the *HYG-LEU2* interval decreased. The Ade$^-$ strain sporulated on minimal KAc exhibited a significantly lower level of crossing over between *HYG* and *LEU2* compared to the Ade$^+$ strain sporulated on both types of KAc ($P < 0.006$, G-test). It was also lower than the level of crossing over in the *HYG-LEU2* interval observed in the Ade$^-$ strain sporulated on complete KAc, however, they were not significantly different ($P = 0.06$, G-test; Table 5.5 and Figure 5.4A).

The *exo1*Δ Ade$^-$ strain sporulated on minimal KAc had significantly lower levels of crossing over in the *HYG-LEU2* interval than the *exo1*Δ Ade$^+$ strain sporulated on complete KAc (1.3 cM compared with 4 cM respectively; $P = 0.0164$, G-test). However, there was no difference compared to the *exo1*Δ Ade$^+$ strain sporulated on minimal KAc (2.4 cM; $P > 0.05$, G-test). The level of crossing over in the *HYG-LEU2* interval in the *exo1*Δ Ade$^-$ strain sporulated on complete KAc was equivalent to that observed in the *exo1*Δ Ade$^+$ strain sporulated under the same conditions ($P > 0.05$, G-test).

The level of crossing over in the *HYG-LEU2* interval exhibited by the *exo1*Δ Ade$^-$ strains was not different to the level of crossing over exhibited by the wild-type Ade$^-$ strains when sporulated on either complete or minimal KAc ($P > 0.05$, G-test).

5.3.5 Crossing Over Was only Affected in Intervals Linked to *HIS4*

Crossing over was also studied at three other intervals to demonstrate that the effect due to adenine limitation was specific to *HIS4*. At *LEU2-MAT* and *CYH2-TRP5*, the levels of crossing over were not different amongst the wild-type strains under any of the conditions tested (Tables 5.5 and 5.6; $P > 0.05$, G-test). At *MET13-CYH2* no difference was observed for crossing over between the Ade$^+$ and Ade$^-$ strains sporulated on complete KAc or on minimal KAc ($P > 0.05$ for both comparisons, G-test; Table 5.6). However, the wild-type Ade$^+$ strain exhibited

Table 5.6 Map Distances for Intervals on Chromosome VII

Genotype	Phenotype	Sporulation Media[1]	Interval							
			MET13-CYH2				*CYH2-TRP5*			
			PD[2]	NPD	TT	cM	PD	NPD	TT	cM
Wild-type[3]	Ade^+	Complete	1160	8	474	15.9	314	174	1216	66.3
Wild-type	Ade^-	Complete	300	1	118	14.8	84	44	289	66.3
Wild-type	Ade^+	Minimal (+ Ura and Lys)	110	0	75	20.3†	50	20	129	62.6
Wild-type	Ade^-	Minimal (+ Ura, Lys and Ade)	141	0	75	17.4	45	15	157	56.9
*exo1*Δ[4]	Ade^+	Complete	352	0	52	6.4	223	6	182	26.5
*exo1*Δ[5]	Ade^-	Complete	542	1	81	7.0	355	9	258	25.1
*exo1*Δ	Ade^+	Minimal (+ Ura and Lys)	160	0	45	11.0	117	6	90	29.6
*exo1*Δ	Ade^-	Minimal (+ Ura, Lys and Ade)	177	3	43	13.7†	119	7	98	31.3

[1] Sporulation media was either supplemented with all nutrients (complete) or only with the nutrients required by the diploid strain (minimal).

[2] PD, NPD and TT are parental ditype, non-parental ditype and tetratype respectively, and map distances (cM) were calculated according to Perkins (1949). Comparisons of the distribution of PD, NPD and TT events were made using a G-test of homogeneity, and significant statistical differences are represented as follows:

* Significant difference between corresponding values for Ade^+ and Ade^- strains sporulated on the same media.

† Significant difference between corresponding values for Ade^+ and Ade^- strains sporulated on different media.

[3] Data from Hoffmann and Borts, unpublished.

[4] Data from Meadows, Hoffmann and Borts, unpublished.

[5] Data from VCD98-1 and VCD132 (Table 5.8) was pooled.

higher levels of crossing over in the *MET13-CYH2* interval after sporulation on minimal KAc compared to sporulation on complete KAc (P = 0.003, G-test). The *exo1*Δ Ade$^+$ and Ade$^-$ strains sporulated on both complete or minimal KAc exhibited equivalent levels of crossing over in the *LEU2-MAT* and *CYH2-TRP5* intervals and formed homogeneous groups (P > 0.05 for both comparisons, G-test; Tables 5.5 and 5.6), as was seen for the wild-type strains. Similarly at *MET13-CYH2*, an effect on crossing over was only observed when different types of medium were used for sporulation of the *exo1*Δ strains (Table 5.6). The *exo1*Δ Ade$^+$ and Ade$^-$ strains sporulated on complete KAc exhibited similar levels of crossing over (P > 0.05, G-test). In contrast, sporulation on minimal KAc caused a 1.7-fold increase in crossing over in the *exo1*Δ Ade$^+$ strain compared to sporulation on complete KAc media, which was borderline to being significant (P = 0.018, G-test). No difference in crossing over in the *MET13-CYH2* interval was observed between the Ade$^+$ and Ade$^-$ *exo1*Δ strains sporulated on minimal KAc (P > 0.05, G-test). In the *LEU2-MAT*, *MET13-CYH2* and *CYH2-TRP5* intervals, the *exo1*Δ strains exhibited reduced levels of crossing over in comparison to the corresponding wild-type strain sporulated on the same kind of media (P ≤ 0.0018, G-test) as expected due to the role of Exo1p in crossing over (Khazanehdari and Borts, 2000; Kirkpatrick *et al.*, 2000; Meadows, Hoffmann and Borts, unpublished; Tsubouchi and Ogawa, 2000; This study, Chapter 4).

5.3.6 NMS and Crossing Over at *HIS4* are Directly Associated

As discussed in Chapter 4, 58.9% of NMS events at *HIS4* were associated with a crossover event between *NAT-HYG* in the wild-type Ade$^+$ strain, and this was not altered in the *exo1*Δ Ade$^+$ strain (P > 0.05, G-test; Table 5.9; Meadows, Hoffmann and Borts, unpublished). Altering the ability of strains to synthesise adenine or using different sporulation media also did not affect the association between NMS and crossing over. The Ade$^+$ and Ade$^-$ wild-type strains sporulated on either complete or minimal KAc exhibited associations of NMS with crossing over that were not different to one another (P > 0.05, G-test). The *exo1*Δ Ade$^+$ strain sporulated on complete and minimal KAc and the *exo1*Δ Ade$^-$ strain sporulated on minimal KAc also exhibited similar levels of association between NMS and crossing over, and were not significantly different from one another (P > 0.05, G-test). Although in the *exo1*Δ Ade$^-$ strain sporulated on complete KAc 38.3%

Table 5.7 Map Distances for Intervals on Chromosome III in the Ade⁻ *exo1*Δ Strains Sporulated on Complete KAc

	Interval											
	NAT-HYG				*HYG-LEU2*				*LEU2-MAT*			
Strain	PD[1]	NPD	TT	cM	PD	NPD	TT	cM	PD	NPD	TT	cM
VCD98 – 1	197	0	28	6.2	208	0	16	6.2	174	1	48	12.1
VCD98 – 2	102	0	39	13.8	129	0	10	3.6	120	1	19	8.9
VCD98 – 3	83	0	32	13.9	104	0	8	3.6	83	2	27	17.4
VCD132	355	0	37	4.7	356	0	31	4.0	301	2	85	12.5

[1] PD, NPD and TT are parental ditype, non-parental ditype and tetratype respectively, and map distances (cM) were calculated according to Perkins (1949).

Table 5.8 Map Distances for Intervals on Chromosome VII in the Ade⁻ *exo1*Δ Strains Sporulated on Complete KAc

	Interval							
	MET13-CYH2				*CYH2-TRP5*			
Strain	PD[1]	NPD	TT	cM	PD	NPD	TT	cM
VCD98 – 1	195	1	33	8.5	132	5	88	26.2
VCD98 – 2	126	0	16	5.6	9	3	59	27.3
VCD98 – 3	105	0	11	4.7	69	2	43	24.1
VCD132	347	0	48	6.1	223	4	170	24.4

[1] PD, NPD and TT are parental ditype, non-parental ditype and tetratype respectively, and map distances (cM) were calculated according to Perkins (1949).

association between NMS and crossing over was observed, this was not different to the *exo1*Δ Ade$^+$ strain sporulated on complete KAc ($P > 0.05$, G-test). However, it was significantly different to the level of association observed in the *exo1*Δ strains sporulated on minimal KAc ($P \leq 0.0024$, G-test). The associations between NMS at *HIS4* and crossing over in the *NAT-HYG* interval from the four experiments with the *exo1*Δ Ade$^-$ strains are shown in Table 5.10. When comparing the corresponding wild-type and *exo1*Δ strains (i.e. wild-type Ade$^+$ with *exo1*Δ Ade$^+$ both sporulated on complete KAc), no differences in association were observed ($P > 0.05$, G-test) with the exception of the Ade$^-$ strains sporulated on complete KAc. The 38.3% association observed in the *exo1*Δ Ade$^-$ strain was significantly lower than the level of association observed in the wild-type (58.9%; $P = 0.012$, G-test). It is unclear why the association in the *exo1*Δ Ade$^-$ strain should be affected given that the frequencies of NMS at *HIS4* and crossing over in the *NAT-HYG* interval are not different to those exhibited in the Ade$^+$ *exo1*Δ strain. Overall both gene conversion and crossover events are concomitantly modulated by nutritional status and adenine auxotrophy, and the association of these events is unaffected by this.

Table 5.9 The Association Between Non-Mendelian Segregation and Crossing Over at *HIS4*

Genotype	Phenotype	Sporulation Media	NMS[1]	CO[2]	% NMS with a CO[3] (NMS with a CO/Total NMS)
Wild-type[4]	Ade$^+$	Complete	243	344	58.9 (143 / 243)
Wild-type	Ade$^-$	Complete	113	135	58.4 (66 / 113)
Wild-type	Ade$^+$	Minimal (+ Ura and Lys)	45	56	62.2 (28 / 45)
Wild-type	Ade$^-$	Minimal (+ Ura, Lys and Ade)	105	131	71.4 (75 / 105)
*exo1*Δ[5]	Ade$^+$	Complete	30	45	56.7 (17 / 30)
*exo1*Δ[6]	Ade$^-$	Complete	60	65	38.3 (23 / 60)
*exo1*Δ	Ade$^+$	Minimal (+ Ura and Lys)	34	45	70.6 (24 / 34)
*exo1*Δ	Ade$^-$	Minimal (+ Ura, Lys and Ade)	67	105	77.6 (52 / 67)

[1] Number of tetrads with a non-Mendelian segregation event at *HIS4*. NMS includes gene conversion and post-meiotic segregation events.
[2] Number of tetrads with a crossover in the *NAT-HYG* interval which spans *HIS4*.
[3] The percentage of tetrads which have a NMS event and an associated crossover. Calculated as the number of tetrads with an NMS event with a crossover / number of tetrads with an NMS event.
[4] Data from Hoffmann and Borts, unpublished.
[5] Data from Meadows, Hoffmann and Borts, unpublished.
[6] Data from VCD98-1 and VCD132 (Table 5.10) was pooled.

Table 5.10 The Association Between Non-Mendelian Segregation and Crossing Over *HIS4* in *exo1*Δ Strains Sporulated on Complete KAc

Genotype	NMS[1]	CO[2]	% NMS with a CO[3] (NMS with a CO/Total NMS)
VCD98-1	22	28	31.8 (7 / 22)
VCD98-2	32	39	56.3 (18 / 32)
VCD98-3	27	32	51.9 (14 / 27)
VCD132	38	37	42.1 (16 / 38)

[1] Number of tetrads with a non-Mendelian segregation event at *HIS4*. NMS includes gene conversion and post-meiotic segregation events.
[2] Number of tetrads with a crossover in the *NAT-HYG* interval which spans *HIS4*.
[3] The percentage of tetrads which have a NMS event and an associated crossover. Calculated as the number of tetrads with an NMS event with a crossover / number of tetrads with an NMS event.

5.4 Discussion

5.4.1 How is *HIS4* Activated in the Absence of the Intermediates AICAR and SAICAR?

As discussed in Section 5.1, the histidine and adenine biosynthesis pathways are upregulated when adenine is limiting. When the initial study was performed that demonstrated an increase in NMS events at *HIS4* in an *ade1-1* strain (Abdullah and Borts, 2001), the specific manner in which Bas1p and Bas2p were regulated was unknown. It has since been demonstrated that AICAR and SAICAR are the crucial intermediates that promote the interaction of Bas1p and Bas2p, and this interaction is necessary to promote activation of these biosynthetic pathways (Rebora *et al.*, 2001; Rebora *et al.*, 2005). However, the *ade1-1* mutation prevents the production of AICAR and SAICAR, as *ADE1* is involved in a step upstream of these intermediates (Figure 5.3). Consequently no activation should be observed at *HIS4* in *ade1-1* strains. There are two possible explanations for why events are increased at *HIS4*. The *ade1-1* mutation is a T to C base change at position 799 (Abdullah *et al.*, 2004), that results in an amino acid change from serine to proline. The entire gene is only 921 bases long, and hence the mutation is situated close to the end of *ADE1*, and may not interfere with any active sites within the protein. Hence if this mutation is leaky, some functionality may be retained. However, yeast cells containing the *ade1-1* mutation have the pink/red appearance that is indicative of a defect in producing adenine. This is due to the accumulation of a red pigment which is derived from the intermediate 1-(5′-phosphoribosyl)-5-aminoimidazole (AIR; Figure 5.3). Another possible explanation is that although the adenine pathway is blocked by the *ade1-1* mutation, there could still be some up-regulation of the histidine biosynthesis pathway in the absence of adenine. AICAR is also produced via the histidine biosynthesis pathway, as a by-product of the conversion of phosphoribulosylformimino AICAR-P to imidazole glycerol-P (Figure 5.3). The AICAR produced via this pathway could promote the interaction of Bas1p and Bas2p, causing the increase in NMS observed at *HIS4*. To determine if either of these hypotheses is correct, further experiments need to be performed (see also Chapter 6).

5.4.2 Regulation of Meiotic Events at *HIS4*

Modulation of the frequency of NMS events was achieved by varying a number of conditions including adenine phenotype and the nutritional environment. Adenine auxotrophy increased NMS events occurring at *HIS4*, in agreement with previous observations (Abdullah and Borts, 2001). For the purpose of this discussion, it will be assumed that one of the above hypotheses regarding the production of AICAR/SAICAR in an *ade1-1* mutant is correct and therefore activation of the histidine pathway is occurring through promotion of the Bas1p/Bas2p interaction. If Bas1p/Bas2p complex formation can be activated to different levels, then in cells that are unable to synthesise adenine and in addition are starved for adenine, there could be a further increase in events at *HIS4*. We aimed to test this by using minimal sporulation media. However, as the majority of nutrients were excluded from this media, we propose that Gcn4p was also activated in response to general amino acid starvation. Therefore, using minimal media did not measure an adenine specific effect. Additionally, the minimal media also had to be supplemented with any nutrients that the cells were unable to synthesise. This included adenine for the Ade^- cells, and therefore these strains were not necessarily completely starved for adenine. The Ade^+ strain sporulated on minimal KAc was starved for both adenine and histidine which would activate both the general and basal control systems, leading to the increased NMS observed. However, when using minimal sporulation media, a further increase in NMS was still observed in the Ade^- strains compared to the Ade^+ strains. The Ade^- strains sporulated under starvation conditions exhibit the most NMS at *HIS4* as they are starved for histidine and also cannot synthesise adenine, and therefore there may be a combined response from Bas1p/2p and Gcn4p. It is also possible that the adenine provided may not be sufficient to compensate for an inability to synthesise adenine, as suggested previously (Abdullah and Borts, 2001). Increased activation of the *HIS4* hotspot could also occur directly due to starvation for histidine, which will also activate Gcn4p and consequently upregulate the histidine biosynthesis pathway. As discussed above, this could result in further activation of Bas1p/Bas2p complex formation due to the production of AICAR from the histidine biosynthesis pathway (Figure 5.3). Therefore, NMS at *HIS4* can be modulated both by changing the metabolic state of the cell, as has been shown previously (Abdullah and Borts, 2001), and by limiting

purine and amino acid availability. This work indicates that experiments to measure resection should be performed in Ade$^-$ cells sporulated on minimal KAc.

5.4.3 Modulation of Events in the Absence of Exo1p

In the absence of Exo1p, conflicting results were obtained as to the effects of adenine auxotrophy. From a total of four experiments, in two cases only a small, non-significant, increase in NMS was observed at *HIS4* in Ade$^-$ cells. However, the other two experiments showed a significant increase in NMS. Given that gene conversion at *HIS4* is reduced in the absence of Exo1p (Abdullah *et al.*, 2004; Khazanehdari and Borts, 2000; Meadows, Hoffmann and Borts, unpublished), the lower values of NMS obtained are more consistent with an *exo1*Δ phenotype. One possible explanation for obtaining high levels of NMS is that the DSB events induced by Bas1p/Bas2p are processed independently of Exo1p. However, if the higher frequency of NMS were correct, then the fold increase in NMS observed when comparing sporulation of the Ade$^-$ *exo1*Δ on complete KAc versus minimal KAc is not as large as observed in the wild-type. This would suggest that these starvation induced events are Exo1p-dependent. We hypothesise that the increased NMS observed when sporulating strains on minimal KAc is due to activation of Gcn4p as a result of amino acid starvation. Therefore, as an increase in NMS is observed between the Ade$^+$ *exo1*Δ strains sporulated on minimal KAc compared to complete KAc, it seems unlikely that the equivalent events in the Ade$^-$ *exo1*Δ strains could be dependent on the presence of Exo1p when those in the Ade$^+$ *exo1*Δ strains are clearly not. Therefore we argue that of the four datasets, the two that yield the lower values of NMS (9.6%) are likely to be the correct ones.

Given that there is a 1.9-fold increase in NMS between the Ade$^+$ and Ade$^-$ wild-type strains sporulated on complete KAc, a similar fold increase was expected in the Ade$^-$ *exo1*Δ strain. Therefore the expected frequency of NMS for this strain was 13.5% (7.1% x 1.9). The observed value of 9.6% (VCD98-1 and VCD132 combined) was not different to the frequency of NMS observed in the *exo1*Δ Ade$^+$ strain however it was significantly different to the expected value ($P < 0.01$, χ^2 test). This suggests that the events that are normally induced in the absence of adenine are dependent on Exo1p for processing.

Increases in NMS frequency were observed when the *exo1*Δ strains were sporulated on minimal KAc. Additionally, the frequency of NMS events observed in the *exo1*Δ strains was approximately half of the corresponding frequency observed in the wild-type. This suggests that half of these events are normally processed by Exo1p, as observed when comparing the wild-type and *exo1*Δ Ade^+ strains sporulated on complete KAc. However, when the *exo1*Δ Ade^- cells were sporulated on minimal KAc a further increase in NMS was observed in comparison to the *exo1*Δ Ade^+ strain sporulated on minimal KAc. If the events induced by Bas1p/Bas2p are dependent on Exo1p, this increase in NMS observed in the Ade^- *exo1*Δ cells must be occurring independently of Bas1p/Bas2p. As mentioned earlier, we propose that sporulation on minimal KAc will activate Gcn4p in response to amino acid starvation, and increase the number of DSBs made at *HIS4*. Since purine limitation also stimulates Gcn4p activity, the additional auxotrophy for adenine might further activate Gcn4p as well as Bas1p/Bas2p. As Gcn4p is already activated due to sporulation under starvation conditions, it is possible that it is Gcn4p that is activated further in response to the additional auxotrophy for adenine in these conditions rather than Bas1p/Bas2p. In contrast, under non-starvation conditions Bas1p/Bas2p are primarily activated in response to adenine auxotrophy. This could explain the difference between the effects on NMS observed when sporulating Ade^- *exo1*Δ strains on the different types of sporulation media. Therefore we suggest that starving cells for amino acids during sporulation does increase meiotic recombination events even in the absence of Exo1p. This suggests that the initiation of DSBs is increased when cells are sporulated on minimal KAc, and that these conditions might be useful in an *exo1*Δ background for analysis of the resection of the *HIS4* DSB.

5.4.4 Crossing Over was Also Modulated Under the Different Conditions

As NMS of the *his4-ATC* allele increased, a corresponding increase was observed in crossing over in the *NAT-HYG* interval that spans *HIS4*. A correlation between the two is expected as both the NMS at *HIS4* and the majority of crossover events in the *NAT-HYG* interval will be initiated from the same break that is regulated by Bas1p-Bas2p and Gcn4p. Additionally, an NMS event and a crossover event can arise from the same DSB. As crossing over increases in the *NAT-HYG* interval, a concomitant decrease is observed in the *HYG-LEU2* interval. This is to be expected

due to the effects of interference, whereby the presence of one crossover limits the presence of a second crossover in the immediate vicinity. Another possibility is that the frequencies of the DSBs themselves are regulated. Increasing the frequency of DSBs at one site may lead to a concomitant reduction at an adjacent site (Wu and Lichten, 1995). Bas1p/Bas2p are only involved in the regulation of some DSB hotspots, particularly those associated with genes in the adenine and histidine biosynthesis pathways (Arndt *et al.*, 1987; Daignan-Fornier and Fink, 1992; Denis *et al.*, 1998; Springer *et al.*, 1996; Tice-Baldwin *et al.*, 1989). Consistent with this, crossing over in the *LEU2-MAT* and *CYH2-TRP5* intervals is unaffected by adenine auxotrophy or by starvation for amino acids. A small increase was observed in the *MET13-CYH2* interval, when sporulation occurred under starvation conditions. This may be due to the up-regulation of multiple biosynthesis pathways by Gcn4p in response to amino acid starvation (reviewed in Hinnebusch, 2005). If a gene involved in one of these pathways is situated within this interval, then an increase in recombination may be expected due to stimulation of DSB production. However, this increase was not consistently observed.

Despite the variation in NMS at *HIS4* observed in the *exo1*Δ Ade$^-$ strains, for all *exo1*Δ strains studied the same trend was observed between NMS and crossing over in the *NAT-HYG* interval as in the wild-type: as NMS increased, so did crossing over. The only difference is that fewer events occur in the absence of Exo1p (Khazanehdari *et al.*, 2000; Kirkpatrick *et al.*, 2000; Meadows, Hoffmann and Borts, unpublished) due to the reduced processing of DSB ends (see Chapter 4). This causes the *his4-ATC* mismatch to be included in heteroduplex DNA less frequently than in the wild-type. Additionally this reduces crossing over, potentially due to less stable strand invasions being made by the shorter 3′ single-stranded DNA overhangs. Therefore fewer gene conversion and crossover events are observed in the absence of Exo1p. As some gene conversion does still occur, this suggests that a gradient of resection exists.

As observed in Chapter 4, the absence of Exo1p does not affect the association between NMS at *HIS4* and crossovers in the *NAT-HYG* interval. This means that the proportion of DSBs that give rise to a gene conversion and crossover event

simultaneously is not affected. It has been demonstrated recently that crossover events are more likely to be associated with longer tracts of DNA synthesis than non-crossover events (Terasawa *et al.*, 2007). Therefore, if the resection extends far enough to produce a gene conversion, then the tract of hDNA may already be a considerable length. This makes it more likely that the intermediate formed is stable enough to give rise to a crossover also. Therefore, although gene conversion is reduced in the absence of Exo1p, the gene conversion events that do occur are just as likely to be associated with a crossover as they would be in a wild-type background. Additionally, altering NMS by changing sporulation media or the ability to synthesise adenine does not affect this association.

5.5 Future Work

To determine the functionality of the *ade1-1* allele, it would be of interest to delete the entire *ADE1* gene, and observe whether there is a difference in the level of gene conversion of *HIS4* compared to using the *ade1-1* allele. If the *ade1-1* allele is leaky and partly functional, then a deletion of *ADE1* should significantly reduce NMS compared to the frequencies observed when using this allele. If the *ade1-1* allele is non-functional then no difference should be observed. Secondly, it would be of interest to test mutants of different genes in the adenine biosynthesis pathway. If the *ade1-1* allele does block this pathway and the increased events are a result of the production of AICAR through the histidine biosynthesis pathway, then mutating *ADE17* or *ADE12* should increase events at *HIS4* further. As *ADE17* and *ADE12* act at steps after the production of AICAR and SAICAR, they should accumulate these intermediates. Consequently up-regulation could still occur, and even more of an increase in events at *HIS4* would be observed. If the *ade1-1* mutation is leaky and the adenine pathway is still partly active, then less of a difference should be observed.

Chapter 6: Processing of the *HIS4* Double-Strand Break by Exo1p

Chapter 6: Processing of the *HIS4* Double-Strand Break by Exo1p

6.1 Introduction

6.1.1 The Polarity Gradient at *HIS4*

As discussed previously (Chapter 1), DNA mismatches in heteroduplex DNA (hDNA) formed during meiotic recombination give rise to non-Mendelian segregation (NMS) events. Gene conversions (GC) arise as a result of repair of these events whilst post-meiotic segregation (PMS) events occur as a result of failure to repair these mismatches. Several loci exhibit a gradient of NMS events along the length of the gene. The high end of the gradient is adjacent to the double-strand break (DSB) that initiates recombination, and the frequency of gene conversion decreases with distance away from the hotspot. One such DSB hotspot is *HIS4*, and at this locus the gradient proceeds from the 5′ to the 3′ end of the gene (Alani *et al.*, 1994; White *et al.*, 1992). A number of models have been proposed to explain the presence of a polarity gradient, as discussed in Chapter 1. A central feature to all of these models is the presence of tracts of hDNA produced between homologous chromosomes during meiotic recombination. The distance that hDNA extends is not clear. In a *Saccharomyces cerevisiae* S288c background it has been shown that a proportion of hDNA formed spans the entire *HIS4* gene, which is 2.4 kb in length (Detloff *et al.*, 1992; Porter *et al.*, 1993). However, in a *S. cerevisiae* Y55 background only short gene conversion tracts were detected, where less than 10% of gene conversion tracts extended as far as 1.6 kb (from the start codon) in the *HIS4* gene (Hoffmann *et al.*, 2005).

6.1.2 MMR Proteins are Involved in hDNA Repair During Meiosis

A number of mismatch repair proteins are involved in the repair of hDNA containing mismatches. In the absence of Pms1p, Mlh1p or Msh2p an increase in PMS events is observed (Alani *et al.*, 1994; Hunter and Borts, 1997; Kramer *et al.*, 1989; Prolla *et al.*, 1994; Reenan and Kolodner, 1992; Wang *et al.*, 1999; Williamson *et al.*, 1985), indicative of a failure to repair mismatches. An *mlh1Δpms1Δ* double mutant has levels of PMS events equivalent to either single mutant indicating that they function in the same pathway for meiotic repair (Prolla *et al.*, 1994).

As no increase in PMS events was observed in an *exo1*Δ mutant it was concluded that Exo1p is not involved in the repair of mismatches during meiosis (Khazanehdari and Borts, 2000; Kirkpatrick *et al.*, 2000; Meadows, Hoffmann and Borts, unpublished). However, as discussed in Chapters 4 and 5 gene conversion is reduced in a locus specific manner in the absence of Exo1p. At *HIS4*, the total NMS events are reduced approximately two-fold compared to wild-type whereas at other intervals there is no decrease (Khazanehdari and Borts, 2000; Meadows, Hoffmann and Borts, unpublished; Chapter 4). This reduction in gene conversion occurs along the entire length of the *HIS4* gradient, although the gradient itself is maintained (Khazanehdari and Borts, 2000).

6.1.3 Processing of Double-Strand Break Ends

The effect on the *HIS4* polarity gradient observed in the absence of Exo1p could arise as a result of a defect in the processing of DSB ends. Less resection could result in shorter tracts of hDNA being produced, due to the presence of a shorter invading 3′ end. Consequently, there is less opportunity for distal mismatches to become incorporated into hDNA and be subjected to repair. In order to test this Tsubouchi and Ogawa (2000) measured the formation and processing of DSBs at *HIS4::LEU2*. In the absence of Exo1p, the disappearance of DSBs was delayed compared to wild-type by 1-2 hours. To further characterise this effect a mutant of Dmc1p was used, as these strains are blocked at the strand invasion stage and accumulate hyper-resected DSBs (Bishop *et al.*, 1992; reviewed in Masson and West, 2001). These single-stranded tails appear as a long smear by Southern blot analysis (Tsubouchi and Ogawa, 2000). However, in the *exo1*Δ*dmc1*Δ mutant discrete bands were observed, indicative of a defect in the generation of the ssDNA tails (Tsubouchi and Ogawa, 2000). This is consistent with a role for Exo1p in the resection of the 5′ ends of DSBs to leave 3′ single stranded overhangs. However, there are two possible explanations. Either fewer molecules are resected overall, or all molecules are still resected, but that resection is shorter than in the wild-type (Khazanehdari and Borts, 2000). As the single-stranded tails detected by Tsubouchi and Ogawa (2000) could not be quantified, it is unknown which of these hypotheses is correct. With the current genetic studies it is also impossible to distinguish between the two.

6.1.4 Resection Length and Crossover Events

A number of studies have recently provided evidence for a direct relationship between resection length, DNA synthesis and crossing over. A mutant of *POL3* was identified that exhibited no mitotic defects but did reduce gene conversion during meiosis (Maloisel *et al.*, 2004). *POL3* encodes the catalytic subunit of DNA polymerase δ, and the identified mutant lacked the last four amino acids at the C-terminus. The *pol3-ct* (**C**-terminal **t**runcation) mutant exhibited a distance specific effect on gene conversion at *HIS4*. Markers close to the 5′ end of *HIS4* exhibited wild-type levels of gene conversion. Markers further away however, showed reduced levels of gene conversion and this reduction in events increased with distance (Maloisel *et al.*, 2004). Gene conversion was affected in a similar manner at two other loci studied, and therefore it is not a locus-specific effect. This suggests that the initiation of DSBs occurs normally, whereas the amount of hDNA formed is shorter than in a wild-type (Maloisel *et al.*, 2004). Additionally, crossing over was affected by *pol3-ct*, however, interference was unaffected. The spore viability of the mutant was also wild type, indicating that the defect in crossing over was not affecting chromosome segregation to a great extent (Maloisel *et al.*, 2004). During the processing of DSBs, the amount of hDNA formed may depend on the length of the single stranded 3′ tail, and this will be determined by the amount of resection that takes place. However, Maloisel *et al.* (2004) proposed that the length of hDNA formed is increased by Polδ through DNA synthesis. Therefore in the *pol3-ct* mutant, hDNA length is limited possibly due to a reduced processivity of the enzyme (Maloisel *et al.*, 2004). Shorter tracts of DNA synthesis may prevent stabilisation of the recombination intermediate. This may result in displacement of the invading strand and utilisation of the synthesis-dependent strand annealing (SDSA) pathway for repair, which leads to the production of non-crossovers (Allers and Lichten, 2001; reviewed in Paques and Haber, 1999). This would reduce the frequency of crossing over as was observed (Maloisel *et al.*, 2004). This also suggests that crossovers are predominantly associated with longer tracts of hDNA as opposed to shorter tracts. It was also proposed that Polδ may function in the Mus81p/Mms4p pathway for crossing over, as interference was not affected in *pol3-ct* (Maloisel *et al.*, 2004).

In contrast, in the absence of Mlh2p gene conversion events were increased at the majority of loci studied (Abdullah *et al.*, 2004). Therefore it was proposed that Mlh2p is involved in the regulation of hDNA tract length, and that in the absence of Mlh2p over-replication of DNA occurs. This results in the production of DNA flaps which require a flap endonuclease to remove. This could be either Exo1p or Mus81p/Mms4p depending on the polarity of the flap (Abdullah *et al.*, 2004). It is possible that Polδ and Mlh2p function together in the coordination of hDNA length and DNA replication.

As discussed earlier, gene conversion events can be associated with either crossovers or non-crossovers, although they arise via different pathways (reviewed in Paques and Haber, 1999). A study of the production of meiotic recombination-related DNA synthesis (MRDS) in association with either crossover or non-crossover products revealed some differences between the two (Terasawa *et al.*, 2007). Yeast strains were modified to incorporate analogues of thymidine during DNA replication, which were detectable by antibodies. MRDS tracts of approximately 1.9 kb were detected in association with crossover events whereas with non-crossover events the tracts were only approximately 0.8 kb. This phenomenon has also been observed in mammalian systems. Analysis of sperm DNA has revealed that the conversion tracts associated with a crossover event are longer than those that are not associated with a crossover event (Jeffreys and Neumann, 2002; Jeffreys and May, 2004). Consistent with these observations, the tract lengths of MRDS were reduced in a *mer3*Δ mutant (Terasawa *et al.*, 2007), which reduces crossing over without a concomitant effect on non-crossover events (Mazina *et al.*, 2004). Consequently, the coordination of DNA synthesis and hDNA length will have an impact on the production of crossovers and non-crossovers.

6.1.5 Aims

1. To use heteroalleles to analyse the length of gene conversion tracts of *HIS4* in wild-type and *exo1*Δ strains.

2. To test the hypothesis that long gene conversion tracts are more likely to be associated with a crossover.

6.2 Materials and Methods

6.2.1 Strains

All haploid strains used in this chapter were derived from H394 (*MATa* line) or H330 (*MATα* line; Borts and Haber, 1989; Lichten *et al.*, 1987). Therefore all haploids of the same mating type are congenic, but the diploids are isogenic. These strains were used because they exhibit higher levels of NMS at *HIS4* (Abdullah and Borts, 2001; Abdullah *et al.*, 2004). The *his4::Cla*I allele is a 2 bp insertion at the *Cla*I restriction site (532 bp; Borts and Haber, 1989), and the *his4::Xho*I (Borts *et al.*, 1990), *his4::Eco*RI (Borts and Haber, 1989) and *his4::Bgl*II (Khazanehdari and Borts, 2000) alleles are 4 bp insertions at the restriction sites (96, 718 and 1688 bp respectively; Figure 6.1). All of these insertions ablate the respective restriction sites. The diploid strains used for analysis are in Table 6.1.

6.2.2 Strain Construction

To introduce the *his4::Cla*I allele, the plasmid pRHB12 (Table 2.1) containing this allele and *URA3* as a selectable marker, was digested using *Sac*I and transformed into a His^+ strain. Transformants were selected on uracil omission media (Chapter 2, 1.4), purified and replicated to 5′-FOA to select for loss of the plasmid. This "pop-in pop-out" method requires two rounds of recombination to firstly integrate the desired allele and secondly to remove the *URA3* gene and plasmid backbone. All other required combinations of alleles in the haploid strains were obtained by mating and dissection to obtain segregants (Chapter 2, 2.8).

6.2.3 Random Spore Experiments

All experiments were performed as described in Chapter 2, 2.10. The raw data for these experiments can be found in Appendix III (Table A3.1).

6.2.4 Recombination Analysis

From each set of random spore experiments, approximately 200 haploid spore colonies were picked from media selecting for canavanine and cycloheximide resistance, to YEPD. A further 200 colonies were picked from media that also selected for histidine prototrophy (Chapter 2, 1.4). The patches were replicated to omission media or drug-containing media as required, to score the genetic markers.

Table 6.1 Strains

Strain Name	Haploid Parents		Genotype[1]
VCD162	VC289	Wild-type	*his4::XhoI FUS::HYG LEU2 MATα ADE1 TRP5 cyh2 met13-4 CAN1*
	VC284		*RRP7::NAT his4::EcoRI leu2-k MATa ade1-1 trp5-1 CYH2 MET13 can1*
VCD169	VC304	Wild-type	*his4::XhoI FUS1::HYG LEU2 MATα ade1-1 TRP5 cyh2 met13-4 CAN1*
	VC284		*RRP7::NAT his4::EcoRI leu2-k MATa ade1-1 trp5-1 CYH2 MET13 can1*
VCD177	VC329	Wild-type	*his4::ClaI FUS1::HYG leu2-r MATα ADE1 TRP5 cyh2 met13-4 CAN1*
	VC328		*RRP7::NAT his4::EcoRI LEU2 MATa ade1-1 trp5-1 CYH2 MET13 can1*
VCD183	VC336	Wild-type	*his4::ClaI FUS1::HYG leu2-r MATα ade1-1 TRP5 cyh2 met13-4 CAN1*
	VC328		*RRP7::NAT his4::EcoRI LEU2 MATa ade1-1 trp5-1 CYH2 MET13 can1*
VCD180	VC333	Wild-type	*his4::BglII FUS1::HYG leu2-r MATα ADE1 TRP5 cyh2 met13-4 CAN1*
	VC328		*RRP7::NAT his4::EcoRI LEU2 MATa ade1-1 trp5-1 CYH2 MET13 can1*
VCD174	VC320	Wild-type	*his4::BglII FUS1::HYG leu2-r MATα ade1-1 TRP5 cyh2 met13-4 CAN1*
	VC328		*RRP7::NAT his4::EcoRI LEU2 MATa ade1-1 trp5-1 CYH2 MET13 can1*

Table 6.1 Continued

Strain Name	Haploid Parents	Genotype[1]
VCD163	VC290	*exo1Δ::kanMX4* *his4::XhoI* *FUS1::HYG* *LEU2* *MATα* *ADE1* *TRP5* *cyh2* *MET13* *CAN1*
	VC285	*RRP7::NAT* *his4::EcoRI* *leu2-k* *MATa* *ade1-1* *trp5-1* *CYH2* *met13-2* *can1*
VCD165	VC305	*exo1Δ::kanMX4* *his4::XhoI* *FUS1::HYG* *LEU2* *MATα* *ade1-1* *TRP5* *cyh2* *MET13* *CAN1*
	VC285	*RRP7::NAT* *his4::EcoRI* *leu2-k* *MATa* *ade1-1* *trp5-1* *CYH2* *met13-2* *can1*
VCD178	VC330	*exo1Δ::kanMX4* *his4::ClaI* *FUS1::HYG* *leu2-r* *MATα* *ADE1* *TRP5* *cyh2* *met13-4* *CAN1*
	VC327	*RRP7::NAT* *his4::EcoRI* *LEU2* *MATa* *ade1-1* *trp5-1* *CYH2* *MET13* *can1*
VCD184	VC339	*exo1Δ::kanMX4* *his4::ClaI* *FUS1::HYG* *leu2-r* *MATα* *ade1-1* *TRP5* *cyh2* *met13-4* *CAN1*
	VC327	*RRP7::NAT* *his4::EcoRI* *LEU2* *MATa* *ade1-1* *trp5-1* *CYH2* *MET13* *can1*
VCD176	VC319	*exo1Δ::kanMX4* *his4::BglII* *FUS1:HYG* *leu2-r* *MATα* *ADE1* *TRP5* *cyh2* *met13-4* *CAN1*
	VC327	*RRP7::NAT* *his4::EcoRI* *LEU2* *MATa* *ade1-1* *trp5-1* *CYH2* *MET13* *can1*
VCD175	VC321	*exo1Δ::kanMX4* *his4::BglII* *FUS1::HYG* *leu2-r* *MATα* *ade1-1* *TRP5* *cyh2* *met13-4* *CAN1*
	VC327	*RRP7::NAT* *his4::EcoRI* *LEU2* *MATa* *ade1-1* *trp5-1* *CYH2* *MET13* *can1*

[1] All strains were also *lys2-d/lys2-c* and *ura3/ura3*.

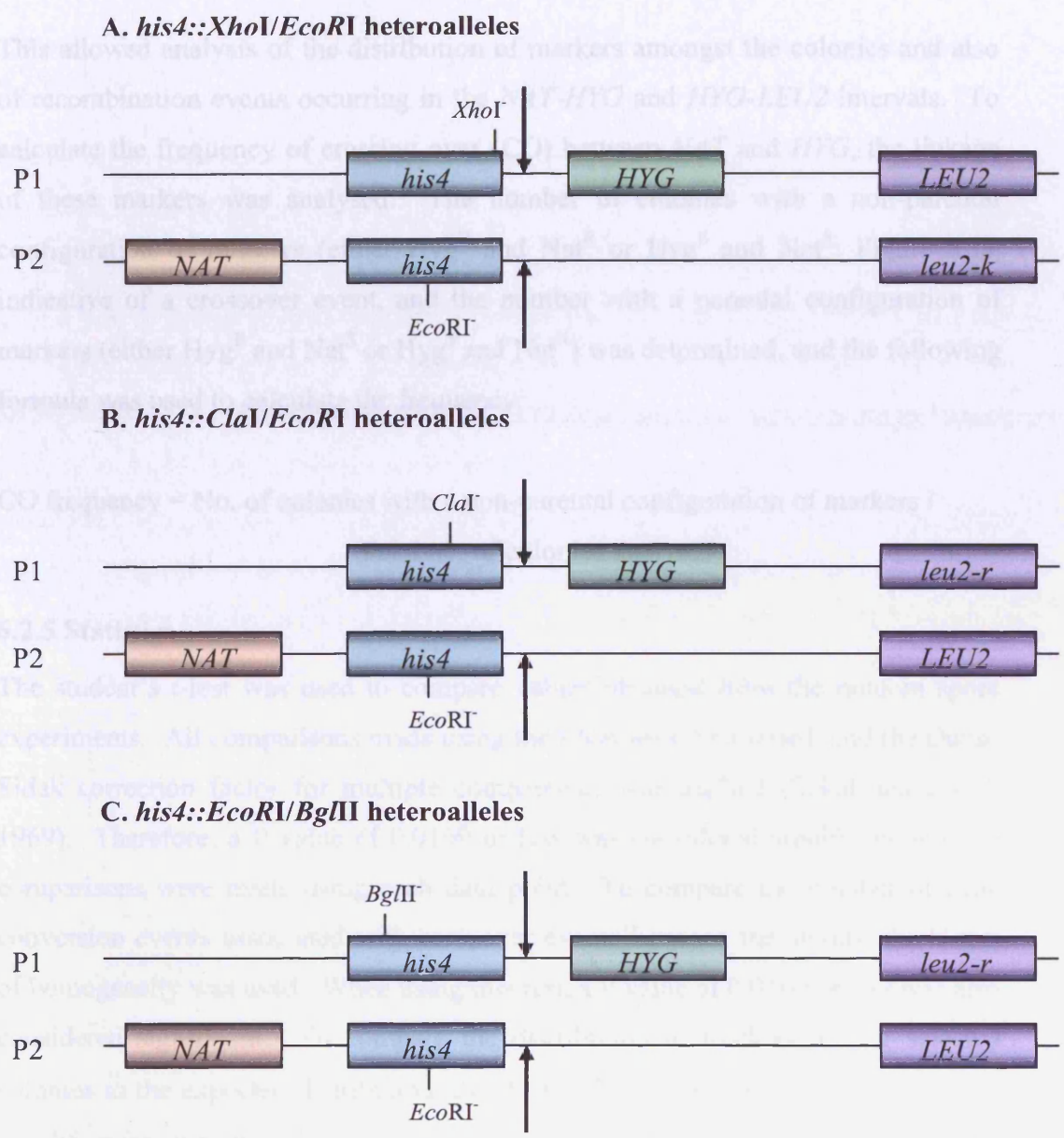


Figure 6.1 Linkage of Alleles on Chromosome III
Three strains were used containing different pairs of *his4* heteroalleles (A, B and C). P1 and P2 are chromosomes from parent 1 and parent 2 respectively. The positions of the mutated restriction sites used to analyse gene conversion in the three diploids are represented. All four mutations result in an auxotrophic phenotype for histidine. In all experiments, one of the parental haploid strains contained a *HIS4* gene with a mutated *Eco*RI restriction site (Parent 2). This was crossed to a second haploid strain containing either a mutated *Xho*I site, *Cla*I site or *Bgl*II site (Parent 1). The *Xho*I site is 96 bp into the open reading frame (ORF), the *Cla*I site is 532 bp, the *Eco*RI site is 718 bp and the *Bgl*II site is 1688 bp with respect to the start codon. All distances are relative to the start of the *HIS4* open reading frame. *HIS4* is 2.4 kb. *NAT* and *HYG* were always present on different parental chromosomes to one another however the linkage of the *LEU2* allele with *HYG* varied. The arrows represent the position of the DSB made in relation to *HIS4*. This break can be made on either of the two homologous chromosomes.

This allowed analysis of the distribution of markers amongst the colonies and also of recombination events occurring in the *NAT-HYG* and *HYG-LEU2* intervals. To calculate the frequency of crossing over (CO) between *NAT* and *HYG*, the linkage of these markers was analysed. The number of colonies with a non-parental configuration of markers (either Hyg^R and Nat^R or Hyg^S and Nat^S; Figure 6.1), indicative of a crossover event, and the number with a parental configuration of markers (either Hyg^R and Nat^S or Hyg^S and Nat^R) was determined, and the following formula was used to calculate the frequency:

CO frequency = No. of colonies with a non-parental configuration of markers / Total no. of colonies analysed

6.2.5 Statistics

The student's t-test was used to compare values obtained from the random spore experiments. All comparisons made using the t-test were two-tailed, and the Dunn-Sidak correction factor for multiple comparisons was applied (Sokal and Rohlf, 1969). Therefore, a P-value of 0.0169 or less was considered significant as three comparisons were made using each data point. To compare the number of gene conversion events associated with crossover events between the strains, the G-test of homogeneity was used. When using this test, a P value of 0.0169 or less was also considered significant. To compare the distribution of markers among selected colonies to the expected distribution, the Pearson's χ^2 test was used, and a P-value of 0.05 or less was considered significant. A test for binomial distribution was used to determine the probability of observing the frequency of gene conversion events changing in the same direction in the absence of Exo1p compared to wild-type in all experiments performed.

6.3 Results

6.3.1 The Frequency of Gene Conversion Events Decreases with Distance from the *HIS4* Double-Strand Break

To determine the effect of distance from the *HIS4* DSB on the frequency of gene conversion in *exo1*Δ strains compared to wild-type, *his4* heteroalleles were used that covered different regions of *HIS4* (Figure 6.1). To restore histidine prototrophy through gene conversion, the tract of hDNA has to terminate between the mutations present in *his4*. Therefore this provides a basic method of measuring the length of resection tracts. When 5′-3′ resection of the DNA results in inclusion of one of the mutated restriction sites in the heteroduplex region, gene conversion in one direction can replace the deleted region with wild-type information and restore functionality of *HIS4*. Therefore, colonies with an ability to synthesise histidine were selected (Table 6.2). The first restriction site of each pair of heteroalleles used was positioned with increasing distance away from the *HIS4* DSB and consequently the hDNA tract would have to extend further to contain these mismatches. Strains auxotrophic and prototrophic for adenine were also used to see if increased events were observed when the strains were unable to synthesise adenine, as discussed in Chapter 5.

In the wild-type, a lower frequency of GC events was obtained when using the *his4::Eco*RI/*Bgl*II heteroalleles compared to the *his4::Xho*I/*Eco*RI heteroalleles (Table 6.2). However, this difference was only significant in the Ade$^-$ wild-type strain ($P = 0.0053$, t-test), where a 2.1-fold decrease was observed between heteroalleles. Statistical comparisons were not made between the frequencies of GC events obtained when using the *his4::Cla*I/*Eco*RI heteroalleles compared to the other pairs of heteroalleles, as very low frequencies of GC were obtained for this interval. This was due to the smaller distance between the *Cla*I and *Eco*RI restriction sites compared to between *Xho*I and *Eco*RI, and *Eco*RI and *Bgl*II, which greatly reduces the opportunity for hDNA to terminate in that interval. No significant differences were observed between the corresponding Ade$^+$ and Ade$^-$ wild-type strains for any of the heteroalleles used ($P \geq 0.02$, t-test). As a whole the data is consistent with the expectations from a gradient of resection.

Table 6.2 Gene Conversion Between Different *his4* Heteroalleles

Strain	Heteroalleles[1]	Ade Phenotype	GC Events ± SD (%)[2]
Wild-type	*Xho*I / *Eco*RI	+	2.12 ± 0.52
	*Xho*I / *Eco*RI	-	2.44 ± 0.33
	*Cla*I / *Eco*RI	+	0.21 ± 0.03
	*Cla*I / *Eco*RI	-	0.28 ± 0.02
	*Eco*RI / *Bgl*II	+	1.15 ± 0.37
	*Eco*RI / *Bgl*II	-	1.14 ± 0.24
exo1Δ	*Xho*I / *Eco*RI	+	1.52 ± 0.33
	*Xho*I / *Eco*RI	-	2.10 ± 0.92
	*Cla*I / *Eco*RI	+	0.10 ± 0.02
	*Cla*I / *Eco*RI	-	0.17 ± 0.03
	*Eco*RI / *Bgl*II	+	0.56 ± 0.21
	*Eco*RI / *Bgl*II	-	0.88 ± 0.19

[1] Three pairs of *his4* heteroalleles were used to assay the amount of gene conversion at different positions along the *HIS4* gene (Figure 6.1).

[2] The average and standard deviation of GC events were calculated from three independent experiments. The percentage of His^+ events was calculated by:

(number of histidine prototrophs / total number of colonies) x 100

6.3.2 Gene Conversion Events are Reduced in the Absence of Exo1p

To study the function of Exo1p in the processing of DSBs, the same *his4* heteroalleles were used to study gene conversion in *exo1*Δ strains. As discussed in Chapters 4 and 5, gene conversion is reduced approximately 2-fold at *his4-ATC* in the absence of Exo1p (Meadows, Hoffmann and Borts, unpublished).

In the *exo1*Δ strains, the frequency with which gene conversion occurred also decreased with increasing distance from the initiating DSB (Table 6.2). However, the frequency of GC events obtained using the *his4::Eco*RI/*Bgl*II heteroalleles was only significantly lower than the frequency obtained using the *his4::Xho*I/*Eco*RI heteroalleles in the Ade$^+$ *exo1*Δ strain (P = 0.014, t-test). No statistical differences were observed between the frequencies of GC events obtained for the *exo1*Δ Ade$^+$ and Ade$^-$ strains for any of the *his4* heteroalleles used (P > 0.02, t-test).

Although GC events occurred less frequently in the absence of Exo1p compared to wild-type, these differences were only minor and none were statistically significant (P > 0.05, t-test). However, in all six cases the frequency of GC events observed in *exo1*Δ was lower than those observed in the corresponding wild-type strain (Table 6.2), and this is unlikely to have occurred by chance. This was significantly different (P = 0.0156, exact binomial) from the expected distribution due to random variation, where values higher than wild-type would be expected with equal frequency as values lower than wild-type.

6.3.3 Longer Tracts of Resection are More Associated with Crossing Over in the *NAT-HYG* Interval

To determine the association of crossovers with gene conversion events occurring at different distances from the *HIS4* DSB, crossing over was assessed in the *NAT-HYG* interval (Table 6.3). In the wild-type Ade$^+$ strain, 54% of GC events occurring between 96 bp and 718 bp of *HIS4* (*Xho*I and *Eco*RI sites respectively) were associated with a crossover event. The association between GC and crossover events increased when looking at events terminating between 532 bp and 718 bp (*Cla*I and *Eco*RI sites respectively) from the start of the *HIS4* open reading frame (ORF). In the wild-type Ade$^+$ strain, there was 80.5% association between the

Table 6.3 Association of Crossovers in the *NAT-HYG* Interval with Gene Conversion Events at *HIS4*

Heteroalleles	Ade Phenotype	GC with a Crossover at *NAT-HYG*[1] (%)		CO:NCO ratio[2]	
		Wild-type	*exo1*Δ	Wild-type	*exo1*Δ
*Xho*I / *Eco*RI	+	108 (54.0)	75* (37.5)	1.17	0.60
	-	107 (53.5)	56* (28.0)	1.15	0.39
*Cla*I / *Eco*RI	+	161 (80.5)	132* (66.0)	4.13	1.94
	-	155 (77.5)	136* (68.3)	3.44	2.16
*Eco*RI / *Bgl*II	+	165 (82.5)	154 (77.0)	4.71	3.35
	-	153 (76.5)	137 (68.5)	3.26	2.17

[1] The number of colonies with a GC event at *HIS4* and a crossover event in the *NAT-HYG* interval. A total of 200 colonies with a GC event at *HIS4* were scored for crossing over, except for the *exo1*Δ Ade$^-$ strain containing the *Cla*I/*Eco*RI heteroalleles for which only 199 colonies were scored. Comparisons were made between the corresponding values for the wild-type and *exo1*Δ strains using the G-test of homogeneity. Significant differences are denoted by *.

[2] The crossover: non-crossover ratio for *NAT-HYG* was calculated by:
% His$^+$ colonies with a crossover / % His$^+$ colonies without a crossover

events, which was significantly higher than the 54% association obtained for the region between the *Xho*I and *Eco*RI restriction sites ($P = 1.14 \times 10^{-08}$, G-test). A similar level of correlation was observed when covering the region furthermost from the 5′ end of *HIS4*, between 718 bp and 1688 bp (*Eco*RI and *Bgl*II sites respectively). Of the selected colonies with GC events, 82.5% had an associated crossover event in the wild-type Ade^+ strain, which was not different to the frequency of association observed between the *Cla*I and *Eco*RI restriction sites ($P > 0.05$, G-test). No differences were observed in the association of GC events and crossing over between Ade^+ and Ade^- strains in any of the regions studied ($P > 0.05$, G-test). In conclusion, with increasing distance from the *HIS4* DSB (and decreasing levels of gene conversion), the association with crossing over in the *NAT-HYG* interval increases (Figure 6.2).

In the Ade^+ *exo1*Δ mutant, only 37.5% of GC events occurring between the *Xho*I and *Eco*RI restriction sites were associated with a crossover in the *NAT-HYG* interval (Table 6.3). When analysing GC events occurring between the *Cla*I and *Eco*RI restriction sites, an increase in the association between GC events and crossing over was observed as in the wild-type. In the *exo1*Δ Ade^+ strain, 66% association was observed and this was significantly higher than the value obtained when using the *his4::Xho*I/*Eco*RI heteroalleles ($P = 9.27 \times 10^{-09}$, G-test). The association increased again between *Eco*RI and *Bgl*II compared to between *Cla*I and *Eco*RI, with 77% being observed in the *exo1*Δ Ade^+ strain ($P = 0.015$, G-test). No differences were observed in the association of GC events at *HIS4* with crossing over in the *NAT-HYG* interval between the Ade^+ and Ade^- *exo1*Δ strains in any region studied ($P \geq 0.04$, G-test). Therefore, the same correlation was observed as in the wild-type strains, whereby association with crossing over in the *NAT-HYG* interval increased with distance away from the initiating DSB at *HIS4* (Figure 6.2).

The association between gene conversion and crossover events was significantly lower in the *exo1*Δ strains compared to the wild-type between 96 bp and 718 bp of *HIS4*, whether they were Ade^+ or Ade^- ($P = 1.75 \times 10^{-07}$ and 0.0009 respectively, G-test). This is in apparent contradiction with the data observed for the level of association between gene conversion of the *his4-ATC* allele with crossing over in

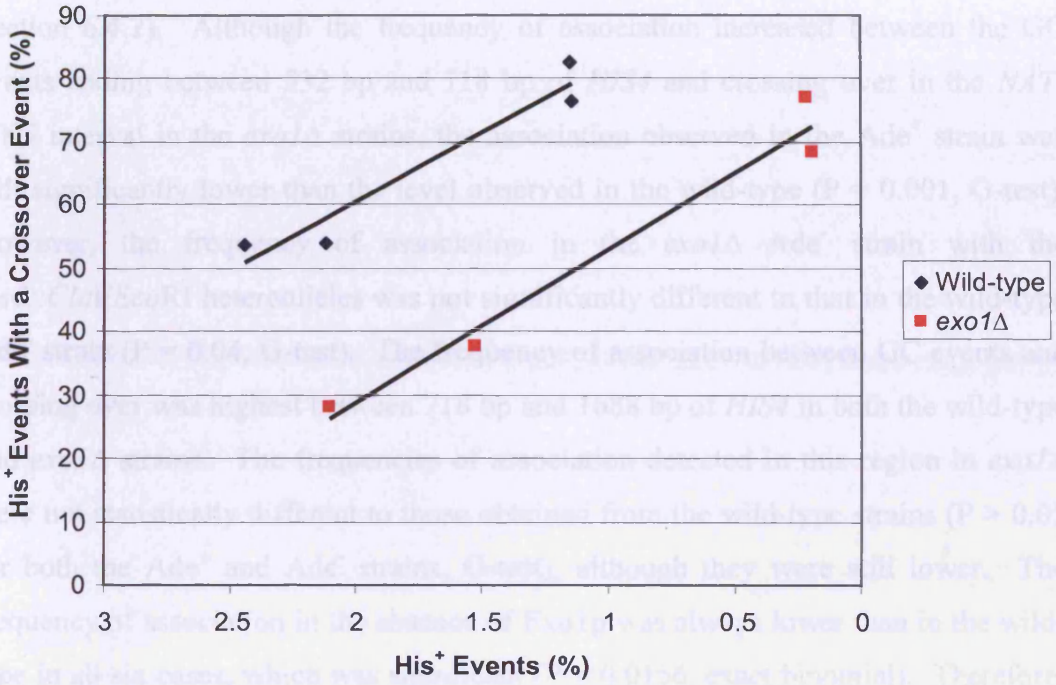


Figure 6.2 The Association Between His^+ Events and Crossing Over in the *NAT-HYG* Interval
The data for the wild-type and *exo1*Δ strains (Ade^+ and Ade^-) with the *his4::XhoI/EcoRI* and *his4::EcoRI/BglII* alleles were plotted. The percentage of His^+ events was plotted against the percentage of those events that also had a crossover in the *NAT-HYG* interval. The x-axis is orientated as to represent increasing distance away from the initiating DSB (from left to right), but is not directly correlated with this.

the wild-type and *exo1*Δ strains (Chapter 4), and this will be discussed later (see Section 6.4.2). Although the frequency of association increased between the GC events ending between 532 bp and 718 bp of *HIS4* and crossing over in the *NAT-HYG* interval in the *exo1*Δ strains, the association observed in the Ade^+ strain was still significantly lower than the level observed in the wild-type (P = 0.001, G-test). However, the frequency of association in the *exo1*Δ Ade^- strain with the *his4::ClaI/Eco*RI heteroalleles was not significantly different to that in the wild-type Ade^- strain (P = 0.04, G-test). The frequency of association between GC events and crossing over was highest between 718 bp and 1688 bp of *HIS4* in both the wild-type and *exo1*Δ strains. The frequencies of association detected in this region in *exo1*Δ were not statistically different to those obtained from the wild-type strains (P > 0.05 for both the Ade^+ and Ade^- strains, G-test), although they were still lower. The frequency of association in the absence of Exo1p was always lower than in the wild-type in all six cases, which was significant (P = 0.0156, exact binomial). Therefore, Exo1p reduces both the frequency of gene conversion events at *HIS4* and the association with crossovers.

6.3.4 Crossovers were Reduced in the *HYG-LEU2* Interval Adjacent to *NAT-HYG*

Crossing over in the adjacent interval, *HYG-LEU2*, was also analysed to determine if the crossovers occurring between *NAT-HYG* were affecting events in this interval (Table 6.4). If increased crossing over occurs in the *NAT-HYG* interval then a concomitant decrease should occur in the *HYG-LEU2* interval due to interference. With increasing distance of the heteroalleles from the *HIS4* DSB, the frequencies of association of GC events with a crossover in the *NAT-HYG* interval increased. Concomitantly a decrease was observed in crossing over in the *HYG-LEU2* interval with increasing distance. In the wild-type Ade^+ strain with the *his4::XhoI/Eco*RI heteroalleles, 54% of GC events were associated with a crossover in the *NAT-HYG* interval whereas 10% of GC events were associated with crossing over in the *HYG-LEU2* interval. In comparison, in the wild-type Ade^+ strain with the *his4::Eco*RI/*Bgl*II heteroalleles, 82.5% of GC events were associated with a crossover in the *NAT-HYG* interval whereas 4.5% of GC events were associated with a crossover in the *HYG-LEU2* interval. The same was observed for the wild-type Ade^- strain. Therefore, when a higher frequency of crossing over occurred in

Table 6.4 Crossovers in the *HYG-LEU2* Interval

Heteroalleles	Ade Phenotype	GC with a Crossover at *NAT-HYG*[1] (%)		GC with a Crossover at *HYG-LEU2*[2] (%)	
		Wild-type	*exo1*Δ	Wild-type	*exo1*Δ
*Xho*I / *Eco*RI	+	108 (54.0)	75* (37.5)	20 (10.0)	3* (1.5)
	-	107 (53.5)	56* (28.0)	12 (6.0)	3* (1.5)
*Cla*I / *Eco*RI	+	161 (80.5)	132* (66.0)	8 (4.0)	12 (6.0)
	-	155 (77.5)	136* (68.3)	7 (3.5)	5 (2.5)
*Eco*RI / *Bgl*II	+	165 (82.5)	154 (77.0)	9 (4.5)	11 (5.5)
	-	153 (76.5)	137 (68.5)	7 (3.5)	12 (6.0)

[1] Data reproduced from Table 6.3.

[2] The number of colonies with a GC event at *HIS4* and a crossover event in the *HYG-LEU2* interval. A total of 200 colonies with a GC event at *HIS4* were scored for crossing over, except for the *exo1*Δ Ade$^-$ strain containing the *Cla*I/*Eco*RI heteroalleles for which only 199 colonies were scored. Comparisons were made between the corresponding values for the wild-type and *exo1*Δ strains using the G-test of homogeneity. Significant differences are denoted by *.

the *NAT-HYG* interval, crossing over in the *HYG-LEU2* interval was reduced. However, amongst the wild-type strains none of the frequencies of association of GC events at *HIS4* with crossing over in the *HYG-LEU2* interval were statistically different from one another ($P \geq 0.02$, G-test).

For the *exo1*Δ strains with the *his4::Xho*I/*Eco*RI heteroalleles, a very low level of crossing over was observed in the *HYG-LEU2* interval, in both the Ade^+ and Ade^- strains (1.5% for both). In the *exo1*Δ strains with the *his4::Cla*I/*Eco*RI and *his4::Eco*RI/*Bgl*II heteroalleles, more crossover events were observed in the *HYG-LEU2* interval. However, not all of these differences were significant. The frequency of association in the *exo1*Δ Ade^+ strain containing the *his4::Cla*I/*Eco*RI heteroalleles was significantly higher than the association observed using the *his4::Xho*I/*Eco*RI heteroalleles ($P = 0.014$, G-test). Additionally, the *exo1*Δ Ade^- strain containing the *his4::Eco*RI/*Bgl*II heteroalleles had an association between GC and crossover events in the *HYG-LEU2* interval that was significantly higher than the association obtained in the *exo1*Δ Ade^- strain containing the *his4::Xho*I/*Eco*RI heteroalleles ($P = 0.014$, G-test). No differences were observed between Ade^+ and Ade^- strains for any of the pairs of heteroalleles studied ($P \geq 0.08$, G-test).

6.3.5 The Distribution of Phenotypes Reflects the Linkage of Markers

The phenotypes of colonies from the random spore experiments were tested to follow the segregation of several markers: *LEU2*, *ADE1*, *TRP5*, *MET13*, *NAT* and *HYG* (Table 6.5). All of the diploids studied were heterozygous for these markers, and therefore without any selection they should exhibit 1:1 segregation (For example there should be equal numbers of Ade^+ and Ade^- colonies out of the total tested). Consistent with this, a 1:1 distribution of Ade^+ and Ade^- colonies was observed in all of the wild-type and *exo1*Δ strains that were heterozygous for *ADE1* ($P \geq 0.05$, χ^2 test; Table 6.5). As these experiments involved the selection of resistance to cycloheximide, it was expected that the markers *TRP5* and *MET13* would be affected due to their proximity to *CYH2* on chromosome VII. With the exception of the Ade^+ and Ade^- *exo1*Δ strains containing the *his4::Xho*I/*Eco*RI heteroalleles, *cyh2* (conferring resistance to cycloheximide) was always associated with *TRP5* and *met13-4* (Table 6.1). Therefore, selection for cycloheximide resistance should bias the frequency towards Trp^+ and Met^- colonies. This was

Table 6.5 Phenotypes of Wild-Type and *exo1*Δ Colonies

Strain	Heteroalleles	Ade[1]	Media[2]	Phenotypes[3]													
				His^+	His^-	Leu^+	Leu^-	Ade^+	Ade^-	Trp^+	Trp^-	Met^+	Met^-	Nat^R	Nat^S	Hyg^R	Hyg^S
Wild-type	*Xho*I / *Eco*RI	+	CC	5	195	124	76*	114	86	125	75*	29	171*	100	100	115	85*
			HCC	200	0	107	93	104	96	133	67*	22	178*	23	177*	103	97
		-	CC	4	196	141	59*	0	200	146	54*	34	166*	91	109	142	58*
			HCC	200	0	114	86	0	200	161	39*	29	171*	27	173*	110	90
	*Cla*I / *Eco*RI	+	CC	0	200	119	81*	106	94	138	62*	38	162*	114	86	95	105
			HCC	200	0	155	45*	96	104	143	57*	27	173*	26	174*	51	149*
		-	CC	0	200	112	88	0	200	130	70*	36	164*	118	82*	91	109
			HCC	200	0	144	56*	0	200	121	79*	32	168*	36	164*	59	141*
	*Eco*RI / *Bgl*II	+	CC	5	195	128	72*	98	102	130	70*	46	154*	111	89	92	108
			HCC	200	0	45	155*	111	89	110	90	20	175*	183	17*	162	38*
		-	CC	3	197	146	54*	0	200	135	65*	30	170*	115	85*	82	118*
			HCC	200	0	46	154*	0	200	121	79*	21	179*	190	10*	159	41*

Table 6.5 Continued

Strain	Heteroalleles	Ade[1]	Media[2]	Phenotypes[3]													
				His^+	His^-	Leu^+	Leu^-	Ade^+	Ade^-	Trp^+	Trp^-	Met^+	Met^-	Nat^R	Nat^S	Hyg^R	Hyg^S
*exo1*Δ	*Xho*I / *Eco*RI	+	CC	3	197	128	72*	92	108	171	29*	197	3*	93	107	126	74*
			HCC	200	0	137	63*	92	108	186	14*	193	7*	17	183*	134	66*
		-	CC	4	194	125	73*	0	198	164	34*	191	7*	91	107	124	74*
			HCC	200	0	162	38*	0	200	183	17*	191	9*	23	177*	161	39*
	*Cla*I / *Eco*RI	+	CC	0	200	120	80*	103	97	171	29*	10	190*	118	82*	103	97
			HCC	200	0	138	62*	102	98	168	32*	13	187*	20	180*	66	134*
		-	CC	0	200	123	77*	0	200	162	38*	7	193*	119	81*	86	114
			HCC	199	0	135	64*	0	199	151	48*	11	188*	33	166*	61	138*
	*Eco*RI / *Bgl*II	+	CC	0	200	110	90	101	99	166	34*	13	187*	114	86	106	94
			HCC	200	0	50	150*	108	92	162	38*	11	189*	187	13*	159	41*
		-	CC	2	198	124	76*	0	200	171	29*	18	182*	121	79*	88	112
			HCC	200	0	70	130*	0	200	173	27*	12	188*	193	7*	140	60*

[1] The strains were either prototrophic (+) or auxotrophic (-) for adenine.

[2] Two types of media were used. One type of media selected for resistance to canavanine and cycloheximide (CC), whereas the second type selected for resistance to canavanine and cycloheximide, and the ability to synthesise histidine (HCC).

[3] The number of colonies are given which could / could not grow in the absence of certain amino acids (+/-) and in the presence of certain drugs (R/S). Approximately 200 colonies of each strain were tested. * represent distributions that were significantly different from 50:50. Comparisons were made using the χ^2 test.

observed in all of the relevant strains (Table 6.5; $P \leq 0.0004$, χ^2 test). In the *exo1*Δ strains containing the *his4::XhoI/EcoRI* heteroalleles, where the linkage of *MET13* and *cyh2* was different (Table 6.1), the expected bias was still observed (more Met^+ than Met^- colonies; $P \leq 4.5 \times 10^{-39}$, χ^2 test).

In the absence of selection for histidine prototrophy there should be no bias for markers present on chromosome III (Figure 6.1). However, a 1:1 segregation of *LEU2*, *HYG* and *NAT* was not always observed (Table 6.5). In the majority of the strains analysed, more Leu^+ colonies were obtained ($P \leq 0.007$, χ^2 test; Table 6.5). Only the wild-type Ade^- strain containing the *his4::ClaI/EcoRI* heteroalleles and the *exo1*Δ Ade^+ strain with the *his4::EcoRI/BglII* heteroalleles gave rise to equal numbers of Leu^+ and Leu^- colonies ($P \geq 0.09$, χ^2 test). The Ade^- wild-type and *exo1*Δ strains containing the *his4::ClaI/EcoRI* or the *his4::EcoRI/BglII* heteroalleles also gave rise to more colonies that were resistant to nourseothricin (Nat) than were sensitive ($P \leq 0.03$, χ^2 test), as did the Ade^+ *exo1*Δ strain containing the *his4::ClaI/EcoRI* heteroalleles ($P = 0.01$, χ^2 test). More hygromycin (Hyg) resistant colonies were obtained from the wild-type and *exo1*Δ strains containing the *his4::XhoI/EcoRI* heteroalleles ($P \leq 0.03$, χ^2 test), whereas the Ade^- wild-type strain containing the *his4::EcoRI/BglII* heteroalleles gave rise to more Hyg^S colonies ($P = 0.01$, χ^2 test). The skewed distribution in the number of colonies with resistance and sensitivity to hygromycin and nourseothricin were all consistent with the linkage to *LEU2* (Figure 6.1 and Table 6.5).

Different patterns emerged from colonies that were selected as being prototrophic for histidine in addition to having resistance to canavanine and cycloheximide. With selection for His^+ colonies, it was expected that the distribution of colonies with resistance to either hygromycin or nourseothricin would also be skewed, as *NAT*, *HIS4* and *HYG* are closely linked on chromosome III (Figure 6.1). In all of the strains tested, the distribution of the colony phenotypes for *TRP5* and *MET13* were consistent with the expectations based on the relative linkage of these markers with *CYH2* on chromosome VII (Table 6.5).

On chromosome III, the cassettes conferring resistance to nourseothricin and

hygromycin are on different parental chromosomes (Figure 6.1). Therefore, in the absence of a recombination event between the two markers, a given colony would be resistant to either nourseothricin or hygromycin, but not both. In the wild-type and *exo1*Δ strains containing the *his4::ClaI/Eco*RI heteroalleles, more colonies obtained were Nat^S than Nat^R, and more colonies were also Hyg^S than Hyg^R ($P \leq 1.52 \times 10^{-06}$, χ^2 test). From the wild-type and *exo1*Δ strains containing the *his4::Eco*RI/*Bgl*II heteroalleles, more colonies were Nat^R than Nat^S, and more were also Hyg^R ($P \leq 1.54 \times 10^{-08}$, χ^2 test). These patterns were inconsistent with complete linkage, indicating that crossing over was occurring between these markers. In the wild-type and *exo1*Δ strains containing the *his4::Xho*I/*Eco*RI heteroalleles, more colonies were Nat^S ($P \leq 5.5 \times 10^{-25}$, χ^2 test). In the wild-type strains containing these heteroalleles, the number of Hyg^R colonies obtained was roughly equal to the number of Hyg^S colonies obtained ($P \geq 0.16$, χ^2 test), as the frequency of association between gene conversion events and crossovers was lower in these strains. However, the *exo1*Δ strains containing these heteroalleles gave rise to more Hyg^R colonies ($P \leq 1.5 \times 10^{-06}$, χ^2 test), which was consistent with the linkage between *NAT* and *HYG* and lower levels of crossing over. Whether more Leu^+ or more Leu^- colonies were obtained was always consistent with the linkage of *LEU2* to *HYG*. No differences were observed between any of the corresponding Ade^+ and Ade^- strains.

6.3.6 Not All Crossover and Non-Crossover Marker Configurations were Equally Associated with Gene Conversion

The configuration of the markers that flank *HIS4*, *NAT* and *HYG*, was determined in colonies selected for a gene conversion event at *HIS4* (Table 6.6). This provided further information on the nature of the event that gave rise to the gene conversion that restored histidine prototrophy, such as on which parental strand the DSB was made (see Section 6.4.4). Colonies that were Nat^S and Hyg^R or Nat^R and Hyg^S were non-recombinant (parental; Table 6.6). Colonies that were Nat^S and Hyg^S or Nat^R and Hyg^R were recombinant (non-parental), and must have had a crossover event between *NAT* and *HYG*. In the wild-type strains containing the *his4::Xho*I/*Eco*RI heteroalleles, approximately equal numbers of His^+ colonies had a parental or non-parental configuration of the markers. However, the majority of crossovers were of

the Nat^S Hyg^S configuration and the majority of non-crossovers were of the Nat^S Hyg^R configuration (45.5% and 43.0% respectively in the wild-type Ade^+ strain; Table 6.6). In the wild-type strains containing the *his4::ClaI/Eco*RI heteroalleles, there was an increase in colonies with a non-parental configuration of the markers (as discussed earlier). As observed for strains containing the *his4::XhoI/Eco*RI heteroalleles, the majority of crossovers also had the Nat^S Hyg^S configuration of markers (71.0% compared with 9.5% with the Nat^R Hyg^R configuration in the wild-type Ade^+ strains; Table 6.6). In the wild-type strains containing the *his4::Eco*RI/*Bgl*II heteroalleles, the majority of colonies also had a non-parental configuration of markers. However, whereas the main non-parental configuration of markers obtained in the strains containing the *his4::XhoI/Eco*RI or the *his4::ClaI/Bgl*II heteroalleles was Nat^S Hyg^S, in these strains the opposite was observed, with the majority being Nat^R Hyg^R (77.5% in the wild-type Ade^+ strain; Table 6.6). Therefore, there is a favoured crossover configuration associated with a His^+ gene conversion and this configuration depends on the heteroalleles utilised. The data suggests that the gene situated proximal to the 3′ end of *HIS4*, and that is associated with the restriction site marker in *HIS4* that is proximal to the initiating DSB, is most likely to be associated with the His^+ gene conversion (See Figure 6.3, Discussion).

In the *exo1*Δ strains containing the *his4::XhoI/Eco*RI heteroalleles, more colonies had a parental configuration of linkage (Figure 6.1 and Table 6.6). This is consistent with the reduction in crossing over observed in the absence of Exo1p (Khazanehdari and Borts, 2000; Kirkpatrick *et al.*, 2000; Meadows, Hoffmann and Borts, unpublished). However, the main parental configuration of markers obtained was Nat^S Hyg^R (60.5% in the *exo1*Δ Ade^+ strain; Table 6.6), consistent with the observations in the corresponding wild-type strains. In the *exo1*Δ strains containing either the *his4::ClaI/Eco*RI or the *his4::Eco*RI/*Bgl*II heteroalleles, more colonies had a non-parental configuration of markers. Consistent with the wild-type, the main configuration of markers obtained in the *exo1*Δ strains containing the *his4::ClaI/Eco*RI heteroalleles was Nat^S Hyg^S (61.5% for the *exo1*Δ Ade^+ strains; Table 6.6), whereas in the strains containing the *his4::Eco*RI/*Bgl*II heteroalleles, the main crossover configuration obtained was Nat^R Hyg^R (75.0% for the *exo1*Δ Ade^+

Table 6.6 Crossover and Non-Crossover Configurations of *NAT* and *HYG* Associated with Gene Conversion at *HIS4*

Genotype	Heteroalleles	Ade[1]	Parental[2]		Non-Parental[3]		Total[4]	Crossover Frequency[5] (%)
			$Nat^R Hyg^S$	$Nat^S Hyg^R$	$Nat^R Hyg^R$	$Nat^S Hyg^S$		
			No. (%)	No. (%)	No. (%)	No. (%)		
Wild-type	*Xho*I / *Eco*RI	+	6 (3.0)	86 (43.0)	17 (8.5)	91 (45.5)	200	54.0
		-	5 (2.5)	88 (44.0)	22 (11.0)	85 (42.5)	200	53.5
	*Cla*I / *Eco*RI	+	7 (3.5)	32 (16.0)	19 (9.5)	142 (71.0)	200	80.5
		-	11 (5.5)	34 (17.0)	25 (12.5)	130 (65.0)	200	77.5
	*Eco*RI / *Bgl*II	+	28 (14.0)	7 (3.5)	155 (77.5)	10 (5.0)	200	82.5
		-	39 (19.5)	8 (4.0)	151 (75.5)	2 (1.0)	200	76.5
*exo1*Δ	*Xho*I / *Eco*RI	+	4 (2.0)	121 (60.5)	13 (6.5)	62 (31.0)	200	37.5
		-	3 (1.5)	141 (70.5)	20 (10.0)	36 (18.0)	200	28.0
	*Cla*I / *Eco*RI	+	11 (5.5)	57 (28.5)	9 (4.5)	123 (61.5)	200	66.0
		-	18 (9.0)	45 (22.6)	18 (9.0)	118 (59.3)	199	68.3
	*Eco*RI / *Bgl*II	+	37 (18.5)	9 (4.5)	150 (75.0)	4 (2.0)	200	77.0
		-	58 (29.0)	5 (2.5)	135 (67.5)	2 (1.0)	200	68.5

[1] The strains were either prototrophic (+) or auxotrophic (-) for adenine.
[2] Parental configurations of *NAT* and *HYG* are indicative of a non-crossover event associated with a gene conversion of *HIS4*.
[3] Non-parental configurations of *NAT* and *HYG* are indicative of a crossover event associated with a gene conversion at *HIS4*.
[4] The total number of colonies analysed with a gene conversion at *HIS4*.
[5] The crossover frequency is calculated by:

$$\text{Crossover frequency} = NP_1 + NP_2 / \text{Total number of colonies analysed}$$

Where NP_1 is the number of colonies that are Nat^R and Hyg^R, and NP_2 is the number of colonies that are Nat^S and Hyg^S.

strain). No differences were observed between the corresponding Ade^+ and Ade^- stains. This suggests that the manner in which the recombination intermediates are processed affects the likelihood of a gene conversion event occurring in conjunction with a crossover, and this varies depending on the heteroalleles utilised. As shown in Figure 6.3 (Section 6.4), gene conversion events that produce His^+ colonies can only arise under certain circumstances.

6.4 Discussion

6.4.1 Gene Conversion Decreases with Distance from the DSB

Previously it has been shown that a gradient of gene conversion occurs at *HIS4*, with high levels being observed at the 5′ end and low levels at the 3′ end (Abdullah *et al.*, 2004; Alani *et al.*, 1994; Khazanehdari and Borts, 2000; White *et al.*, 1992). It has been suggested that this gradient reflects a gradient of resection of DSBs (Khazanehdari and Borts, 2000), and the data obtained here is in agreement with this. During meiosis, conversion of either of the ablated restriction enzyme sites, using the homologue as a template, would restore functionality to *HIS4*. Fewer gene conversion events occurred between the *his4* heteroalleles distal to the DSB (*his4::Eco*RI/*Bgl*II) compared with those proximal to the DSB (*his4::Xho*I/*Eco*RI). This was true in both the wild-type and *exo1*Δ strains. From these experiments, it cannot be determined which of the ablated restriction sites was converted and consequently restored functionality to *HIS4*. However, the DSB repair model predicts that the DNA strand which receives the DSB will be repaired using the donor strand as a template, resulting in conversion of markers included in the hDNA. Additionally, the exact length of the resection tract that produced the gene conversion event could not be determined. However, to produce a gene conversion event that restores histidine prototrophy, the tract of hDNA had to end between the two restriction sites being utilised. Few gene conversion events were detected in the strains containing the *his4::Cla*I/*Eco*RI heteroalleles due to the small distance between the two restriction enzyme sites (184 bp). Between the *Xho*I and *Eco*RI, and the *Eco*RI and *Bgl*II sites, there was 620 bp and 972 bp respectively. Therefore, the likelihood that a resection tract would end between the *Cla*I and *Eco*RI restriction sites was much reduced compared to between the other sites. Additionally, the close proximity of the sites increases the probability of them both being included in hDNA and being co-converted (reviewed in Paques and Haber, 1999). If both markers on a single DNA strand were co-converted the resultant colony would not be His^+ and therefore would not be recovered in this experiment.

6.4.2 The Association of Gene Conversion and Crossover Events Increases with Distance from the *HIS4* DSB

There is some evidence that meiotic crossover events are more likely to be

associated with longer tracts of hDNA (Abdullah *et al.*, 2004; Jeffreys and Neumann, 2002; Jeffreys and May, 2004; Maloisel *et al.*, 2004; Terasawa *et al.*, 2007). In this study, when using the *his4::XhoI/EcoRI* heteroalleles, approximately 50% of the gene conversion events at *HIS4* were crossover associated whereas the other half were associated with non-crossovers. This is consistent with the frequency of association observed between gene conversion of the *his4-ATC* allele and crossing over in the *NAT-HYG* interval (Chapters 4 and 5). In contrast, a higher percentage of His^+ colonies that arose from gene conversion events between either the *ClaI* and *EcoRI* or the *EcoRI* and *BglII* restriction sites had an associated crossover event in the *NAT-HYG* interval. The bias towards crossover association when using the latter heteroalleles may be due to the relative positions of the alleles used in these assays in relation to the *HIS4* DSB. The region between the *ClaI* and *EcoRI* restriction sites is also inside the region covered by the *XhoI* and *EcoRI* restriction sites, yet gene conversion events occurring in this region still exhibited higher levels of associated crossovers. This suggests that hDNA tracts that do not extend as far as 500 bp into *HIS4* (the *ClaI* restriction site is at 532 bp; Figure 6.1) are less likely to be associated with a crossover. On the other hand, tracts of hDNA which extend beyond 500 bp into *HIS4* are much more likely to be associated with a crossover event in the *NAT-HYG* interval. This suggests that shorter tracts of hDNA are less frequently associated with crossovers than longer tracts, and that there may be a critical length that is required for the stabilisation of recombination intermediates to favour crossover production.

This trend was also observed in the absence of Exo1p. Gene conversion events in the *exo1*Δ mutant containing the *his4::XhoI/EcoRI* heteroalleles had lower levels of association with crossovers in the *NAT-HYG* interval than the wild-type strain. This confirms the fact that Exo1p has roles in maintaining normal levels of gene conversion and crossing over (Khazanehdari and Borts, 2000; Kirkpatrick *et al.*, 2000; Tsubouchi and Ogawa, 2000). However, the frequency of association observed here was considerably lower than that observed between crossing over and gene conversion of the *his4-ATC* allele in *exo1*Δ, where the association was not different to that observed in the wild-type (Chapters 4 and 5). When using the *his4-ATC* allele, to produce a His^+ gene conversion the hDNA tract only has to reach that marker (situated at 2 bp into *HIS4* ORF). However, the hDNA tract may also

extend a lot further into the gene. In this situation, the detected crossovers will be associated with hDNA tracts of a range of lengths. Consequently the frequency of association will be an average of different frequencies of association. However, when using the *his4::XhoI/Eco*RI heteroalleles, the hDNA tract has to end between the two markers (96 bp and 718 bp in the *HIS4* ORF respectively) and therefore measures the crossover association in that region. This may explain the differences observed between the different alleles.

In the *exo1*Δ mutant containing either the *his4::ClaI/Eco*RI or the *his4::Eco*RI/*Bgl*II heteroalleles, the association between gene conversion and crossing over was almost as high as in the wild-type strains containing these heteroalleles. Therefore, when tracts of hDNA extend beyond 532 bp (the position of the *Cla*I restriction enzyme site; Figure 6.1), and therefore are long tracts, the propensity of a gene conversion event to be associated with a crossover is much higher, as was observed in the wild-type. This is in agreement with the observations of Terasawa *et al.* (2007). However, gene conversion events were reduced in all three intervals analysed in the absence of Exo1p. Therefore the manner in which the crossover association is maintained must be an Exo1p-independent mechanism. It was proposed in Chapter 4 that the reduced processing of DSBs in the absence of Exo1p reduced the stabilisation of strand invasion intermediates. This in turn would reduce the production of crossovers. The crossover associations observed in the strains containing the *his4::XhoI/Eco*RI heteroalleles support this idea. However, when the hDNA tracts extend further due to the processing of DSBs independently of Exo1p, higher levels of crossing over are supported. This suggests that recombination intermediates that arise from DSBs at *HIS4*, which are processed in the absence of Exo1p (and produce long tracts of hDNA) are not dependent on the presence of Exo1p for their resolution. However, the frequencies of crossover association are not as high as in the wild-type, even when using the *his4::ClaI/Eco*RI or the *his4::Eco*RI/*Bgl*II heteroalleles. This suggests that a subset of these events would normally be resolved as a crossover in an Exo1p-dependent manner. It has been demonstrated that reducing the frequency of DSBs increased the amount of crossing over, a mechanism that has been termed crossover homeostasis (Martini *et al.*, 2006). Therefore it is also possible that the longer hDNA tracts are driven towards crossover production to make up for the reduction

in crossover association with the shorter tracts of hDNA in the absence of Exo1p. Although in this situation it may be expected that more crossovers would occur than in the wild-type, this might not be possible as Exo1p may also be required for the resolution of some of these recombination intermediates as crossovers. Overall, the absence of Exo1p affects two aspects of meiotic recombination; the number of gene conversion events is reduced and to varying degrees (depending on the distance from the DSB) the association between gene conversion and crossing over is also reduced.

6.4.3 Are the Random Spores Really Random?

One issue with these experiments is the nature of the 'random' spores obtained. The phenotypes of the spores should be equally represented in the absence of any selection. For the marker *ADE1*, there was no bias towards either Ade^+ or Ade^- colonies, which indicates that these experiments do give rise to random spores. However, analysis of other phenotypes of the colonies selected for on the basis of resistance to canavanine and cycloheximide revealed that the phenotypes conferred by markers on chromosome III (*LEU2*, *HYG* and *NAT*) were not equally present in colonies. In all of the strains analysed, more colonies were Leu^+ than Leu^-, potentially indicating that being able to synthesise leucine gave a positive growth advantage to colonies on this selective medium. Additionally, whether strains gave rise to more colonies that were sensitive or resistant to hygromycin and nourseothricin varied. This depended on the linkage of *LEU2* with *HYG* and *NAT* (Table 6.1). If leucine prototrophy does confer a selective advantage, then it might be expected that the results obtained in these experiments were influenced by this. However, analysis of the phenotypes of colonies selected for resistance to canavanine and cycloheximide and the ability to synthesise histidine do not support this. Firstly, in strains containing the *his4::Xho*I/*Eco*RI heteroalleles, approximately equal numbers of Leu^+ and Leu^- colonies were obtained. Secondly, when using the *his4::Eco*RI/*Bgl*II heteroalleles, more colonies with a gene conversion event at *HIS4* were Leu^-, not Leu^+. This is consistent with the linkage of the *leu2-r* allele with *HYG* in these strains (Figure 6.1). Together these results suggest that if leucine prototrophy does confer an advantage, it is not biasing the His^+ colonies selected for in these experiments.

6.4.4 Particular Crossover Configurations are More Associated with His^+ Gene Conversions

From the colonies selected for histidine prototrophy (i.e. have had a gene conversion event that restored histidine prototrophy), the pattern of association with resistance to hygromycin and/or nourseothricin with a gene conversion provides information about the mechanics of the processing of recombination intermediates required to bring about these events (Figure 6.3). In these experiments, the parental strains used were either Nat^R and Hyg^S or Nat^S and Hyg^R. Any colonies obtained that were either resistant or sensitive to both drugs (non-parental configuration) is indicative of a crossover event. DSBs are made equally frequently on both of the homologous chromosomes, as demonstrated by the presence of equal numbers of 6:2 and 2:6 gene conversions in the wild-type (e.g. Hoffmann *et al.*, 2005). Gene conversion events occurring in the strains containing the *his4::XhoI/Eco*RI heteroalleles were equally associated with either resistance or sensitivity to hygromycin, but more so with sensitivity to nourseothricin. This indicates that DSBs initiated on the chromosome from parent 2 (P2; Figure 6.3A) are more likely to give rise to a gene conversion event that will restore the functionality of *HIS4*. These events were equally associated with crossover and non-crossover events. To produce an event of this type, only the *Xho*I marker must be included in the hDNA. Additionally the invading DNA strand must be lacking the *Xho*I restriction enzyme site (*Xho*I$^-$) so that conversion of the invading strand restores the restriction enzyme site (*Xho*I$^+$; Figure 6.3A-1). In contrast, Nat^R colonies, whether associated with a crossover or not were obtained rarely. This indicates that events including *NAT* are unlikely to have had a gene conversion event. Similar subclasses of events have also been observed in random spore experiments performed by Martini *et al.* (2006), using heteroalleles of *ARG4*. One possible way of obtaining a gene conversion event associated with Nat^R would be if a crossover event not initiated from the *HIS4* DSB, occurred between the 3′ end of *HIS4* and *NAT*. The assays used by Martini *et al.* (2006) included markers flanking *ARG4*, which allowed detection of distal crossovers. They demonstrated that a small number of events in their subclass did arise through the occurrence of a distal crossover, but these were not common (Martini *et al.*, 2006). Therefore there must be a second way of establishing and processing recombination intermediates to produce a His^+ colony with a crossover

A. *his4::Xho*I/*Eco*RI heteroalleles

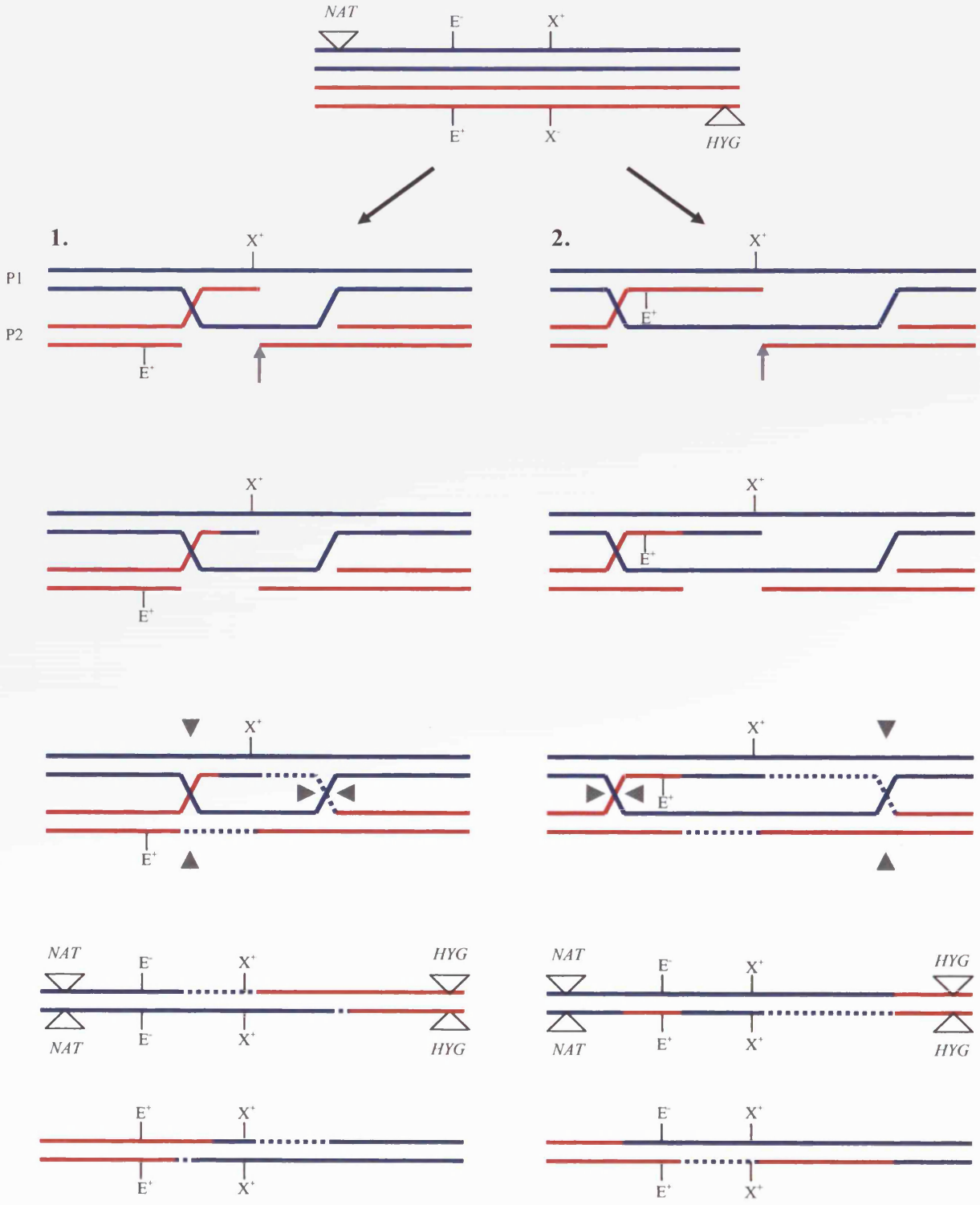


Figure 6.3A Gene Conversion of the *his4::XhoI/Eco*RI Heteroalleles Associated with a Crossover Event

E^+ and X^+ indicate the presence of the *Eco*RI and *Xho*I restriction sites respectively. The grey arrow represents the position of the DSB at *HIS4*, and the DNA strand that the break is made on. The dashed lines indicate newly synthesised DNA. The grey arrowheads indicate the direction of resolution. Only resolution in one direction is shown, however, the alternative direction results in the same configuration of markers. Not drawn to scale.

1. The *his4::Xho*I marker is included in the heteroduplex DNA arising from strand invasion. The 3′ invading end is excised to remove the mismatch, and resynthesis of DNA using the donor strand as a template converts the marker. Resolution of the double Holliday Junction results in wild-type *HIS4* not linked with *NAT* or *HYG*.
2. Both markers are included in the heteroduplex DNA arising from strand invasion, but only the *his4::Xho*I marker is excised. DNA synthesis using the donor strand as a template converts the marker. Resolution of the double Holliday Junction results in wild-type *HIS4* on one DNA strand either linked with both *NAT* and *HYG* or linked with neither.

The processing of recombination intermediates, to give rise to a His^+ gene conversion, as shown in **1** occurs more frequently as the majority of recombinant colonies were sensitive to Hyg and Nat.

resulting in resistance to both Hyg and Nat. We propose that inclusion of both the *Xho*I and *Eco*RI markers within hDNA could result in association with *NAT* (Figure 6.3A-2). However, it would depend on only the *Xho*I marker being converted, as if both were converted simultaneously the *HIS4* gene would still be mutant. In conclusion, only the production of a DSB on the *Xho*I$^-$ parental strand will lead to the production of a gene conversion event that restores histidine prototrophy.

Due to the higher frequency of crossing over associated with gene conversion events, the colonies obtained from strains containing the *his4::Cla*I/*Eco*RI heteroalleles were more often sensitive to both Nat and Hyg. This indicates that in this situation a DSB made on the chromosome from P2 (Figure 6.3B) is also more likely to result in a gene conversion that restores the wild-type *HIS4* gene. Nat^R colonies were obtained infrequently, as was observed when using the *his4::Xho*I/*Eco*RI heteroalleles. The events leading to gene conversion and crossing over from these two pairs of heteroalleles are likely to be similar due to the relative positions of the markers. Both include *Eco*RI, and *Cla*I is situated between the *Xho*I and *Eco*RI markers. Consequently, as with the *his4::Xho*I/*Eco*RI heteroalleles, the presence of the mutated *Cla*I marker in the 3′ invading end (*Cla*I$^-$) and its subsequent repair using the donor strand (*Cla*I$^+$) as a template will result in gene conversion to His$^+$ (Figure 6.3B-1). To produce a gene conversion event associated with resistance to nourseothricin the equivalent situation as described for the *his4::Xho*I/*Eco*RI heteroalleles has to occur (Figure 6.3B-2). Both markers must be included in the hDNA but only the *Cla*I$^-$ marker must be converted. Again some of these events may arise through a distal crossover event. Gene conversion events occurred with a much lower frequency when using these heteroalleles due to the short distance between the restriction sites. This decreases the chance that the hDNA tract will terminate in that region, but also increases the likelihood that co-conversion of the markers will occur, which would result in the production of a His$^-$ colony.

His$^+$ colonies from the strains containing the *his4::Eco*RI/*Bgl*II heteroalleles were more often associated with hygromycin resistance. Therefore gene conversion events that restored histidine prototrophy were more likely to result from breaks initiated on the chromosome from parent 1 (P1; Figure 6.3C). The gene conversion

B. *his4::Cla*I/*Eco*RI heteroalleles

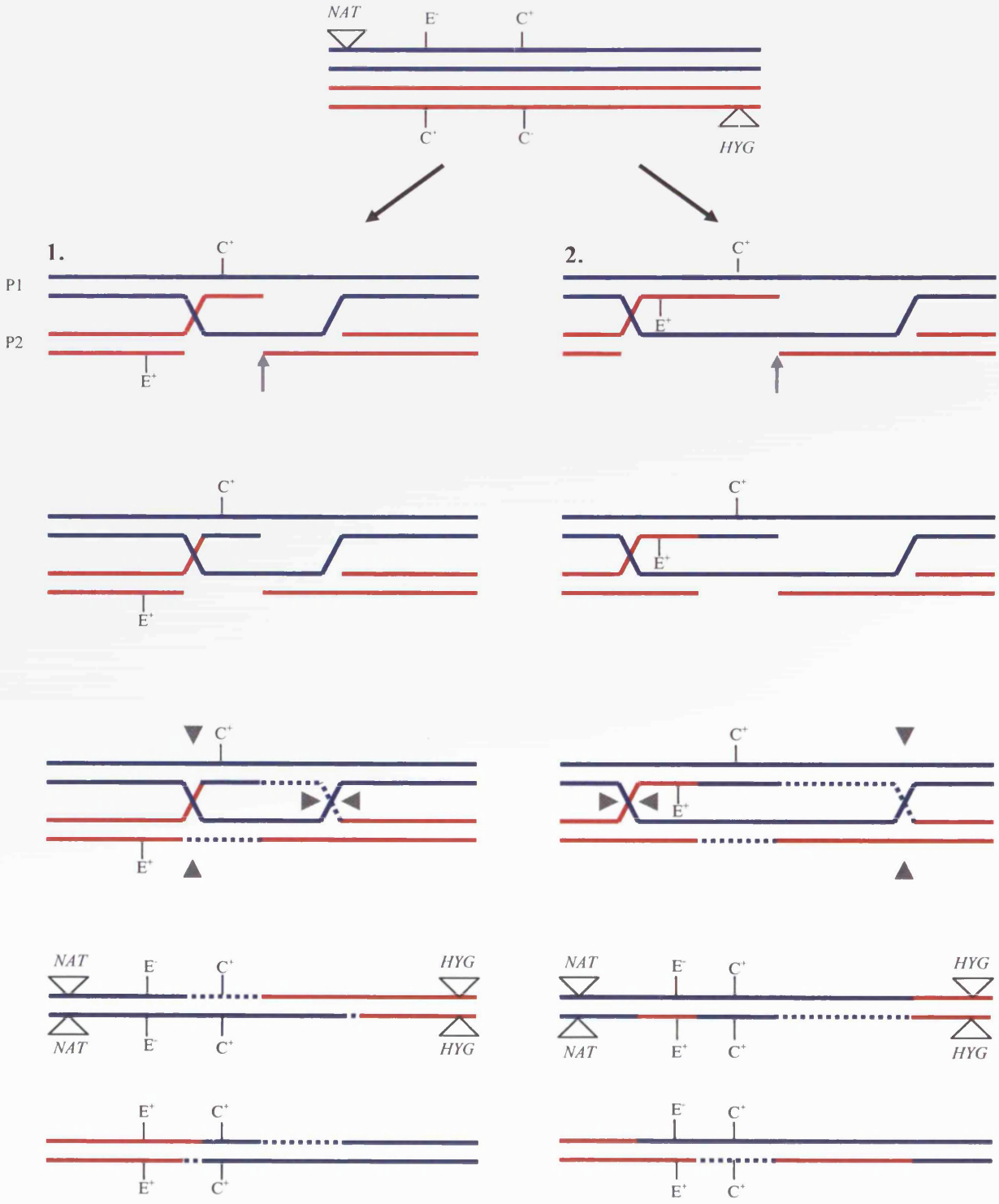


Figure 6.3B Gene Conversion of the *his4::ClaI/EcoRI* Heteroalleles Associated with a Crossover Event

E^+ and C^+ indicate the presence of the *Eco*RI and *Cla*I restriction sites respectively. The grey arrow represents the position of the DSB at *HIS4*, and the DNA strand that the break is made on. The dashed lines indicate newly synthesised DNA. The grey arrowheads indicate the direction of resolution. Only resolution in one direction is shown, however, the alternative direction results in the same configuration of markers. Not drawn to scale.

1. The *his4::Cla*I marker is included in the heteroduplex DNA arising from strand invasion. The 3′ invading end is excised to remove the mismatch, and resynthesis of DNA using the donor strand as a template converts the marker. Resolution of the double Holliday Junction results in wild-type *HIS4* not linked with *NAT* or *HYG*.
2. Both markers are included in the heteroduplex DNA arising from strand invasion, but only the *his4::Cla*I marker is excised. DNA synthesis using the donor strand as a template converts the marker. Resolution of the double Holliday Junction results in wild-type *HIS4* on one DNA strand either linked with both *NAT* and *HYG* or linked with neither.

The processing of recombination intermediates, to give rise to a His^+ gene conversion, as shown in **1** occurs more frequently as the majority of recombinant colonies were sensitive to Hyg and Nat.

events were highly associated with crossover events and therefore a large number of colonies were also resistant to nourseothricin. Gene conversion events associated with a crossover resulting in the presence of both *NAT* and *HYG* arise through the inclusion of only the *Eco*RI marker in the hDNA (Figure 6.3C-1). The mutated site must be present on the 3′ invading strand (*Eco*RI$^-$) so that conversion restores the wild-type information (*Eco*RI$^+$). In this situation, events not including *NAT* were less likely to be associated with a gene conversion event and consequently were not recovered in these experiments. Instead these events may arise when both markers are included in the hDNA but only the proximal marker, *Eco*RI, is converted (Figure 6.3C-2).

In all three situations, whether only one marker or both were included in the hDNA, resolution of the double Holliday Junctions in either orientation would give the same outcome. That is, the association with the flanking markers, *NAT* and *HYG*, is unaltered. Additionally when only one of the *his4* markers was included in the hDNA, regardless of the pair of heteroalleles being used, resolution of the events results in both DNA strands containing the necessary information to constitute a wild-type *HIS4* gene (Figure 6.3). In contrast, resolution of the events where both markers are included in the hDNA do not have the information to constitute a wild-type *HIS4* gene on both DNA strands. After sporulation, mitotic divisions of growth will separate these DNA strands. When only one DNA strand contains the wild-type *HIS4* information a sectored colony will be produced. These events are rarer as the marker configurations associated with these His$^+$ gene conversions were obtained infrequently. In conclusion, to produce a gene conversion event that will restore histidine prototrophy, initiation has to occur on the DNA strand that contains the marker that is the closest to the *HIS4* DSB.

In most cases the same patterns of marker association were observed for *exo1*Δ. However, when using the *his4::Xho*I/*Eco*RI heteroalleles more gene conversion events were associated with a parental configuration of flanking markers due to the reduction in crossing over in the absence of Exo1p. This supports the theory that shorter lengths of resection, and consequently hDNA, are less capable of stabilising recombination intermediates to ensure crossover formation. Nat^R colonies however

C. *his4::Eco*RI/*Bgl*II heteroalleles

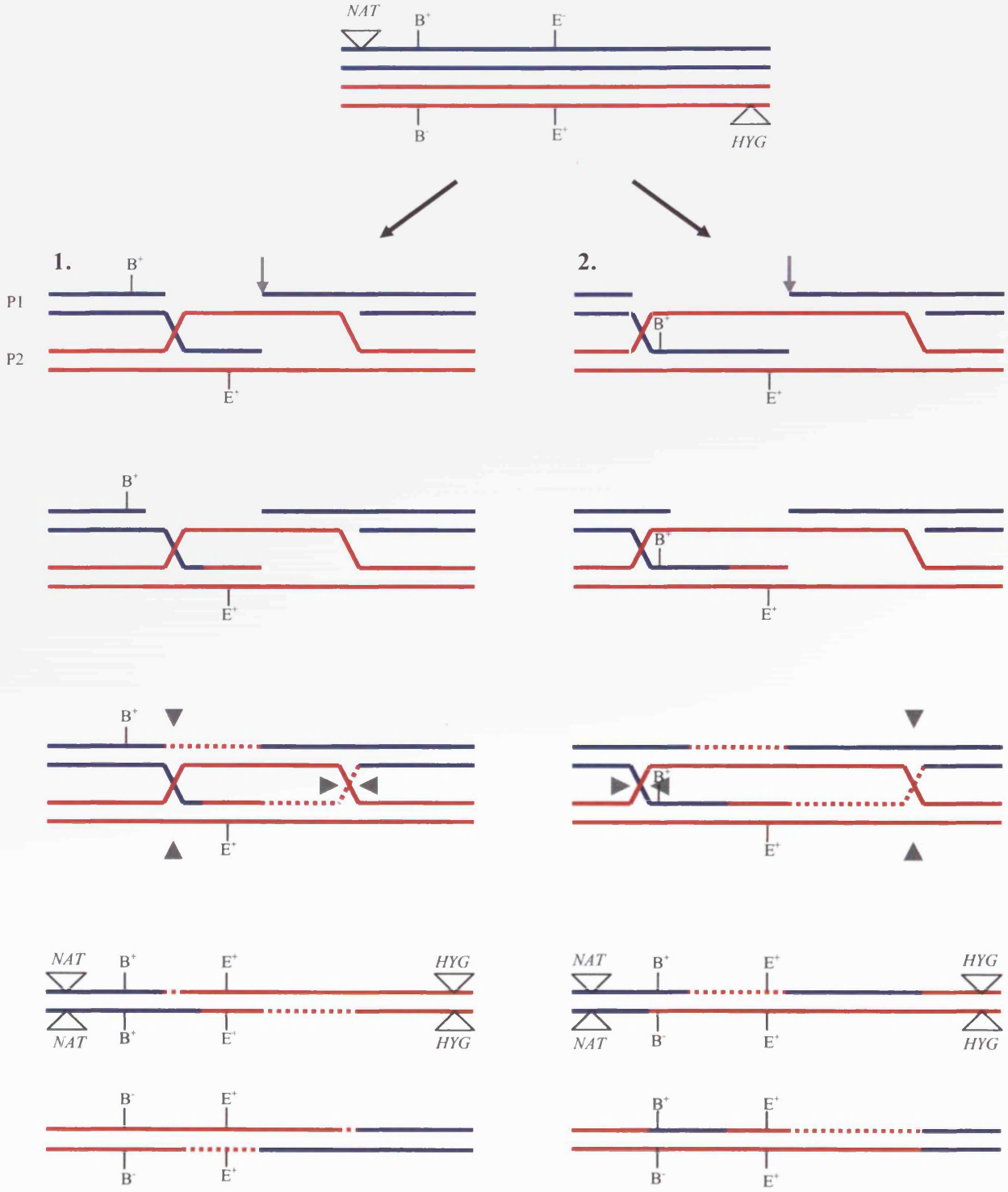


Figure 6.3C Gene Conversion of the *his4::Eco*RI/*Bgl*II Heteroalleles Associated with a Crossover Event

B^+ and E^+ indicate the presence of the *Bgl*II and *Eco*RI restriction sites respectively. The grey arrow represents the position of the DSB at *HIS4*, and the DNA strand that the break is made on. The dashed lines indicate newly synthesised DNA. The grey arrowheads indicate the direction of resolution. Only resolution in one direction is shown, however, the alternative direction results in the same configuration of markers. Not drawn to scale.

1. The *his4::Eco*RI marker is included in the heteroduplex DNA arising from strand invasion. The 3′ invading end is excised to remove the mismatch, and resynthesis of DNA using the donor strand as a template converts the marker. Resolution of the double Holliday Junction results in wild-type *HIS4* linked with *NAT* and *HYG*.
2. Both markers are included in the heteroduplex DNA arising from strand invasion, but only the *his4::Eco*RI marker is excised. DNA synthesis using the donor strand as a template converts the marker. Resolution of the double Holliday Junction results in wild-type *HIS4* on one DNA strand either linked with both *NAT* and *HYG* or linked with neither.

The processing of recombination intermediates, to give rise to a His^+ gene conversion, as shown in **1** occurs more frequently as the majority of recombinant colonies were resistant to Hyg and Nat.

were still obtained infrequently as in the wild-type. When using the *his4::ClaI/EcoRI* or the *his4::EcoRI/BglII* heteroalleles, the events obtained were reflective of the observations made in the wild-type strains, as there was also an increase in the crossover association even in the absence of Exo1p.

6.4.5 Adenine Auxotrophy does not Increase Gene Conversion Events in Strains Auxotrophic for Histidine

In Chapter 5, an increase in the frequency of gene conversion events of a single allele at *HIS4* was observed in the Ade^- strains compared to the Ade^+ strains. However, in the experiments reported here no differences were observed in the levels of gene conversion between Ade^+ and Ade^- strains, in either the wild-type or *exo1*Δ background. As discussed in Chapter 5, the strains that were auxotrophic for adenine contain a mutation in the *ADE1* gene that should prevent the production of the intermediates AICAR and SAICAR (Figure 5.3), which are responsible for promoting the interaction between Bas1p and Bas2p (Rebora *et al.*, 2001; Rebora *et al.*, 2005). Consequently, the interaction of Bas1p and Bas2p should not be activated in this situation. It was hypothesised in Chapter 5, that the observed increase in gene conversion events at *HIS4* in the Ade^- strain was a result of upregulation of AICAR production via the histidine biosynthesis pathway. The fact that no increase is observed in these experiments supports this hypothesis. The Ade^- strains used are also His^-, with a mutation in each *HIS4* allele that blocks the histidine biosynthesis pathway upstream of *HIS7*, which is responsible for AICAR production. Therefore, because the strains cannot synthesise AICAR, they do not have the ability to upregulate either of these pathways.

6.5 Future Work

It would be of interest to perform further experiments to determine the effect that Exo1p has on the gradient of gene conversion at *HIS4*. This would lead to a better understanding of the precise role(s) of Exo1p. To improve genetic methods of determining the gradient, crosses containing a single *his4* marker would be more useful in determining the end point of resection tracts. Markers at short intervals spanning the length of *HIS4* would be required to give a detailed analysis of the range of resection tracts in both a wild type situation and then in *exo1*Δ mutants.

Physical experiments could also be performed to analyse the gradient. One method of monitoring the length of resection tracts would be to use loss of restriction enzyme sites, as DNA that has been resected and is therefore single-stranded will no longer be digested. A second method would be to use quantitative amplification of single-stranded DNA (QAOS; Booth *et al.*, 2001). This could be used to amplify the regions that are resected during processing of the *HIS4* DSB.

Chapter 7: Discussion

Chapter 7: Discussion

7.1 Mlh3p Functions in the Msh4p-Msh5p Crossover Pathway

This study provides further evidence that Mlh3p is involved in the promotion of crossovers in concert with Mlh1p, as illustrated by the *mlh1Δmlh3Δ* double mutant. Previously Mlh1p and Mlh3p have been shown to interact with one another (Flores-Rozas and Kolodner, 1998; Wang *et al.*, 1999), and also with Msh4p in mammalian cells (Santucci-Darmanin *et al.*, 2000; Santucci-Darmanin *et al.*, 2002). Together this suggests that Mlh3p functions in the Msh4p-Msh5p pathway for crossing over. Mlh3p foci do not appear until early-mid pachytene (Kolas *et al.*, 2005; Lipkin *et al.*, 2002) when double Holliday Junctions (dHJs) are being formed (reviewed in Zickler and Kleckner, 1999). However, the crossover/non-crossover decision is thought to be made prior to strand invasion. Therefore, it is likely that Mlh3p is involved in the stabilisation and/or resolution of recombination intermediates rather than their distribution. This is supported by the good spore viability and relatively low levels of chromosome mis-segregation. Additionally, in the absence of Mlh3p Mlh1p foci do not form, indicating that Mlh3p is also required for recruitment of Mlh1p (Lipkin *et al.*, 2002; reviewed in Svetlanov and Cohen, 2004).

The contribution of specific proteins to interference is difficult to establish, as even in the absence of a functional interfering crossover pathway, there is a second pathway that produces crossover events. Therefore, if the Msh4p/Msh5p crossover pathway is defective, the resolution of the intermediates that would normally proceed through this pathway will depend on whether they are directed to the SDSA non-crossover pathway or are resolved by Mus81p/Mms4p to become a crossover. It is also possible that a subset of these intermediates will fail to be resolved and result in spore death. Nonetheless a number of these intermediates are still resolved as a crossover. As a consequence, although interference will be weakened due to the presence of fewer crossovers, it may not be abolished (Figure 7.1). It has been observed that chromosome size influences the level of meiotic recombination and also the strength of interference (Kaback *et al.*, 1989; Kaback *et al.*, 1999). More crossover events per kb occur on smaller chromosomes, and interference is also weaker. Consistent with this, the Msh4p-Msh5p pathway that displays interference,

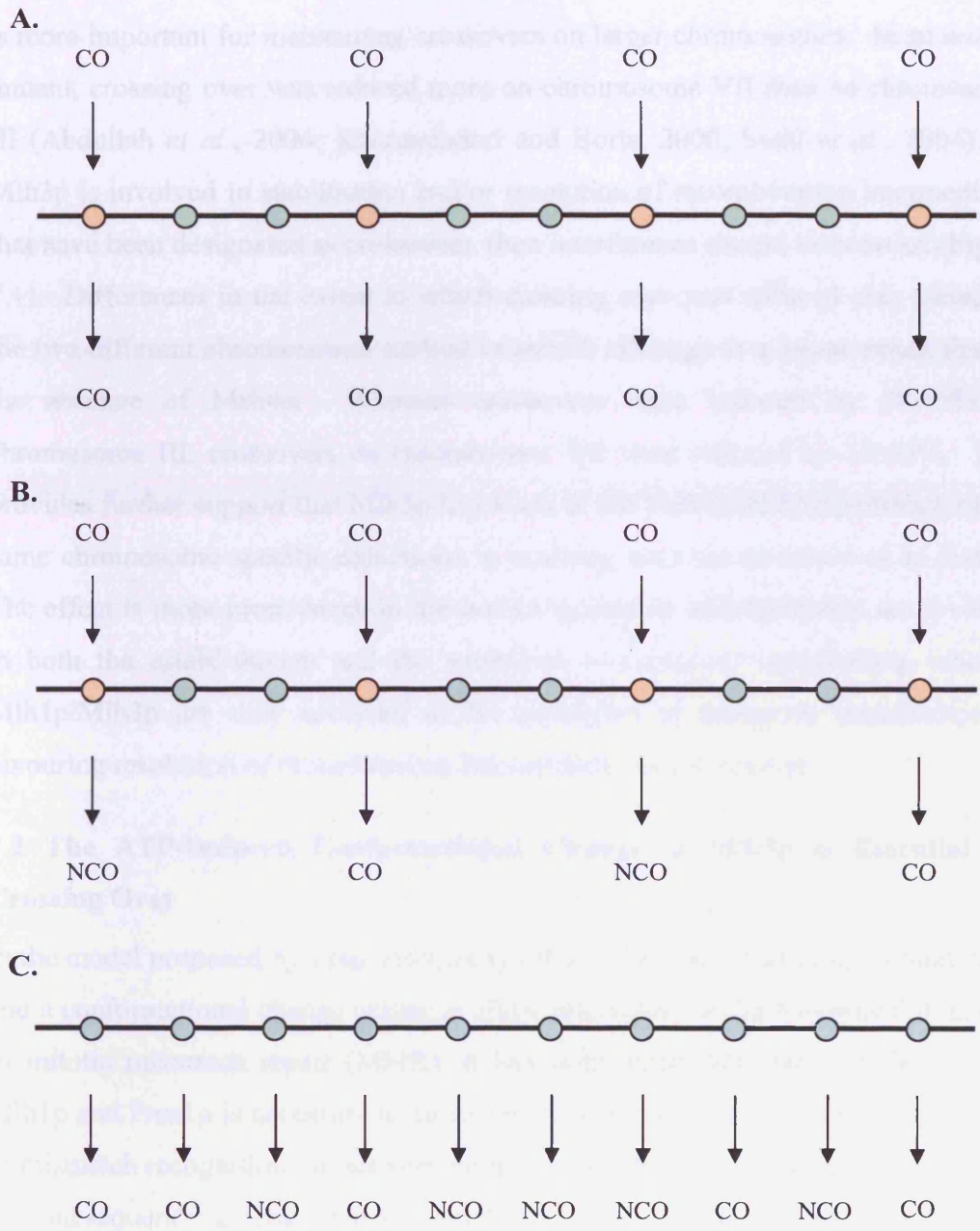


Figure 7.1 Crossover Distribution

A. In the wild-type a number of DSB precursors are designated to become a crossover (orange). These are interspersed by a number of DSBs which will become non-crossovers (green).

B. In the absence of Mlh1p/Mlh3p, crossover designation by Msh4p/Msh5p will still occur, however, resolution will be defective. Random resolution either as a non-crossover (SDSA pathway) or a crossover (Mus81p/Mms4p) will allow the production of some crossovers and their distribution will not be as equally spaced across the chromosome.

C. In the absence of Msh4p/Msh5p, precursors will not be designated to become a crossover or non-crossover. Therefore, crossovers can occur much closer together than would happen normally.

is more important for maintaining crossovers on larger chromosomes. In an *msh4*Δ mutant, crossing over was reduced more on chromosome VII than on chromosome III (Abdullah *et al.*, 2004; Khazanehdari and Borts, 2000; Stahl *et al.*, 2004). If Mlh3p is involved in stabilisation and/or resolution of recombination intermediates that have been designated as crossovers, then interference should be reduced (Figure 7.1). Differences in the extent to which crossing over was reduced also varied on the two different chromosomes studied in *mlh3*Δ although to a lesser extent than in the absence of Msh4p. Whereas crossovers were reduced by 38-45% on chromosome III, crossovers on chromosome VII were reduced by 51-53%. This provides further support that Mlh3p functions in the Msh4p-Msh5p pathway, as the same chromosome-specific reductions in crossing over are observed as in *msh4*Δ. The effect is more pronounced in the *msh4*Δ mutant as Msh4p/Msh5p are involved in both the establishment and the execution of crossover interference, whereas Mlh1p/Mlh3p are only involved in the execution of crossover interference by favouring resolution of recombination intermediates as a crossover.

7.2 The ATP-Induced Conformational Change in Mlh3p is Essential for Crossing Over

In the model proposed by Tran and Liskay (2000), the MutL homologues bind ATP, and a conformational change occurs to allow interaction of the N-terminal domains. In mitotic mismatch repair (MMR), it has been suggested that ATP binding by Mlh1p and Pms1p is necessary to allow recruitment of factors required downstream of mismatch recognition. It has been demonstrated that upon binding of ATP, MutL can subsequently activate the endonuclease MutH, which is required for strand discrimination (Ban *et al.*, 1999).

When Mlh3p can bind ATP but cannot hydrolyse it, the crossover function is unaffected. However, when Mlh3p is unable to bind, and consequently hydrolyse ATP, crossing over is affected to the same extent as in the null mutant. This indicates that ATP binding is absolutely required for this function. This also suggests that once the conformational change has been made, the consequent interactions that are necessary for crossing over can occur, and an inability to hydrolyse ATP does not affect this. As Mlh1p can still hydrolyse ATP, this may be sufficient to release the two proteins from the heterodimer. This may also aid in

maintaining function. However, the *mlh1 mlh3* double ATP hydrolysis mutant does decrease crossing over, indicating that at least some recycling of the complex is important for function. This also suggests that the availability of Mlh1p and Mlh3p is limiting for function. Not only is crossing over affected in the double ATP hydrolysis mutant but a small defect in meiotic mismatch repair was also detected. Therefore, not only do these mutations impair the functioning of the Mlh1p-Mlh3p complex in crossing over, they also affect the Mlh1p specific, Mlh3p-independent functions during meiotic recombination.

In order to further characterise Mlh3p, we analysed the effects of the mutations G97A and G97V. Residue G97 is situated in the GYRGDAL box in Mlh3p (GFRGEAL in the other MutL homologues), which forms the lid of the ATP-binding pocket (Ban and Yang, 1998; Ban *et al.*, 1999). This motif is important for providing contact with ATP, but it also forms part of the N-terminal interaction domain between MutL homologues (Ban and Yang, 1998; Ban *et al.*, 1999; Tran and Liskay, 2000). Mutating this residue to alanine only resulted in a mild effect on mitotic MMR, whereas the meiotic functions of Mlh3p were unaffected. In contrast, the *mlh3-G97V* mutant was defective for mitotic MMR and for crossing over. Similar effects were also observed for the corresponding mutations in Mlh1p; *mlh1-G98A* and *mlh1-G98V* (Hoffmann *et al.*, 2003; Pang *et al.*, 1997; Tran and Liskay, 2000). The valine substitution in *mlh3-G97V* may cause more local disruption to the conformation of the ATP-binding site due to its bulkier side-chain, which will reduce ATP binding more than the alanine substitution. This could explain why *mlh3-G97V* exhibits more mutant phenotypes than *mlh3-G97A*. Interestingly, *mlh1-G98V* was shown to disrupt the interaction between Mlh1p and Pms1p, whereas *mlh1-G98A* did not (Pang *et al.*, 1997). This could be due to either disrupted ATP binding that prevents a conformation change and hence interaction, or through directly affecting the interaction without affecting ATP binding. Given that Mlh1p is thought to interact with Mlh2p, Mlh3p and Pms1p in the same manner, then disruption of the interaction with Pms1p is also likely to disrupt the interaction with the other MutL homologues. Hence, this corresponding mutation in Mlh3p is also likely to disrupt the interaction with Mlh1p. Therefore, we propose that the defect in crossing over observed in the *mlh3-G97V* mutant is caused by the inability to interact with Mlh1p. Although crossing over is unaffected in the *mlh3-*

G97A mutant, a small increase in mutation rate was observed that may be due to a reduced ability to bind ATP. Presumably *mlh3-G97A* retains the ability to interact with Mlh1p as crossing over is unaffected. This suggests that mismatch repair is more sensitive to reduced ATP binding than crossing over is. This was also demonstrated by the *mlh1-G98A* mutant, as although *mlh1-G98A* did not affect the interaction with Pms1p, both mitotic and meiotic MMR was affected (Hoffmann *et al.*, 2003; Pang *et al.*, 1997).

7.3 ATPase Mutants of Mlh3p and Pms1p have Different Functional Consequences

Equivalent ATP binding and hydrolysis mutants of Mlh1p and Pms1p exhibit different effects on mitotic mutation rate (Hall *et al.*, 2003; Tran and Liskay, 2000). ATP binding by Mlh1p is essential, whilst binding of ATP by Pms1p is not. This is illustrated by the fact that the *mlh1* mutants display a more severe phenotype than the *pms1* mutants. This suggests that Mlh1p can compensate for the absence of Pms1p better than Pms1p can for the absence of Mlh1p (Tran and Liskay, 2000). Additionally Mlh1p has a higher affinity for ATP than Pms1p (Hall *et al.*, 2002). Therefore it is possible that Mlh1p binds ATP first, followed by Pms1p, and that these changes coordinate different downstream events (Hall *et al.*, 2003). This situation is not seen in the Mlh1p-Mlh3p heterodimer. ATP binding mutants of Mlh1p and Mlh3p are equally defective in crossing over, indicating that they are both required for coordination of all subsequent events. The particular conformation adopted by Mlh1p and Mlh3p upon binding of ATP may allow their specific interaction with one another and/or with other recombination intermediates to provide stabilisation and/or recruitment of downstream factors. A second possibility is that these proteins are directly involved in the resolution of recombination intermediates. It has recently been shown that hPMS2 has latent endonuclease activity, and the active site for this activity is highly conserved in the homologues of hPMS2 and hMLH3, but not hMLH1 or hPMS1 (Kadyrov *et al.*, 2006). If Mlh3p possesses this activity, a specific conformation might be necessary to perform this activity, the production of which may be dependent on ATP-binding. Solving the crystal structure of a heterodimer of Mlh1p-Mlh3p would reveal whether this complex has the ability to bind to recombination intermediates.

The double ATP hydrolysis mutant of Mlh1p and Pms1p was phenotypically null (Tran and Liskay, 2000), unlike the double ATP hydrolysis mutant of Mlh1p and Mlh3p. This suggests that recycling of Mlh1p/Pms1p complexes during mitotic MMR is more important than recycling of Mlh1p/Mlh3p complexes during meiotic recombination. This may be due to the relative number of structures needing to be dealt with (i.e. replication errors compared with crossover intermediates), and/or the expression levels of these genes during the mitosis versus meiosis.

7.4 The 5′-3′ Exonuclease and the Flap Endonuclease Activities of Exo1p are Required for Meiotic Recombination

In the absence of Exo1p crossing over is reduced at all intervals that have been tested, whereas gene conversion is only affected at certain loci. Analysis of point mutations in Exo1p that are defective for different nuclease activities has given some insight into the requirements of Exo1p for meiotic recombination (summarised in Table 7.1). Exo1p appears to have at least two roles; an early role in the 5′-3′ resection of the *HIS4* DSB, and a later role in flap removal from recombination intermediates, which is necessary for crossover formation. A role in resection of the *HIS4* DSB is indicated by the reduction of gene conversion events observed in the *exo1* mutant that is defective for the 5′-3′ exonuclease activity, but is functional for the flap endonuclease activity. The reduction in gene conversion implies that less hDNA is being produced as a consequence of less resection. Crossing over in the *NAT-HYG* interval was also reduced in this mutant (Table 7.1). We suggest that the shorter 3′ single-stranded DNA tails produced when less resection occurs may be less capable of forming stable strand invasion intermediates, and consequently reduce crossovers. A decrease in the frequency of crossing over that was intermediate between wild-type and *exo1*Δ levels was also observed in the *LEU2-MAT* interval on chromosome III and in the *CYH2-TRP5* interval on chromosome VII (Table 7.1). This suggests that there are one or more DSB(s) that are normally resected by Exo1p in these intervals. As crossing over is not reduced to the same extent as in the null mutant, Exo1p may be required for the resection of a smaller proportion of breaks at these DSBs than at *HIS4*. This suggests that the dependence on Exo1p for resection may vary between DSBs.

By comparing the levels of crossing over observed in the 5′-3′ exonuclease mutant

Table 7.1 Summary of the Phenotypes of *exo1* Mutants

Genotype	Crossing Over					NMS at	% Repair	Viability
	NAT-HYG	*HYG-LEU2*	*LEU2-MAT*	*MET13-CYH2*	*CYH2-TRP5*	*HIS4*	*his4-ATC*	
exo1Δ	-	-	-	-	-	-	+	-
mlh1Δ	+	-	-	-	-	++	-	+/-
exo1Δmlh1Δ	-	-	-	-	-	+/-	-	-
exo1-D173A	-	+/-	-	-	-	+/-	-	+/-
exo1-D171A	-	+/-	+/-	+	+/-	-	+	+/-
exo1-E150D	-	+	+/-	+	+/-	-	+	+/-
exo1-S445A-F447A-F448A	+	+	+	+	+	+	+	+

+ represents wild-type phenotype.
- represents mutant phenotype.
+/- represents an intermediate phenotype between wild-type and mutant.

with the *exo1* mutant lacking both nuclease activities, the requirement of the flap endonuclease activity for crossing over can be estimated. Interestingly, whereas the 5′-3′ exonuclease deficient mutant only reduces crossing over in the *NAT-HYG* interval, the *exo1* mutant that is defective for both the 5′-3′ exonuclease activity and the flap endonuclease activity reduces crossing over in all of the intervals studied (Table 7.1). Therefore in at least four of the five intervals studied the flap endonuclease activity of Exo1p is specifically required for the production of wild-type levels of crossing over. Whether the flap endonuclease activity is also required at DSBs that Exo1p is responsible for processing cannot be determined from the results here, as the mutant defective for the flap endonuclease activity is also defective for the 5′-3′ exonuclease activity. However, if the recombination intermediates are destabilised at an early stage when resection is reduced, the intermediate that requires the flap endonuclease activity for processing may not be formed.

Surprisingly the *exo1-D171A* mutant did not exhibit equivalent mutant phenotypes to *exo1-D173A* (Table 7.1), which is defective for the 5′-3′ exonuclease activity and the flap endonuclease activity. This was expected due to the close proximity of the residues and their being situated in a highly conserved region. Instead the phenotypes most closely resembled those of *exo1-E150D*, which is defective for the 5′-3′ exonuclease activity but proficient for the flap endonuclease activity. Therefore we have characterised a new separation of function mutant, based on genetic phenotypes. This also suggests that the substitution of aspartic acid for alanine at position 171 is more important for the resection function of Exo1p than for the removal of flaps from recombination intermediates.

7.5 A Model for the Removal of 5′ Flap Structures by Exo1p

Abdullah *et al.* (2004) proposed that the increased gene conversion observed in the absence of Mlh2p could be explained by increased DNA synthesis, which may increase the amount of hDNA formed (Abdullah *et al.*, 2004). Furthermore, the additional deletion of Mlh2p in an *msh4*Δ, *msh5*Δ or *mlh3*Δ background improved crossing over and viability possibly by stabilising recombination intermediates. It was hypothesised that longer tracts of DNA synthesis would lead to the production

of intermediates containing flaps that would provide substrates for either Mus81p/Mms4p (D-loops and 3′ flaps) or Exo1p (5′ flaps). This could provide an alternative mechanism for the production of crossovers. Interestingly the deletion of Mlh2p in an *exo1*Δ background did not improve crossing over, suggesting that the increases in crossing over observed in *mlh2*Δ*mlh3*Δ, *mlh2*Δ*msh4*Δ and *mlh2*Δ*msh5*Δ mutants are dependent upon the presence of Exo1p. This data supports the model presented below.

DNA synthesis is required to fill in gaps produced by 5′-3′ resection of DSBs (Figure 7.2). However, it is unknown how, or even if, DSB resection and DNA replication are coordinated. If these processes were tightly coordinated, then over-replication would not be expected to occur (Figure 7.2 B-C). If, however, DNA synthesis extends further than the resection of the 5′ end, the excess DNA will need to be removed for resolution of the intermediates to occur (Figure 7.2 D-F). It may be that at some loci these events are highly coordinated whereas at others over-replication is tolerated, or even promoted.

Another possibility is that the amount of DNA synthesis is constant and that when DSBs are resected less the newly synthesised DNA will be longer than required to fill the resected gap (Figure 7.3). As discussed above this leads to the production of flaps. Therefore flap production may occur more frequently in the absence of Exo1p. If DNA is over-replicated, displacement of one end of the DNA strand will have to occur (Figure 7.2 D-E). We propose that displacement of the free 5′ end by the newly synthesised (invading) 3′ end of the same chromatid produces a flap, which can be removed by Exo1p (Figure 7.2 E1). The removal of this flap would be necessary to allow completion of the double Holliday Junction. Formation of the double Holliday Junction would allow resolution of recombination intermediates to proceed via the Msh4p/Msh5p pathway (Figure 7.2 C). In the absence of Exo1p, or the flap endonuclease activity of Exo1p, one of two things may happen. The 5′ flap may get converted to a 3′ flap, which would require a 3′ flap endonuclease for removal, such as Mus81p/Mms4p (Figure 7.2 E2). Another possibility is that strand displacement could occur resulting in collapse of the D-loop as in SDSA (reviewed in Paques and Haber, 1999). This would reduce crossing over, and any flaps that

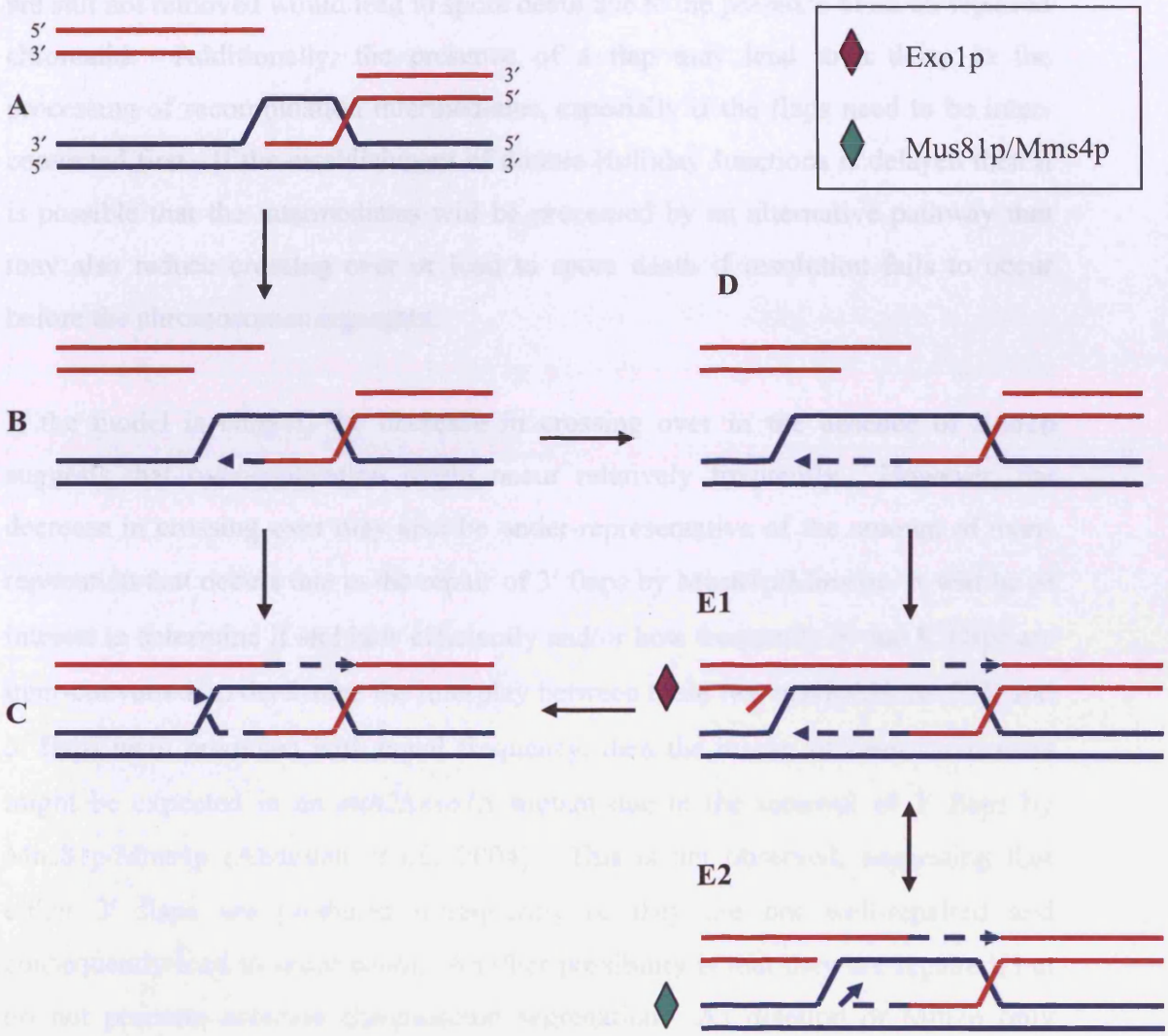


Figure 7.2 Model for the role of Exo1p in Removal of 5′ Flaps

A. **-C**: Coordination of DSB resection and repair DNA synthesis. The 3′ single-stranded overhang invades the homologue, creating a single-end invasion intermediate. This primes DNA synthesis, which is terminated at a distance corresponding to the free 5′ resected end of the sister chromatid. Second end capture completes the formation of a double Holliday Junction.

D. Over-replication of DNA. DNA synthesis extends beyond the free 5′ resected end of the sister chromatid (**D**).

E. Displacement of the 5′ end will produce a 5′ flap which Exo1p can remove (**E1**). Alternatively the over-replicated DNA strand may form the flap, which will require a 3′ flap endonuclease to remove, for example Mus81p/Mms4p (**E2**). The two flaps may be inter-changeable.

Adapted from Abdullah *et al.*, 2004.

are still not removed would lead to spore death due to the presence of an un-repaired chromatid. Additionally, the presence of a flap may lead to a delay in the processing of recombination intermediates, especially if the flaps need to be inter-converted first. If the establishment of double Holliday Junctions is delayed then it is possible that the intermediates will be processed by an alternative pathway that may also reduce crossing over or lead to spore death if resolution fails to occur before the chromosomes segregate.

If the model is correct, the decrease in crossing over in the absence of Exo1p suggests that over-replication might occur relatively frequently. However, the decrease in crossing over may also be under-representative of the amount of over-replication that occurs due to the repair of 3′ flaps by Mus81p/Mms4p. It will be of interest to determine if and how efficiently and/or how frequently 5′ and 3′ flaps are inter-converted, to determine the interplay between these two mechanisms. If 3′ and 5′ flaps were produced with equal frequency, then the rescue of some crossovers might be expected in an *mlh2*Δ*exo1*Δ mutant due to the removal of 3′ flaps by Mus81p/Mms4p (Abdullah *et al.*, 2004). This is not observed, suggesting that either 3′ flaps are produced infrequently or they are not well-repaired and consequently lead to spore death. Another possibility is that they are repaired, but do not promote accurate chromosome segregation. As deletion of Mlh2p only rescued some of the Msh4p-dependent crossovers, this indicates that Mlh2p only controls a subset of the total Msh4p-dependent events (Abdullah *et al.*, 2004). In contrast the requirement for the flap endonuclease activity of Exo1p for crossing over suggests that even in the presence of Mlh2p, some DNA flaps are still produced (Figure 7.3). Additionally, there may be a proportion of events that are consistently not over-replicated and consequently do not require a flap endonuclease for processing. This could explain why some crossing over still occurs in the absence of Exo1p.

7.6 Crossovers are More Frequently Associated with Longer Tracts of hDNA

Due to the increased gene conversion events observed in *mlh2*Δ, Abdullah *et al.* (2004) proposed that more hDNA was being produced in this mutant. As this is sufficient to rescue some crossing over, it also suggests a relationship between

hDNA tract length and these events. In this study, long tracts of hDNA were more frequently associated with crossovers, as determined by gene conversion of heteroalleles present at different distances from the initiating DSB at *HIS4*. This was observed in both wild-type and *exo1*Δ strains. For gene conversion of the heteroalleles to result in restoration of the His^+ phenotype, the hDNA tract must terminate between the two markers (Figure 6.3). As some gene conversion events occurred distal to the DSB in the absence of Exo1p, some 'long' resection must still be occurring. Therefore, there must be another nuclease(s) that is involved in processing of the *HIS4* DSB, as was suggested previously by Tsubouchi and Ogawa (2000).

Furthermore, in the absence of Exo1p there is a small reduction in the association of gene conversion with crossing over compared to wild-type. In light of the model proposed above, if short resection tracts provide more opportunity for over-replication to occur, then it is also possible that longer resection tracts are not associated with over-replication (Figure 7.3). When resection occurs over a larger distance and the tracts of hDNA produced are also long as a result, then it is possible that over-replication is less likely to occur, maybe due to regulation of DNA synthesis by Mlh2p (as discussed above). In this situation DNA flaps would not be formed and there would be no requirement for Exo1p (Figure 7.3B). This could explain why the crossover events associated with longer tracts of hDNA are not decreased to such as large an extent as in the absence of Exo1p.

The tracts of hDNA that give rise to a His^+ phenotype as a result of gene conversion can potentially end anywhere between the markers. Therefore, out of the total gene conversion events recovered a subset of events will have had shorter hDNA tracts than the rest. If there is a critical distinction point between the length of resection/length of hDNA produced and whether this produces a flap structure then the subset with shorter hDNA tracts could still require Exo1p (Figure 7.3A). This could account for the small reduction in crossover association obtained in the absence of Exo1p compared to wild-type. If a range of recombination intermediates are produced, some of which contain flaps, then this also suggests that a gradient of resection occurs at DSBs.

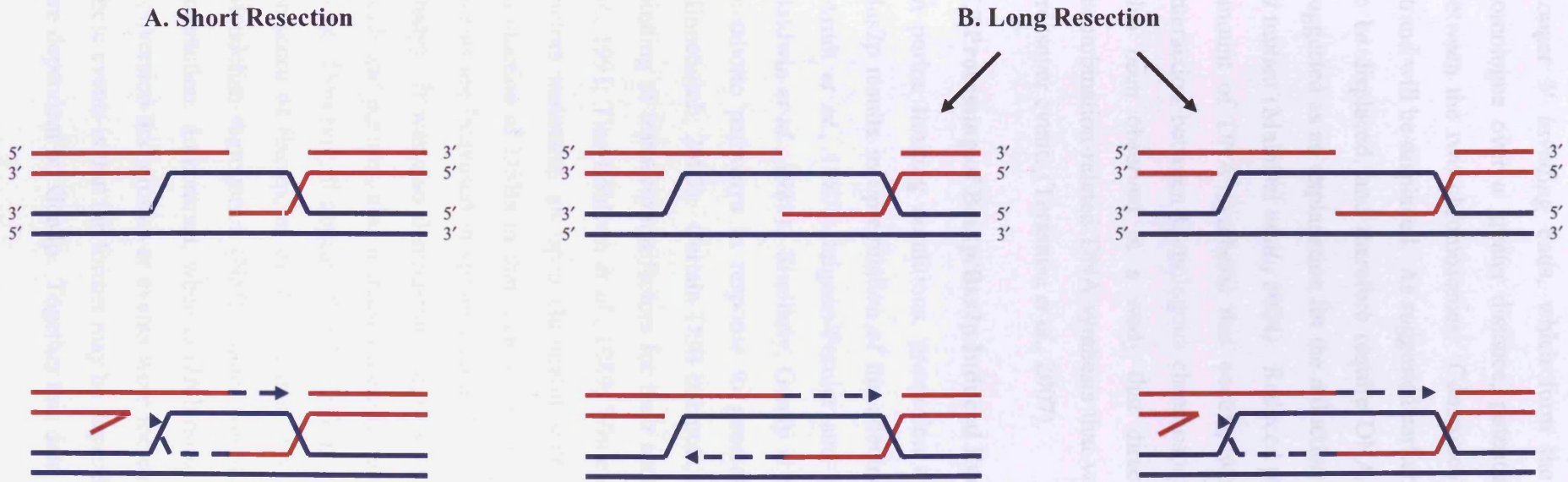


Figure 7.3 Relationship Between Resection Length and DNA Synthesis
If the same amount of DNA synthesis always occurs, then resection length will determine whether DNA flaps are produced. The dashed lines represent newly synthesised DNA. Only 5′ flaps are shown which are removable by Exo1p, however, they may also be converted to 3′ flaps which can be removed by Mus81p/Mms4p.

- **A.** If resection is short, then DNA synthesis to fill in the resultant gap between ends will produce a flap of DNA. These events will require Exo1p for the removal of the flaps.
- **B.** If resection is long, and the 'normal' amount of DNA synthesis occurs then DNA flaps will not be produced and the intermediate will be stable. However, if over-replication occurs, for example in the absence of Mlh2p, then DNA flaps will be produced which require Exo1p.

Longer 3′ invading ends, which form the tracts of hDNA, interact with the homologue over a greater distance, potentially forming more stable interactions between the two chromosomes. Consequently, it is less likely that the invading strand will be displaced. As suggested earlier, shorter invading ends are more likely to be displaced, and therefore require DNA synthesis to be stabilised. This was suggested as an explanation for the reduction in crossing over observed in the *pol3-ct* mutant (Maloisel *et al.*, 2004). Reduced processivity in *pol3-ct* could reduce the amount of DNA synthesis that occurs, which may reduce the stability of the interaction between homologous chromosomes (Maloisel *et al.*, 2004). This has also been observed in a study that directly measured the length of meiotic recombination-related DNA synthesis that was associated with crossover and non-crossover events (Terasawa *et al.*, 2007).

7.7 Processing of Bas1p/Bas2p Induced Events is Exo1p-Dependent

In purine limiting conditions, promotion of the interaction between Bas1p and Bas2p results in upregulation of the adenine and histidine biosynthesis pathways (Arndt *et al.*, 1987; Daignan-Fornier and Fink, 1992; Denis *et al.*, 1998; Tice-Baldwin *et al.*, 1989). Similarly, Gcn4p is involved in the upregulation of several metabolic pathways in response to general amino acid starvation (reviewed in Hinnebusch, 2005). Certain DSB hotspots, such as *HIS4*, are dependent upon the binding of transcription factors for their activity (Arndt and Fink, 1986; Devlin *et al.*, 1991; Tice-Baldwin *et al.*, 1989; White *et al.*, 1993). Binding of transcription factors maintains an open chromatin state, which is thought to allow increased production of DSBs in that region. At *HIS4*, both gene conversion and crossover events are increased in strains auxotrophic for adenine (Abdullah *et al.*, 2001; this study). It was also demonstrated in this study that complete starvation for amino acids and purines, also increased meiotic recombination events at *HIS4* in the wild-type. However, it appears that Bas1p/Bas2p induced events are dependent on the presence of Exo1p, as *exo1*Δ mutants only exhibited a marginal increase in non-Mendelian segregation (NMS) under conditions that should increase Bas1p/Bas2p interaction. In contrast, when *exo1*Δ strains were starved for all amino acids, gene conversion and crossover events were increased at *HIS4*. The distinction between these events is that the former may be dependent on Bas1p/Bas2p whereas the latter are dependent on Gcn4p. Together this data suggests that Exo1p is not involved in

the processing of all DSBs made at *HIS4*, as suggested previously (Khazanehdari and Borts, 2000; Tsubouchi and Ogawa, 2000). It is unclear why Bas1p/Bas2p mediated DSBs would specifically require Exo1p for processing. One possibility is that the transcription factors required for *HIS4* hotspot activity also recruit the exonuclease(s) to carry out resection of the DSB. This would suggest that Bas1p/Bas2p recruits Exo1p, and therefore in the absence of Exo1p the DSBs induced by adenine auxotrophy cannot be resected. Conversely, Gcn4p must be able to recruit Exo1p and other exonucleases, which allows DSB processing under conditions of amino acid starvation. This distinction between Gcn4p and Bas1p/Bas2p supports the hypothesis that there are two types of DSB at *HIS4*; those that require Exo1p for resection and those that are Exo1p-independent.

7.8 Conclusions

The work presented here provides evidence for the importance of the roles of Mlh3p and Exo1p in the production and/or processing of recombination intermediates. Mlh3p is as important as Mlh1p in promoting crossover events and ensuring correct chromosome segregation within the context of the Msh4p/Msh5p crossover pathway. Whether the role Mlh3p plays is purely structural, in that recombination intermediates are stabilised in a manner which promotes their resolution as a crossover, or if Mlh3p has an active role in resolution of such intermediates remains to be determined. The results presented here suggest that the conformation of Mlh3p is crucial to its function. It will be of great interest to ascertain if Mlh3p possesses a latent endonuclease activity as has been demonstrated for hPMS2 (Kadyrov *et al.*, 2006), and if so, whether this activity is important for the meiotic functions of Mlh3p.

Exo1p appears to have two distinct but overlapping functions in DSB repair. Firstly Exo1p is involved in resection of some DSBs, although how widespread this function is, is unknown. Although not conclusive, the data presented here support the hypothesis that there are two classes of breaks. If only a subset of DSBs are processed by Exo1p, it would also be interesting to determine if this designated breaks for a specific crossover pathway. If so, this would indicate that the crossover/non-crossover decision is made prior to, or at the time that DSBs are resected. A test of this would be to determine if interference is affected in the

absence of Exo1p. If interference is unaffected, this would suggest that the resection of DSBs at *HIS4* by Exo1p is not specific to those that will proceed down the Msh4p/Msh5p crossover pathway. The presence of shorter 3′ tails could affect either the Msh4p/Msh5p pathway and/or the Mus81p/Mms4p pathway, as both require strand invasion of the homologue (de los Santos *et al.*, 2001; de los Santos *et al.*, 2003; Hunter and Kleckner, 2001; Osman *et al.*, 2003; Szostak *et al.*, 1983). However, it is unknown if intermediates in the Mus81p/Mms4p pathway require extensive hDNA for stabilisation.

Additionally, Exo1p is involved in the removal of flap structures produced during meiotic recombination, and this ability to remove flaps is essential for some crossover formation. This suggests that there are recombination intermediates produced during DSB repair that have yet to be identified physically. How frequently flaps are produced during meiotic recombination remains to be determined.

We also find support for the preferential association of crossover events with longer tracts of hDNA. We suggest that the length of resection of DSBs and the amount of DNA synthesis primed by the 3′ invading end together determines the length of hDNA tracts. How these events are coordinated and/or regulated remains to be elucidated. It is unclear whether mechanisms are in place to prevent over-replication of DNA, and if there is a mechanism to deal with these events once they arise. It is also possible that both of these mechanisms occur in a locus-specific manner. If this model is correct, the requirement of the flap endonuclease activity of Exo1p for wild-type levels of crossing over suggests that over-replication occurs routinely to produce DNA flaps. This may arise from two different circumstances: when 'shorter' resection occurs, but also when over-replication of hDNA occurs in conjunction with 'longer' resection events. As mentioned earlier, it is unknown to what extent either of these events occur.

This study provides some insight into the functions of two proteins in meiotic recombination. Increasing the understanding of this process has a number of important implications. Crossover events that occur during meiotic recombination

ensure chromosome segregation at the first division of meiosis. Defects in the processing of recombination intermediates can also lead to errors in segregation at the second division of meiosis. Failure of gametes to receive the correct number of chromosomes can reduce the viability of gametes, which consequently can lead to infertility if it happens on a large scale. In addition, gametes that are viable but contain the wrong number of chromosomes can lead to disease in the offspring. Therefore, understanding the mechanisms that ensure crossovers are made and also distributed correctly for segregation is extremely important to increase our understanding of the causes of these problems.

Appendices

Table A1.1 Oligonucleotide Sequences

Name	Sequence	Use
General :		
H2	CGGCGGGAGATGCAATAGG	Internal to the hphMX4 cassette, to confirm insertion.
H3	TCGCCCGCAGAAGCGCGGCC	
K2	TTCAGAAACAACTCTGGCGCA	Internal to the kanMX4 cassette, to confirm insertion.
K3	CATCCTATGGAACTGCCTCGG	
U2	ACTGGTATATGATTTTGTGGAC	Internal to the *KlURA3* cassette, used to confirm insertion.
U3	GAAGCGTACCAAAAGAGAATC	
Chapter 3:		
MLH3 5'	ATGAGCCAGCATATTAGGAAATTAGATTCTGATGTTTCTGAAAGCGTACGCTGC AGGTCGACGG	To amplify hphMX4 from pAG32, for deletion of *MLH3*.
MLH3 3'	TTACTTCAATTCTGCAATGGGTACCATAGAAGGTCTCCCGTGGGATCGATGAATT CGAGCTCGT	
MLH3-E31/N35-CORE.F	CTTAAATCTCAGGCATGCACGGTATCGCTAGCATCAGCGGTTAGAGAGCTCGTTT TCGACACTGG	To amplify kanMX4/*KlURA3* from pCORE to delete E31-N35 in *MLH3*.
MLH3-E31/N35-CORE.R	GAGGTCGATCATGACGTCGATAGTGGTAGCGTGTGCATCTACAGATCCTTACCA TTAAGTTGATC	
MLH3-GYRGDAL-CORE.F	TCCAAAATACGAAAGATGAATGATTTAGTAACGATGAAAACCTACCGTACGCTG CAGGTCGAC	To amplify kanMX4/*KlURA3* from pCORE to delete G94-L100 in *MLH3*.
MLH3-GYRGDAL-CORE.R	TTTCTTGGAACAAACAAACAGATTAGAGACATTAGAAATGCTATATCCTTACCA TTAAGTTGATC	

Name	Sequence	Use
MLH3 A1	ATGCGTGACGTTGAATCGAG	To confirm the insertion of cassettes into *MLH3*.
MLH3 A4	TCTGAACAGAACCACAGTCCA	
E31/N35 insert A4	CAATATATTTAGGTCACTTCG	
MLH3-E31A-IRO.F	TAGATTCTGATGTTTCTGAAAGACTTAAATCTCAGGCATGCACGGTATCGCTAGC ATCAGCGGTTAGAGCAATAGTTCAA	To introduce the mutation E31A into *MLH3*.
MLH3-E31A-IRO.R	GCTCAAATTAGGGAGGTCGATCATGACGTCGATAGTGGTAGCGTGTGCATCTAC AGAATTTTGAACTATTGCTCTAACCG	
MLH3-N35A-IRO.F	CTGATGTTTCTGAAAGACTTAAATCTCAGGCATGCACGGTATCGCTAGCATCAGC GGTTAGAGAAATAGTTCAAGCTTCT	To introduce the mutation N35A into *MLH3*.
MLH3-N35A-IRO.R	TGCAAAGCTCAAATTAGGGAGGTCGATCATGACGTCGATAGTGGTAGCGTGTGC ATCTACAGAAGCTTGAACTATTTCTC	
MLH3-G97A-IRO.F	CACAAAATTATACTTCCAAAATACGAAAGATGAATGATTTAGTAACGATGAAAA CCTACGGTTACAGAGCAGACGCCCTA	To introduce the mutation G97A into *MLH3*.
MLH3-G97A-IRO.R	AGAGTTGTAATCCTTTTTCTTGGAACAAACAAACAGATTAGAGACATTAGAAAT GCTATATAGGGCGTCTGCTCTGTAAC	
MLH3-G97V-IRO.F	CACAAAATTATACTTCCAAAATACGAAAGATGAATGATTTAGTAACGATGAAAA CCTACGGTTACAGAGTAGACGCCCTA	To introduce the mutation G97V into *MLH3*.
MLH3-G97V-IRO.R	AGAGTTGTAATCCTTTTTCTTGGAACAAACAAACAGATTAGAGACATTAGAAAT GCTATATAGGGCGTCTACTCTGTAAC	
MLH3 F2	GCGTTTATTTGCGAGCGCCA	For sequencing of *MLH3* to confirm the insertion of point mutations.
MLH3 R8	GCTATATAGGGCGTCTCCTC	
MLH3 F3	CGACGTCATGATCGACCTCC	
MLH3 R7	GCTGGTGCTTTGTTAACCCC	

Name	Sequence	Use
Chapter 4:		
E1 -100.F	CAGGTATATCTATATGCTCTC	Internal to the *EXO1* ORF (except E1 – 100.F), to confirm insertion of the *GAL1-I-SceI kanMX4/KlURA3* cassette from pGSKU or for sequencing to confirm the insertion of mutations.
E1 + 300.F	GCCATAGCTGAAAGACTGTG	
E1 + 403.R	ACCGTTTAGCTTACAGTAGCA	
E1 + 672.F	CGGTTGTGACTATACAAATGGA	
E1 + 787.R	GTATGTGTCTGGTATCATCAG	
E1 + 1054.F	CCATATGATTTTCACCAACCTC	
E1 + 1164.R	GGAAGAACGATTCTATTGGTTTG	
E1 + 1410.F	AGAGGACACAAACTCACAATCT	
E1 + 1512.R	GAAACCTCTTCACTCAGGTTG	
E1 + 1764.F	CAGCGGAGTTAATGCAAATAG	
E1 + 1861.R	TTTACCATCGTTATCGTCGCA	
EXO1 S445-F448 CORE SCE1.F	TTAAGTAATGCCAATGTAGTCCAAGAAACGTTGAAGGATACAAGATAGGGATAA CAGGGTAATTTGGATGGACGCAAAGAAGT	To amplify the *GAL1-I-SceI kanMX4/KlURA3* cassette from pGSKU.
EXO1 S445-F448 CORE SCE1.R	GCCTTTTTCTTTGAAGTTTTCCACAACAGTCATGGAGGGTTTATTTTCGTACGCTG CAGGTCGAC	
EXO1-MLH1 interaction IRO.F	TGGCAATTAAGCGTAGGAAATTAAGTAATGCCAATGTAGTCCAAGAAACGTTGA AGGATACAAGAGCCAAAGCTGCTAAT	To amplify the *EXO1* ORF from pEAM67 and concomitantly introduce the mutations S445A, F447A and F448A.
EXO1-MLH1 interaction IRO.R	AAAATCCTGTATGCTGTCGCCTTTTTCTTTGAAGTTTTCCACAACAGTCATGGAG GGTTTATTAGCAGCTTTGGCTCTTG	
EXO1 Adaptamer A	AATTCCAGCTGACCACCATGTGGGTATCCAAGGTCTTCTTCC	To amplify a mutated *EXO1* ORF from pEAM69, pEAM71 or pJM3.
EXO1 Adaptamer B	GATCCCCGGGGAATTGCCATGAAAATTATCGCGACATATTTCTAAACATTCTCC	

Name	Sequence	Use
Adaptamer a	CATGGTGGTCAGCTGGAATTCGATCATGTAGTTTCTGGTT	To amplify partial fragments of *KlURA3* from pWJ716.
Adaptamer b	CATGGCAATTCCCGGGGATCGTGATTCTGGGTAGAAGATCG	
Internal 5' KlURA3	CTTGACGTTCGTTCGACTGATGAGC	
Internal 3' KlURA3	GAGCAATGAACCCAATAACGAAATC	
LYS2 (1-21).F	ATGACTAACGAAAAGGTCTGG	To amplify *LYS2* from genomic DNA for replacement of the *lys2-d* allele.
LYS2 (4158-4179).R	TTAAGCTGCTGCGGAGCTTCC	
Chapter 5:		
EXO1-KANMX4.F	ATTAAAATAAAAGGAGCTCGAAAAAACTGAAAGGCGTAGAAAGGACGTACGCT GCAGGTCGACGG	To amplify *kanMX4* from pFA6-kanMX4, for deletion of *EXO1*.
EXO1-KANMX4.R	ATTTGAAAAATATACCTCCGATATGAAACGTGCAGTACTTAACTTGATCGATGA ATTCGAGCTCGT	

Table A2.1 Raw Data from the Fluctuation Tests in Chapter 3

Strain	Experiment	Media[1]	Dilutions[2]	Colony[3]: 1	2	3	4	5	6	7	8	9	10	11
Wild-type (EY39)	1	COM	10^{-05}	1381	-	1412	1332	1795	1219	1658	1147	-	905	1308
			10^{-06}	187	214	184	157	199	175	180	143	213	185	68
			10^{-07}	15	18	27	23	16	10	20	13	29	9	13
			10^{-08}	1	1	3	5	0	2	3	5	7	6	31
		LYS	Neat	22	13	51	6	17	21	121	34	18	47	26
			10^{-01}	2	3	7	2	3	5	11	8	3	4	3
	2	COM	10^{-05}	1113	878	-	1065	1210	-	1034	1056	492	673	571
			10^{-06}	164	0	139	151	145	143	92	94	35	86	122
			10^{-07}	12	8	12	16	23	24	8	16	7	6	9
			10^{-08}	2	2	1	5	2	1	0	2	1	0	0
		LYS	Neat	32	21	26	17	23	18	20	14	36	39	16
			10^{-01}	4	1	0	1	3	1	6	0	2	1	0
	3	COM	10^{-05}	-	1680	1920	1827	1373	1156	-	1013	1651	758	-
			10^{-06}	285	114	190	240	132	111	229	106	213	173	253
			10^{-07}	18	13	23	26	9	11	26	11	9	22	21
			10^{-08}	10	3	0	1	1	4	8	1	4	4	2
		LYS	Neat	22	13	39	17	55	17	106	22	34	13	18
			10^{-01}	1	0	4	1	3	1	11	5	4	2	2

Strain	Experiment	Media[1]	Dilutions[2]	Colony[3]: 1	2	3	4	5	6	7	8	9	10	11
*mlh3*Δ (VC29)	1	COM	10^{-05}	-	2005	-	1797	1996	-	1796	1656	1446	1885	-
			10^{-06}	234	192	260	202	189	244	169	161	163	195	298
			10^{-07}	19	20	29	18	20	34	24	15	20	23	17
			10^{-08}	2	1	2	3	1	2	0	2	7	1	3
		LYS	Neat	236	223	162	288	230	630	134	189	-	279	144
			10^{-01}	20	25	32	22	22	68	11	18	173	28	21
	2	COM	10^{-05}	1939	2076	1956	1804	1012	1828	1860	1676	1524	1668	1424
			10^{-06}	294	230	236	228	197	214	166	200	199	190	176
			10^{-07}	32	28	30	12	35	47	13	14	13	29	28
			10^{-08}	1	1	1	4	1	1	0	2	1	2	1
		LYS	Neat	221	228	199	244	230	165	168	409	294	472	363
			10^{-01}	29	26	27	26	29	25	13	73	49	49	28
	3	COM	10^{-05}	1556	1812	1376	1692	1388	1460	1344	1756	1268	1192	1500
			10^{-06}	225	147	170	199	180	191	184	158	149	164	146
			10^{-07}	13	30	23	19	19	16	14	18	9	12	20
			10^{-08}	1	0	0	1	1	3	0	5	0	4	5
		LYS	Neat	157	182	166	118	-	244	179	176	136	203	193
			10^{-01}	11	13	13	17	127	28	15	17	20	28	26

Strain	Experiment	Media[1]	Dilutions[2]	Colony[3]: 1	2	3	4	5	6	7	8	9	10	11
mlh3-G97A (VC163)	1	COM	10^{-05}	2564	1980	1868	2496	2064	1496	1372	1536	1772	1988	1956
			10^{-06}	282	226	270	222	233	150	131	214	334	186	168
			10^{-07}	50	40	29	14	15	15	13	9	15	29	23
			10^{-08}	1	19	3	0	5	1	1	0	6	2	0
		LYS	Neat	80	136	129	77	90	352	87	193	191	86	137
			10^{-01}	13	19	26	1	14	60	8	17	39	5	16
	2	COM	10^{-05}	1332	124	1288	1524	1620	1044	1320	1353	1948	2300	1740
			10^{-06}	144	133	111	137	140	132	179	180	264	333	165
			10^{-07}	21	18	18	6	17	17	18	18	32	38	20
			10^{-08}	0	2	1	2	1	6	4	4	8	2	0
		LYS	Neat	131	133	335	122	610	128	597	104	166	153	115
			10^{-01}	16	18	32	16	57	24	48	12	18	18	21
	3	COM	10^{-05}	1548	1244	892	916	1128	1232	1608	1640	864	1208	1420
			10^{-06}	181	202	153	141	127	143	140	164	108	112	174
			10^{-07}	25	22	23	9	12	10	15	13	13	6	11
			10^{-08}	0	1	0	0	1	0	1	0	1	0	4
		LYS	Neat	76	113	95	67	119	123	443	123	71	183	104
			10^{-01}	5	14	6	6	7	11	44	14	9	21	20

Strain	Experiment	Media[1]	Dilutions[2]	Colony[3]: 1	2	3	4	5	6	7	8	9	10	11
mlh3-G97V (VC136)	1	COM	10^{-05}	1252	1040	1356	992	1676	1164	1094	1484	1100	1247	1486
			10^{-06}	168	115	150	136	137	143	136	148	154	160	168
			10^{-07}	23	13	16	14	8	21	15	30	7	14	13
			10^{-08}	1	3	0	1	1	3	7	0	4	2	1
		LYS	Neat	17	95	131	224	117	97	123	502	135	139	160
			10^{-01}	11	9	20	21	20	13	14	65	18	14	13
	2	COM	10^{-05}	1369	1176	1035	1604	1141	1076	1100	1240	1108	1028	1336
			10^{-06}	192	165	122	158	159	109	112	196	195	129	150
			10^{-07}	20	10	12	53	12	19	27	29	9	19	15
			10^{-08}	20	8	1	2	2	10	5	1	16	7	5
		LYS	Neat	121	170	160	159	135	134	113	114	158	101	376
			10^{-01}	5	34	12	10	12	14	11	13	16	31	25
	3	COM	10^{-05}	1066	1040	1124	970	1252	884	342	1332	1035	1076	86
			10^{-06}	129	125	112	118	119	131	150	115	113	113	67
			10^{-07}	23	10	7	8	8	11	9	15	2	11	18
			10^{-08}	1	1	4	0	0	0	0	4	0	0	0
		LYS	Neat	133	99	303	152	133	297	21	122	198	132	117
			10^{-01}	17	127	25	21	21	27	14	5	-	18	12

[1] Colonies were plated on complete media (COM) to obtain total number of cells and on lysine omission media (LYS) to detect mutation events.

[2] The total volume of cells from dilutions 10^{-05}-10^{-08} was plated on COM (200 µl), and 50 µl (out of total of 200 µl) of the neat and 10^{-01} dilution was plated on LYS.

[3] For each test, 11 colonies were used. Any boxes containing a dash (-) indicates that there were too many colonies to be counted accurately.

Table A2.2 Raw Data from the Fluctuation Tests in Chapter 4

Strain	Experiment	Media[1]	Dilutions[2]	Colony[3]: 1	2	3	4	5	6	7	8	9	10	11
Wild-type (EY39)[4]	1	COM	10^{-05}	-	-	-	-	-	-	-	-	-	-	-
			10^{-06}	304	309	445	361	359	239	255	347	195	265	330
			10^{-07}	34	16	48	139	41	45	18	22	25	28	46
			10^{-08}	1	2	9	0	5	6	3	6	0	4	1
		CAN	Neat	-	-	-	-	-	-	-	-	-	-	-
			10^{-01}	82	634	18	4	41	24	21	18	25	35	24
	2	COM	10^{-05}	-	-	-	-	-	-	-	-	-	-	-
			10^{-06}	165	201	194	222	159	161	190	165	136	152	189
			10^{-07}	30	19	15	23	25	16	15	20	20	14	13
			10^{-08}	2	1	0	2	1	3	7	2	3	2	4
		CAN	10^{-02}	-	-	-	-	-	-	-	-	-	-	-
			10^{-01}	24	65	47	45	26	25	37	42	30	20	26
	3	COM	10^{-05}	-	-	-	-	-	-	-	-	-	-	-
			10^{-06}	279	213	183	159	161	162	148	149	150	114	169
			10^{-07}	-	27	24	16	11	16	28	26	10	13	26
			10^{-08}	7	4	7	2	0	5	4	5	2	1	6
		CAN	10^{-02}	-	-	2	-	-	-	-	-	-	-	-
			10^{-01}	23	28	23	20	37	22	30	42	65	31	30

Strain	Experiment	Media[1]	Dilutions[2]	Colony[3]: 1	2	3	4	5	6	7	8	9	10	11
*exo1*Δ (VC178)	1	COM	10^{-05}	1508	3044	2500	1876	1541	1538	1632	1578	1490	1876	2320
			10^{-06}	220	288	286	207	160	177	201	174	185	143	260
			10^{-07}	11	42	23	19	12	14	18	10	30	14	36
			10^{-08}	3	5	1	2	4	0	1	1	1	0	2
		LYS	Neat	213	239	176	341	515	295	347	192	202	218	265
			10^{-01}	20	29	32	34	40	32	38	20	23	20	13
		CAN	Neat	-	-	-	-	-	-	-	-	-	-	-
			10^{-01}	283	225	215	204	214	207	195	189	197	1516	12
	2	COM	10^{-05}	2196	2088	1872	1840	1624	1212	1188	1204	2026	2068	1696
			10^{-06}	209	203	171	171	164	180	155	148	220	185	141
			10^{-07}	26	21	25	21	33	15	14	24	24	26	18
			10^{-08}	4	1	8	2	2	2	0	5	2	0	0
		LYS	Neat	243	-	-	204	-	-	-	-	185	-	248
			10^{-01}	27	33	100	17	22	25	50	26	16	21	20
		CAN	10^{-01}	233	160	183	157	175	203	186	202	402	176	179
			10^{-02}	30	12	18	22	14	18	14	27	42	23	17

Strain	Experiment	Media[1]	Dilutions[2]	Colony[3]: 1	2	3	4	5	6	7	8	9	10	11
exo1-S445A-F447A-F448A (VC310)	1	COM	10^{-05}	1081	1220	948	1222	956	831	1198	1135	1624	1068	1369
			10^{-06}	130	116	106	111	92	119	101	133	167	105	124
			10^{-07}	11	12	3	9	7	7	7	12	19	11	13
			10^{-08}	5	3	1	3	1	2	5	0	1	1	0
		LYS	Neat	42	29	56	25	63	64	49	231	30	39	117
			10^{-01}	5	5	3	2	12	4	0	25	8	10	13
		CAN	10^{-01}	-	-	-	-	-	-	-	-	-	-	-
			10^{-02}	25	19	25	24	26	25	30	20	16	43	53
	2	COM	10^{-05}	1124	1110	965	1151	1084	1658	1464	2012	1964	1632	1668
			10^{-06}	162	159	135	135	158	196	186	152	158	176	167
			10^{-07}	6	21	13	31	22	28	8	30	17	12	7
			10^{-08}	3	0	0	0	1	0	0	2	1	0	0
		LYS	Neat	93	73	59	35	39	30	28	33	51	26	29
			10^{-01}	18	11	-	2	1	1	3	0	6	2	0
		CAN	10^{-01}	26	211	16	26	19	35	26	80	13	24	29
			10^{-02}	3	18	2	3	9	5	4	8	2	5	4

[1] Colonies were plated on complete media (COM) to obtain total number of cells and on lysine omission media (LYS) or canavanine containing media (CAN) to detect mutation events.

[2] The total volume of cells from dilutions 10^{-05}-10^{-08} was plated on COM (200 µl), 50 µl (out of the total 200 µl) of the neat and 10^{-01} dilution was plated on LYS, and 100 µl (out of the total 200 µl) of the 10^{-01}-10^{-02} dilutions was plated on CAN.

[3] For each test, 11 colonies were used. Any boxes containing a dash (-) indicates that there were too many colonies to be counted accurately.

[4] The data from fluctuation tests on the wild-type using the CAN1 assay was obtained from M. Almeida (unpublished). The wild-type data for reversion to lysine prototrophy in Table A2.1 was used for comparisons with the *exo1* mutants.

Table A3.1 Raw Data from the Random Spore Experiments in Chapter 6

Strain	Ade Phenotype	Heteroalelles	Experiment	Media[1]	Dilution[2]	Number of colonies[3] Plate 1	Plate 2
Wild-type	+	*Xho*I / *Eco*RI	1	CC	10^{-02}	4012	-
					10^{-03}	479	-
					10^{-04}	33	-
					10^{-05}	1	-
				HCC	Neat	1660	1508
					10^{-01}	92	136
					10^{-02}	19	18
			2	CC	10^{-02}	4420	-
					10^{-03}	458	-
					10^{-04}	67	-
					10^{-05}	7	-
				HCC	Neat	2972	2532
					10^{-01}	222	206
					10^{-02}	18	17
			3	CC	10^{-02}	4340	-
					10^{-03}	517	-
					10^{-04}	65	-
					10^{-05}	0	-
				HCC	Neat	2732	2796
					10^{-01}	220	208
					10^{-02}	25	22

Strain	Ade Phenotype	Heteroalelles	Experiment	Media[1]	Dilution[2]	Number of colonies[3] Plate 1	Plate 2
Wild-type	-	*Xho*I / *Eco*RI	1	CC	10^{-02}	2764	-
					10^{-03}	281	-
					10^{-04}	37	-
					10^{-05}	6	-
				HCC	Neat	1456	1418
					10^{-01}	156	173
					10^{-02}	11	12
			2	CC	10^{-02}	4980	-
					10^{-03}	621	-
					10^{-04}	43	-
					10^{-05}	4	-
				HCC	Neat	3548	3316
					10^{-01}	383	374
					10^{-02}	45	64
			3	CC	10^{-02}	-	-
					10^{-03}	681	-
					10^{-04}	60	-
					10^{-05}	6	-
				HCC	Neat	3896	4628
					10^{-01}	363	349
					10^{-02}	40	41

Strain	Ade Phenotype	Heteroalelles	Experiment	Media[1]	Dilution[2]	Number of colonies[3] Plate 1	Plate 2
Wild-type	+	*Cla*I / *Eco*RI	1	CC	10^{-03}	1697	-
					10^{-04}	194	-
					10^{-05}	27	-
					10^{-06}	1	-
				HCC	Neat	874	768
					10^{-01}	80	75
					10^{-02}	6	5
			2	CC	10^{-03}	2039	-
					10^{-04}	245	-
					10^{-05}	20	-
					10^{-06}	3	-
				HCC	Neat	1064	1042
					10^{-01}	110	-
					10^{-02}	11	9
			3	CC	10^{-03}	2120	-
					10^{-04}	232	-
					10^{-05}	24	-
					10^{-06}	2	-
				HCC	Neat	1249	1419
					10^{-01}	125	103
					10^{-02}	15	12

Strain	Ade Phenotype	Heteroalelles	Experiment	Media[1]	Dilution[2]	Number of colonies[3] Plate 1	Plate 2
Wild-type	-	*Cla*I / *Eco*RI	1	CC	10^{-03}	2624	-
					10^{-04}	264	-
					10^{-05}	23	-
					10^{-06}	5	-
				HCC	Neat	1504	1837
					10^{-01}	213	181
					10^{-02}	20	13
			2	CC	10^{-03}	1686	-
					10^{-04}	193	-
					10^{-05}	25	-
					10^{-06}	5	-
				HCC	Neat	1233	1272
					10^{-01}	109	117
					10^{-02}	12	10
			3	CC	10^{-03}	1041	-
					10^{-04}	130	-
					10^{-05}	9	-
					10^{-06}	2	-
				HCC	Neat	761	783
					10^{-01}	73	71
					10^{-02}	15	7

Strain	Ade Phenotype	Heteroalelles	Experiment	Media[1]	Dilution[2]	Number of colonies[3] Plate 1	Plate 2
Wild-type	+	*EcoR*I / *Bgl*II	1	CC	10^{-02}	8208	-
					10^{-03}	720	-
					10^{-04}	52	-
					10^{-05}	6	-
				HCC	Neat	1598	1798
					10^{-01}	153	135
					10^{-02}	23	10
			2	CC	10^{-03}	1043	-
					10^{-04}	105	-
					10^{-05}	18	-
					10^{-06}	1	-
				HCC	Neat	-	-
					10^{-01}	413	408
					10^{-02}	38	39
			3	CC	10^{-03}	842	-
					10^{-04}	70	-
					10^{-05}	8	-
					10^{-06}	2	-
				HCC	Neat	2204	2192
					10^{-01}	234	236
					10^{-02}	22	27

Strain	Ade Phenotype	Heteroalelles	Experiment	Media[1]	Dilution[2]	Number of colonies[3] Plate 1	Plate 2
Wild-type	-	*EcoR*I / *Bgl*II	1	CC	10^{-02}	-	-
					10^{-03}	1048	-
					10^{-04}	147	-
					10^{-05}	11	-
				HCC	Neat	2192	2280
					10^{-01}	344	347
					10^{-02}	32	0
			2	CC	10^{-02}	-	-
					10^{-03}	1037	-
					10^{-04}	104	-
					10^{-05}	11	-
				HCC	Neat	2848	3284
					10^{-01}	390	421
					10^{-02}	50	40
			3	CC	10^{-02}	-	-
					10^{-03}	918	-
					10^{-04}	86	-
					10^{-05}	2	-
				HCC	Neat	2649	3300
					10^{-01}	339	368
					10^{-02}	31	32

Strain	Ade Phenotype	Heteroalelles	Experiment	Media[1]	Dilution[2]	Number of colonies[3] Plate 1	Plate 2
*exo1*Δ	+	*Xho*I / *Eco*RI	1	CC	10^{-02}	3319	-
					10^{-03}	344	-
					10^{-04}	17	-
					10^{-05}	2	-
				HCC	Neat	1748	1093
					10^{-01}	105	101
					10^{-02}	8	12
			2	CC	10^{-02}	2500	-
					10^{-03}	234	-
					10^{-04}	22	-
					10^{-05}	2	-
				HCC	Neat	1208	986
					10^{-01}	80	113
					10^{-02}	10	10
			3	CC	10^{-02}	1575	-
					10^{-03}	171	-
					10^{-04}	16	-
					10^{-05}	6	-
				HCC	Neat	459	433
					10^{-01}	50	49
					10^{-02}	3	5

Strain	Ade Phenotype	Heteroalelles	Experiment	Media[1]	Dilution[2]	Number of colonies[3] Plate 1	Plate 2
*exo1*Δ	-	*Xho*I / *Eco*RI	1	CC	10^{-02}	4108	-
					10^{-03}	322	-
					10^{-04}	26	-
					10^{-05}	2	-
				HCC	Neat	1547	1687
					10^{-01}	142	120
					10^{-02}	17	27
			2	CC	10^{-02}	2068	-
					10^{-03}	321	-
					10^{-04}	37	-
					10^{-05}	2	-
				HCC	Neat	1800	1624
					10^{-01}	107	276
					10^{-02}	17	11
			3	CC	10^{-02}	4020	-
					10^{-03}	463	-
					10^{-04}	50	-
					10^{-05}	3	-
				HCC	Neat	1488	1660
					10^{-01}	163	150
					10^{-02}	20	16

Strain	Ade Phenotype	Heteroalelles	Experiment	Media[1]	Dilution[2]	Number of colonies[3] Plate 1	Plate 2
*exo1*Δ	+	*Cla*I / *Eco*RI	1	CC	10^{-02}	5692	-
					10^{-03}	600	-
					10^{-04}	54	-
					10^{-05}	3	-
				HCC	Neat	154	149
					10^{-01}	18	15
					10^{-02}	3	1
			2	CC	10^{-02}	-	-
					10^{-03}	880	-
					10^{-04}	86	-
					10^{-05}	9	-
				HCC	Neat	154	154
					10^{-01}	18	15
					10^{-02}	3	1
			3	CC	10^{-03}	1114	-
					10^{-04}	130	-
					10^{-05}	11	-
					10^{-06}	1	-
				HCC	Neat	306	318
					10^{-01}	44	26
					10^{-02}	4	2

Strain	Ade Phenotype	Heteroalelles	Experiment	Media[1]	Dilution[2]	Number of colonies[3] Plate 1	Plate 2
*exo1*Δ	-	*Cla*I / *Eco*RI	1	CC	10^{-03}	638	-
					10^{-04}	63	-
					10^{-05}	14	-
					10^{-06}	0	-
				HCC	Neat	200	312
					10^{-01}	14	30
					10^{-02}	3	6
			2	CC	10^{-03}	984	-
					10^{-04}	106	-
					10^{-05}	10	-
					10^{-06}	1	-
				HCC	Neat	399	371
					10^{-01}	32	41
					10^{-02}	1	5
			3	CC	10^{-03}	469	-
					10^{-04}	44	-
					10^{-05}	4	-
					10^{-06}	1	-
				HCC	Neat	223	246
					10^{-01}	23	11
					10^{-02}	15	2

Strain	Ade Phenotype	Heteroalelles	Experiment	Media[1]	Dilution[2]	Number of colonies[3] Plate 1	Plate 2
*exo1*Δ	+	*EcoR*I / *Bgl*II	1	CC	10^{-02}	3696	-
					10^{-03}	447	-
					10^{-04}	50	-
					10^{-05}	2	-
				HCC	Neat	666	718
					10^{-01}	53	71
					10^{-02}	4	7
			2	CC	10^{-02}	-	-
					10^{-03}	609	-
					10^{-04}	44	-
					10^{-05}	6	-
				HCC	Neat	938	957
					10^{-01}	78	86
					10^{-02}	15	9
			3	CC	10^{-02}	-	-
					10^{-03}	954	-
					10^{-04}	60	-
					10^{-05}	3	-
				HCC	Neat	673	768
					10^{-01}	92	79
					10^{-02}	7	11

Strain	Ade Phenotype	Heteroalelles	Experiment	Media[1]	Dilution[2]	Number of colonies[3] Plate 1	Plate 2
*exo1*Δ	-	*EcoR*I / *Bgl*II	1	CC	10^{-02}	3744	-
					10^{-03}	438	-
					10^{-04}	46	-
					10^{-05}	8	-
				HCC	Neat	1011	1083
					10^{-01}	96	99
					10^{-02}	7	9
			2	CC	10^{-02}	4824	-
					10^{-03}	634	-
					10^{-04}	61	-
					10^{-05}	5	-
				HCC	Neat	1144	916
					10^{-01}	73	116
					10^{-02}	11	13
			3	CC	10^{-02}	1816	-
					10^{-03}	216	-
					10^{-04}	26	-
					10^{-05}	1	-
				HCC	Neat	345	305
					10^{-01}	33	42
					10^{-02}	4	4

[1] CC: media selecting for resistance to canavanine and cycloheximide.
HCC: media selecting for resistance to canavanine and cycloheximide, and the ability to synthesise histidine.
[2] On CC media, 200 µl from a total of 550 µl of dilutions $10^{-02} - 10^{-06}$ was plated.
On HCC media, 50 µl from a total of 550 µl of neat solution and dilutions $10^{-01} - 10^{-02}$ was plated.
[3] When selecting for His+ events, due to the small number of colonies obtained, each dilution was plated twice. Only one set was plated to determine the total number of colonies. Any boxes containing a dash (-) for the dilutions on HCC media was due to there being too many colonies to count or that they were too clustered together to count accurately.

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