DOCTOR OF PHILOSOPHY

The cloning and characterisation of Schizosaccharomyces pombe rec20-144

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Abstract

The process of meiosis enables the generation of genetic diversity. Intrinsic to this process is meiotic homologous recombination. Studies to date indicate the greatest part of meiotic recombination is initiated at a limited number of recombinational "hotspots".

In this study we demonstrate that the *Schizosaccharomyces pombe* meiotic recombination mutant *rec20-144* is an allele of *rec10*$^{+}$ and that Rec10 is a component of linear elements. We also determine that a function(s) of Rec10 which is lost in the *rec20-144* mutant is required for the full activation of certain *M26* heptamer-containing recombination hotspots and that this function(s) can be suppressed by a single alteration to the nucleotide context within which an *M26* heptamer is embedded. This demonstrates that the chromosomal context within which a *cis*-activating hotspot element is embedded influences whether some factors are required for full hotspot activation. Finally, as we demonstrate that Rec10 shares structural similarities to *S. cerevisiae* Red1 and has weak amino acid homology to Red1 and *C. elegans* XNP1 a possibility exists that this modulation of hotspot activity may be conserved.

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Acronyms:

AARE	amino acid response element
AE	axial element
APC	anaphase promoting complex
DSB	double stranded break
DSBR	double strand break repair
HAT	Histone acetyl transferase
HDAC	Histone deacetyl complex
HJ	Holliday junction
JM	joint molecule
LE	linear element
MI	meiosis I
MII	meiosis II
NDJ	non disjunction
SC	synaptonemal complex
SCC	sister chromatid cohesion
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEI	single end invasion
SNP	single nucleotide polymorphism

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Chapter 1 Literature review

1.1 Introduction

Life on earth relies upon the remarkable ability of deoxyribonucleic acid (DNA) molecules to faithfully replicate and then pass down to the next generation the genetic information contained within a genome. The fact that this process and evolution can both be achieved appears contradictory. However, eukaryotic organisms have a way of bestowing an "altered" potentially evolutionary genome upon their offspring through the use of a specialised process termed meiosis.

In the following review I will outline meiosis and the molecular processes that make it possible with special emphasis on recombination at specific-loci termed meiotic hotspots. Finally I will discuss the role of the *S. pombe* protein, Rec10 in meiosis and meiotic recombination.

1.2 Meiosis, an overview

Meiosis forms genetically distinct haploid gametes or progeny from diploid precursor cells. In order to achieve this, the meiotic cycle employs a single round of DNA replication followed by two successive rounds of chromosome and nuclear division. In molecular terms, within a diploid precursor cell, pre-meiotic DNA replication creates pairs of homologous chromosomes consisting of two "joined" sister chromatids. The first meiotic division, termed meiosis I or MI, then separates (disjoins) each pair of homologous chromosome and segregates them into two individual nuclei. As MI reduces a diploid chromosomal number to a haploid chromosomal number, it is known as the reductional division. The second meiotic division, meiosis II or MII, then disjoins each homologous chromosome into individual chromatids and then shares them equally into four individual gametes or progeny. Meiosis II is therefore an equational division and resembles a mitotic division. In the following sections, I will outline the various stages and sub-stages of the meiosis I and II highlighting (where applicable) both the cytological observations and the physical interactions taking place between DNA molecules.

1.3 The stages of meiosis

Both meiosis I and II are comprised of a number of sub-stages classified via the cytological observations of both chromosomal appearance and movements which occur during each stage. The stages in the order of which they occur are prophase, metaphase, anaphase and telophase (Figure 1.1). Prophase of meiosis I (prophase I) is especially long and as such has been further divided into five sub-stages. The division between sub-stages is based once more upon the cytological observations and molecular interactions taking place between homologous chromosomes in each sub-stage (Table 1.1). It is during prophase I that homologous chromosomes come together and align (termed pairing), form a common proteinaceious axis (synapse) and in most cases form physical connections (recombine), utilising homologous recombination.

Figure 1.1
The Stages and classification of the stages of meiosis I and II

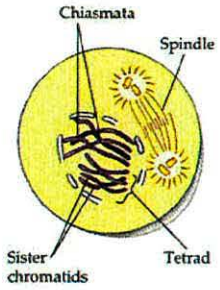
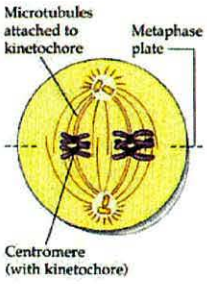
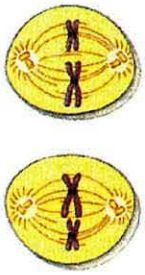
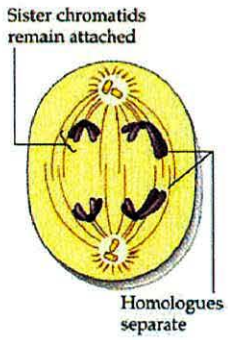
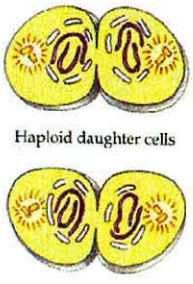
Prophase I 	Homologous chromosomes pair synapse and usually undergo homologous recombination to form joint molecules. (see Table 1.1 for sub-stages of prophase I).	Prophase II 	Chromosomes consisting of two chromatids attached at centromeric regions begin to congress to the metaphase II plate.
Metaphase I 	Pairs of homologous chromosome align at the metaphase plate.	Metaphase II 	Chromosomes align on the metaphase II plate.
Anaphase I 	Recombined homologues disjoin and move to opposite poles.	Anaphase II 	Centromeric cohesion is dissolved and each chromosomes disjoins into two sister chromatids
Telophase I 	Nuclear envelope formation and cytokinesis may take place or the nuclei may directly enter meiosis II.	Telophase II 	4 haploid progeny are formed from individual chromatids
Adapted from (Campbell and Reece, 1996)			

Table 1.1
The Stages and classification of the 5 sub-stages of meiosis prophase I.

Sub-stages of prophase I	Characteristic cytological observations that define the onset of each sub-stage of prophase I.
Leptotene	Chromosomes start to condense. Telomeres begin to cluster at late leptotene as homologue pairing initiates.
Zygotene	Telomere clustering continues leading to formation of the "bouquet structure". Homologue pairing is complete and synapsis initiates.
Pachytene	Synapsis is complete. Reciprocal recombination between homologues form chiasmata (crossovers). The resulting crosslinked structure is called a bivalent.
Diplotene	The bivalents begin to separate but remain attached by the chiasmata
Diakinesis	Chromatids condense further Centrioles/spindle pole bodies begin to migrate to opposite poles of the nucleus. Bivalents congress on the metaphase plate.

1.4 *Schizosaccharomyces pombe* a model eukaryote for the study of meiosis.

S. pombe has many advantages as a model organism and the study of meiosis in *S. pombe* is both relevant and highly amenable. The common *S. pombe* lab strains are predominately isogenic and extremely tractable micro-organisms with a wide range of molecular biological techniques available for their genetic manipulation. In addition the three *S. pombe* chromosomes are similar in structural format to higher eukaryotic chromosomes, especially in centromeric and pericentric regions (Takahashi *et al.*, 1992) and have been fully sequenced (Wood *et al.*, 2002).

S. pombe meiosis can be initiated from cells in either a haploid or diploid state with a high degree of synchronicity via starvation for nutrients (especially nitrogen) and/or through the use of the *pat1-114* allele, a temperature sensitive mutant of *pat1*, a gene that codes for a key meiotic regulatory kinase (Iino and Yamamoto, 1985; Nurse, 1985). Using these techniques, the meiotic gene expression pattern has been characterised (Mata *et al.*, 2002) and many of the proteins expressed have homologues or orthologues in higher eukaryotes (Wood *et al.*, 2002). Meiosis can also be interrupted at various stages through the use of other mutant alleles (Nakaseko *et al.*, 1984; Niwa and Yanagida, 1988; Shimoda *et al.*, 1985) allowing further dissection of the meiotic cycle. Meiotic recombination in *S. pombe* is also highly efficient, with approximately 45 meiotic crossovers taking place distributed in the ratio of 19:15:11 on chromosomes I, II and III respectively (Munz *et al.*, 1989). Finally, the low chromosomal complement of *S. pombe* has allowed the development of screens for meiotic deficient mutant genes (DeVeaux, 1992; Ponticelli and Smith, 1989). The subsequent cloning of many of these genes has greatly enhanced our understanding of both meiotic and other cellular processes in higher eukaryotes (see Chapter 3).

1.5 Commitment to meiosis, in *Schizosaccharomyces pombe*.

In wild type *S. pombe* the change from the mitotic to the meiotic cell cycle requires two conditions namely, heterozygosity (h^+/h^-) at the mating-type (*mat*) locus and starvation for nutrients, especially nitrogen. Diploid cells that are heterozygous at *mat* can directly enter meiosis. However, haploid cells must firstly form a diploid h^+/h^- zygote. This is achieved through a mating of haploid h^+ and h^- cells. Mating itself consists of two basic stages, firstly conjugation (cell fusion) and then karyogamy (nuclear fusion). Once a heterozygous (h^+/h^-) state is present, further nutrient starvation enforces a cell signalling cascade reaction that transiently initiates a G1 arrest, premeiotic DNA synthesis and progress into meiosis.

In molecular terms, nutrient starvation is sensed by unknown mechanism that involves at least one transmembrane G-protein (Git3) (Welton and Hoffman, 2000), its Gα subunit (Gpa2) (Isshiki *et al.*, 1992) and the action of adenylyl cyclase (Cyr1) (Yamawaki-Kataoka and *et al.*, 1989; Young and *et al.*, 1989). Other signaling modules involved in commitment to meiosis are the mTOR-PDK1-S6K1 system (Matsuo *et al.*, 2003) and the MAP kinase cascade (Kanoh *et al.*, 1996; Shiozaki and Russell, 1996; Smith *et al.*, 2002; Takeda *et al.*, 1995). Both of these signalling systems are highly conserved in higher eukaryotes and also involved in response to cellular stresses. A primary outcome of sensing nutrient starvation is the modulation of cAMP concentration to a critical threshold that initiates a transient G1 arrest through inhibition of protein kinase 1a, (Pka1). This transient G1 arrest is then enhanced by a series of feedback reactions which result in a further decrease in the cellular level of cAMP (Mochizuki and Yamamoto, 1992) resulting in the release of transcriptional inhibition (Sugimoto *et al.*, 1991) of the meiotic transcription factor *ste11* (Watanabe *et al.*, 1988) via Rst2 (Higuchi *et al.*, 2002; Kunitomo *et al.*, 2000). Ste11 is a member of the high mobility group (HMG) transcription factors that regulate expression of the *S. pombe* meiotic transcriptome via targeting promoters which contain a 5′ TR-box motif (TTCTTTGTTY) (Sugimoto *et al.*, 1991). Ste11-dependent transcription operates in meiosis via production of Mei3 (McLeod and Beach, 1988), a Pat1 kinase psuedosubstrate (Li and McLeod, 1996; Sato *et al.*, 2002; Wang *et al.*,

1998) and via activation of the pheromone-responsive MAP kinase pathway (Yamamoto *et al.*, 2004). Repression of Pat1 activity by Mei3 and activation of the pheromone-responsive MAP kinase pathway both lead to release of inhibition of transcription of the meiotic DNA replication initiator protein Mei2 (Shimoda *et al.*, 1987). Transcription of *mei2* defines a critical (Watanabe *et al.*, 1988; Yamamoto *et al.*, 2004) but not essential (Peng *et al.*, 2003) point in the initiation of meiotic DNA replication and commitment to meiosis (see Figure 1.2 for diagram of signalling pathways involved in commitment to meiosis in *S. pombe*).

The meiotic transcriptome consists of some 250 upregulated genes which have been sub-dived into categories (Mata *et al.*, 2002). In many of the promoters of the approximately 40 "delayed" genes are sequences enriched in the Ste11 TR-box motif. In the "continuous" group are at least 20 stress response genes (Mata *et al.*, 2002) including the mitogen activated protein kinase (MAPK) homologue Spk1 (Toda *et al.*, 1991). An important part of MAP kinase signalling pathway is its role in stress activation through the modulation of the b-Zip transcription factor Atf1 (Shiozaki and Russell, 1996; Takeda *et al.*, 1995; Wilkinson *et al.*, 1996). Atf1 is a cyclic AMP response element (CRE) binding protein (CREB) (reviewed in (Johannessen *et al.*, 2004; Roesler, 2000). Both Atf1 and its related CREB protein Pcr1 (Watanabe and Yamamoto, 1996) play a crucial role in the activation of the *S. pombe M26* and CRE meiotic recombination hotspots. The importance of these CREB proteins to this study is highlighted in chapters 4, 5, 6, 7 and 8.

Figure 1.2
Diagram of major signaling pathways involved in commitment to meiosis in *S. pombe*.

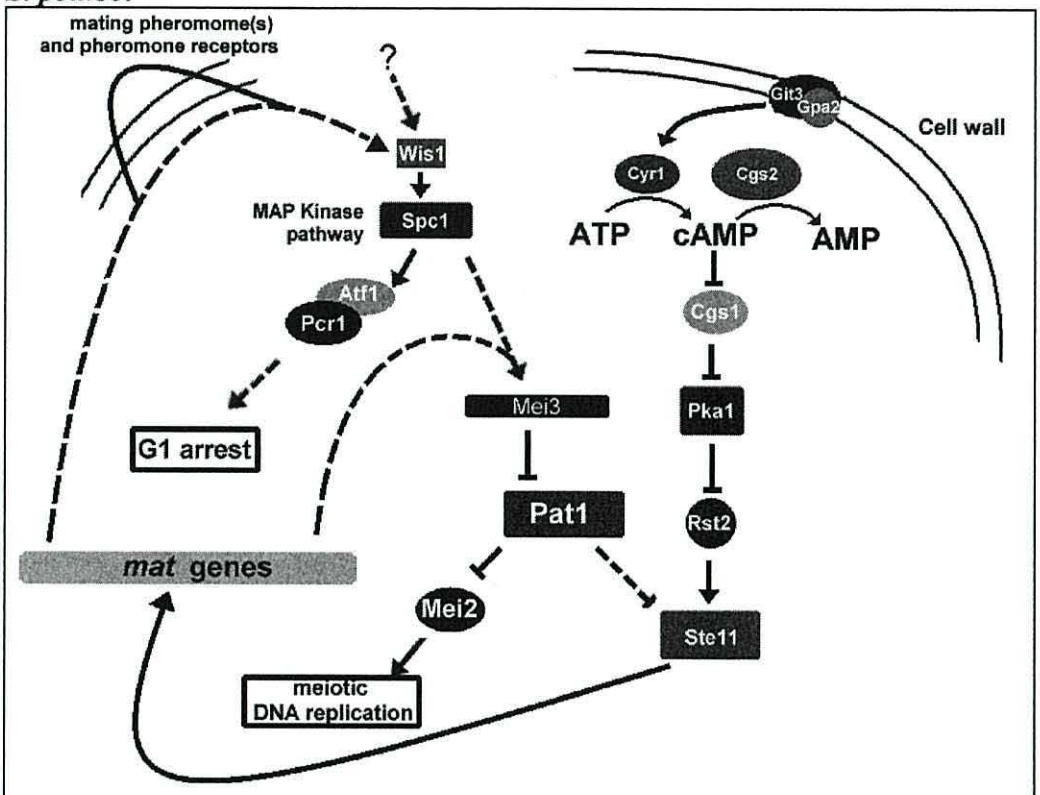


Figure 1.2 represents a diagram of the signalling pathways that are involved in commitment to meiosis in *S. pombe*.
Full lines indicate proven direct interaction
Dotted lines indicate possibility of stages prior to effect on target protein
Arrow heads indicate stimulation
Crossed heads indicate suppression
Joined pathways indicates joint interaction(s) required
See section 1.5 for details and references.

1.6 Meiotic DNA replication, sister chromatid cohesion, homologue pairing and genetic recombination: an integrated process.

A successful meiotic cycle requires a cell faithfully replicates its chromosomes and then correctly segregates them twice before finally producing offspring. To achieve this, meiosis integrates the processes of homologous chromosome pairing and recombination and then the differential loss of meiotic-specific sister chromatid cohesion (SCC). How meiosis integrates these processes is a highly active area of research. Recent studies have shown that initiation of meiotic recombination is linked to the completion of DNA replication (S-phase) at both a regional and a genome wide level (Borde *et al.*, 2000; Murakami *et al.*, 2003). In the following sections I will discuss the aspects of meiotic S-phase, SCC, homologue pairing and homologous recombination and attempt to show these processes are integrated to achieve the faithful segregation of chromosomes during meiosis I and meiosis II.

1.7 Meiotic S-phase.

In the yeast model systems, the nutritional state of a cell entering meiosis has no influence on the length of time it takes to complete meiotic S-phase (Colomina *et al.*, 1999; Hayashi *et al.*, 1998). In addition, the actual speed of a replication fork in mitosis and meiosis appears to be approximately equal (Borde *et al.*, 2000; Williamson *et al.*, 1983). These data would suggest that the duration of meiotic S-phase should be approximately the same as mitotic S-phase. However, this is not the case in either yeast or higher eukaryotes (Cha *et al.*, 2000).

Perhaps to delay replication when DNA is damaged (Shirahige *et al.*, 1998) or to help minimise the number of active replication forks when cells are starved for dNTPs (Santocanale and Diffley, 1998), the length of mitotic S-phase can be modulated by suppression of late-firing replication origins. In *S. cerevisiae*, the *MEC1* checkpoint gene and its downstream target *RAD53* are involved in the control of late origin firing in mitosis and also in meiosis (Cha and Kleckner, 2002). As it appears that the same replication origins and basic replication machinery are employed in both mitotic and meiotic S-phase (Collins and

Newlon, 1994; Forsburg and Hodson, 2000; Grallert and Sipiczki, 1991; Murakami *et al.*, 2003) this suggests that the length of meiotic S-phase may be regulated by the regulation of late firing replication origins. However, which events could initiate this modulation of late origin firing is unknown.

In both yeasts the chromosome segregation patterns of MI and MII requires the establishment of meiotic-specific SCC, the formation of meiotic-specific chromosomal structures and homologous recombination. In addition to Mec1 and Rad53, the evolutionary conserved proteins; Rec8 (Klein *et al.*, 1999), a meiotic-specific cohesin and Spo11 (Bergerat *et al.*, 1997), the endonuclease that initiates meiotic double stranded break (DSB) formation (Keeney *et al.*, 1997), can both individually and synergistically modulate meiotic S-phase (Cha *et al.*, 2000). In *S. cerevisiae*, Rec8 is required for the formation of axial elements the precursors of the lateral elements that form part of a meiotic-specific chromosomal structure called the synaptonemal complex (reviewed in Zickler and Kleckner, 1999). In *S. pombe*, Rec8 is also required for the formation of meiotic-specific chromosomal structures called linear elements, thought to be evolutionary relics of the lateral elements of the SC (Bahler *et al.*, 1993; Lorenz *et al.*, 2004; Molnar *et al.*, 1995; Molnar *et al.*, 2003). In both yeasts failure to create SC or LEs results in increased mis-segregation of chromosomes and loss of spore viability (Klein *et al.*, 1999; Molnar *et al.*, 1995; Molnar *et al.*, 2003; Rockmill and Roeder, 1990).

In summary, why S-phase in meiosis is longer than in mitosis and how this is achieved are still not fully understood. However, evidence suggests that the control of late replication origin firing is used to extend meiotic S-phase in order to allow the formation of meiotic-specific structures that facilitate the interhomologue interactions and homologous recombination that take place during meiosis (Cha *et al.*, 2000; Watanabe and Nurse, 1999; Watanabe *et al.*, 2001). As stated above, some of the proteins required for the formation of these structures are the cohesins.

1.8 Cohesins and SCC formation

Cohesins and SCC play a vital role in the correct segregation of chromosomes in mitosis (Michaelis *et al.*, 1997; Nasmyth, 1999; Nasmyth *et al.*,

2000; Panizza *et al.*, 2000). In meiosis, cohesins are required for meiotic recombination (DeVeaux and Smith, 1994), full hotspot activity (Chapter 5) and segregation of chromosomes during meiosis I and meiosis II (Buonomo *et al.*, 2000; Kitajima *et al.*, 2003a; Watanabe and Nurse, 1999; Yokobayashi *et al.*, 2003). The basic mitotic cohesin complex consists of homologues of at least four proteins; Scc1, Scc3, Smc1, and Smc3 (Reviewed in (Jones and Sgouros, 2001). In *S. cerevisiae* and *S. pombe*, the meiotic cohesin complexes contain both mitotic and meiotic-specific cohesins (Kitajima *et al.*, 2003b; Klein *et al.*, 1999). In *S. cerevisiae* the main difference between the mitotic and meiotic cohesin complexes appears to be that Scc1 is predominately replaced by its meiotic homologue, Rec8 (Klein *et al.*, 1999). In *S. pombe*, two meiotic-specific cohesins are found in cohesin complexes; Rec8 (Parisi *et al.*, 1999) and Rec11 (Ponticelli and Smith, 1989), a homologue of Psc3(Scc3). However, whilst *S. cerevisiae* Rec8 is found along the entire length of meiotic chromosomes (Klein *et al.*, 1999), *S. pombe* Rec8 and Rec11 are predominately confined to certain chromosomal domains namely; centromeric regions (Rec8) and chromosomal arms (Rec11) (Kitajima *et al.*, 2003b; Watanabe and Nurse, 1999). Therefore, in two aspects of meiotic SCC *S. pombe* appears to more closely mimic higher eukaryotes (Pezzi *et al.*, 2000). In addition to the cohesins the formation and maintenance of SCC also requires several other proteins which include Scc2, Scc4 (Ciosk *et al.*, 2000; Furuya *et al.*, 1998), Eco1 (Tanaka *et al.*, 2000a; Toth *et al.*, 1999) and Pds5 (Hartman *et al.*, 2000; Panizza *et al.*, 2000).

In *S. cerevisiae*, the loading of the mitotic cohesin complex onto chromosomes is facilitated by a heterodimer of the Scc2 and Scc4 "Adherin" proteins (Ciosk *et al.*, 2000) and the activation of SCC takes place only during S-phase (Skibbens *et al.*, 1999; Uhlmann and Nasmyth, 1998). In *S. pombe*, Mis4 (Furuya *et al.*, 1998) a homologue of Scc2, is also required for establishment of SCC during S-phase (Tomonaga *et al.*, 2000).

In *S. cerevisiae*, mitotic cohesin complexes form >100 foci (Michaelis *et al.*, 1997; Toth *et al.*, 1999; Uhlmann and Nasmyth, 1998) spread along the length of chromosomes (Guacci *et al.*, 1997; Toth *et al.*, 1999). At higher resolution, analysis of chromosome III has shown cohesion complexes to be located at

approximately 23 locations that closely correspond to peaks of high local AT base composition found at the chromosomal "axis" that lays between sister chromatids (Blat and Kleckner, 1999). These locations probably coincide with regions of convergent actively transcribing genes, which have been shown to play a fundamental role in the final placement of both mitotic and meiotic cohesin complexes (Lengronne *et al.*, 2004).

In mitosis the establishment of SCC takes place during or shortly after S-phase. In *S. pombe*, Rec8 (Parisi *et al.*, 1999; Ponticelli and Smith, 1989) must also be present and activated during meiotic S-phase to enable it to perform its meiotic-specific recombination and chromosomal segregation functions (Watanabe *et al.*, 2001). One process of SCC formation probably involves the catalysed "linkage" of sister chromatids as they emerge from passing replication forks (Toth *et al.*, 1999; Uhlmann and Nasmyth, 1998) facilitated by homologues of Ctf7(Eco1), a chromosome transmission fidelity protein that is active specifically in S-phase (Skibbens *et al.*, 1999). Ctf7 interacts with PCNA, an important component of the replication fork and *ctf7* mutants can be rescued by over expression of PCNA (Skibbens *et al.*, 1999). In addition cohesion is not established in Pol kappa mutants (Wang *et al.*, 2000). In *S. pombe*, the Eco1 homologue, Eso1 possesses an N-terminal domain with a highly homology to the DNA polymerase η (Tanaka *et al.*, 2000a) and recombination plays a role in the initiation of *S. pombe* DNA replication (Segurado *et al.*, 2002). These data suggest that the linking of sister chromatids during DNA replication may be a conserved way of establishing SCC in eukaryotes. However, this is not the only way of establishing SCC. In *S. pombe*, during both mitosis and meiosis, apart from the centromere central core, centromeric SCC is established after DNA replication and is dependent on heterochromatin formation. (Nonaka *et al.*, 2002; Kitajima *et al.*, 2003b). These data highlight possible distinct differences between the nature of how SCC is established at heterochromatic domains and within chromosomal arms.

In summary, the core cohesins and SCC establishment/maintenance proteins have homologues in all the eukaryotic systems studied to date (Ball Jr and Yokomori, 2001; Jones and Sgouros, 2001; Sumara *et al.*, 2000) and it

appears the method(s) of establishing and maintaining SCC during mitosis and meiosis may also be highly conserved (Biggins and Murray, 1999; Darwiche *et al.*, 1999; Losada *et al.*, 1998; Michaelis *et al.*, 1997; OrrWeaver, 1999), (see table 1.2 for a list of cohesins and proteins required for cohesion establishment and maintenance in the yeast model systems). In addition to their roles in SCC meiotic cohesins also play an important role in meiotic recombination.

Table 1.2
Cohesin and cohesion establishment/maintenance proteins in yeast.

Primary functions as	*S. cerevisiae*		*S. pombe*	
	Mitotic	**Meiotic**	**Mitotic**	**Meiotic**
Core members of Cohesin complex	*SCC1*	*REC8*	*rad21*$^+$	*rec8*$^+$
	SCC3	-	*psc3*$^+$	*rec11*$^+$
	SMC1	-	*psm1*$^+$	-
	SMC3	-	*psm3*$^+$	-
Cohesion	*PDS5*	*PDS5?*	*pds5*$^+$	*pds5*$^+$
Loading and maintenance of SCC	*SCC2*	-	*mis4*$^+$	-
	SCC4	-	-	-
Establishment of cohesion	*ECO1*	-	*eso1*$^+$	-

Table 1.2 shows a list of cohesin and cohesin associated proteins from *S. pombe* and *S. cerevisiae*
(-) Indicates no homologue yet discovered.
See section 1.8 for appropriate references

1.9 Cohesins and meiotic recombination

It has been suggested that meiotic recombination may have evolved from the mechanisms and proteins used in mitotic DNA repair (van Heemst and Heyting, 2000). Although this does seem to make a logical progression, there is no direct evidence that mitosis proceeded meiosis in evolutionary terms (Cavalier-Smith, 2002). However, in addition to their role in SCC mitotic cohesins are required for response to DNA damage (Jessberger *et al.*, 1996; Sjogren and Nasmyth, 2001; Sonoda *et al.*, 2001; Tatebayashi *et al.*, 1998) and have been implicated in deciding whether inter-sister or inter-homologue recombination is used to repair this damage (Hartsuiker *et al.*, 2001). The correct segregation of homologues during meiosis I depends upon recombination between homologues and not sister chromatids. The distinctive feature of meiotic recombination is how this interhomologue recombination is preferentially achieved (Schwacha and

Kleckner, 1997) over the mitotic DNA repair pathway that favours recombination between sister chromatids (Petes and Pukkila, 1995). The bias towards interhomologue recombination is established very early in meiosis, prior even to the formation of DSBs (Bishop *et al.*, 1999; Bishop and Zickler, 2004) and enforced by meiotic-specific proteins (Nag *et al.*, 1995; Schwacha and Kleckner, 1997) required for the formation of the SC. One of these proteins is Red1 (Rockmill and Roeder, 1988). In *S. pombe, rec10*$^{+}$ is required for LE formation (Molnar *et al.*, 2003), is a component of LEs and has structural and weak sequence homology with Red1 (Lorenz *et al.*, 2004). These data suggest that one role of the SC and LEs may be the enforcement of interchromosomal recombination. Before chromosomes can undergo efficient interchromosomal recombination they must firstly correctly pair.

1.10 Homologue pairing

The pairing of homologue chromosomes is an important stage in meiotic recombination. Disruptions in pairing for example by preventing telomere attachment to the spindle pole body (SPB) (Cooper *et al.*, 1998; Nimmo *et al.*, 1998) or horsetailing (Yamamoto *et al.*, 1999) result in significant reductions in homologous recombination. Telomeres plays an important role in enabling homologue recognition and pairing in *S. cerevisiae* (Chua and Roeder, 1997; Conrad *et al.*, 1997; Rockmill and Roeder, 1998), *S. pombe* (Cooper *et al.*, 1998; Nimmo *et al.*, 1998; Shimanuki *et al.*, 1997) and higher eukaryotes (Bass *et al.*, 1997; Scherthan *et al.*, 1996). In meiosis telomeres cluster at the nuclear periphery and form a cytologically observable structure known as a "bouquet" (Zickler and Kleckner, 1998). This evolutionary conserved stage of meiotic homologue pairing is seen in budding yeast, (TrellesSticken *et al.*, 1999), fission yeast (Chikashige *et al.*, 1994) and in higher eukaryotes (Bass *et al.*, 2000).

1.11 The bouquet structure and *S. pombe* horsetailing

Exposure to mating pheromone during *S. pombe* meiosis leads to a series of profound nuclear reorganisations. One of these reorganisations involves a switch from a mitotic "Rabl" chromosome configuration (where centromeres are

clustered at the spindle pole body (SPB) and telomeres associated with the nuclear membrane) to a meiosis specific "bouquet" configuration where centromeric SPB clustering is replaced by SPB telomeric clustering. An analogous telomere-centromere switch also occurs in diploid *S. pombe* cells (Watanabe *et al.*, 1997), budding yeast (Jin *et al.*, 1998; TrellesSticken *et al.*, 1999) and higher eukaryotes (Zickler and Kleckner, 1998). The *S. pombe* the telomere-centromere switch takes place in two phases. In the first phase, telomeres associate with the SPB, then in the second phase centromeres dissociate from the SPB (Chikashige *et al.*, 1997). The first phase can take place in the haploid cells in response to mating pheromone, but the second does not take place in haploid cells and probably depends on conjugation-related events (Chikashige *et al.*, 1997). In addition to forming a bouquet structure, immediately following karyogamy (in haploid, zygotic meiosis) and before initiation of premeiotic DNA replication (in diploid, azygotic meiosis) a pronounced cytoplasmic dynein-dependent nuclear movement also occurs (Yamamoto *et al.*, 1999). Due to the extended shape that the nucleus forms during this movement it has been named "horsetailing" (Chikashige *et al.*, 1994). Horsetailing involves a SPB lead "dragging" of the nucleus from one end of the cell via the selective stabilisation and destabilisation of astral microtubules originating from the spindle-pole body (SPB) at an average speed of about 5 μm/minute (Ding and Smith, 1998) and persists throughout DNA replication and into late prophase I (Chikashige *et al.*, 1994). Despite their obvious importance, telomere mediated associations are only one way of initiating homologue pairing. Another important method of homologue pairing involves the association of heterochromatic regions (Renauld and Gasser, 1997) especially in species that do not utilise recombination to achieve chromosomal segregation in meiosis I (Merrill *et al.*, 1992; Wolf, 1994).

In summary the bouquet appears to be an important method of aligning chromosomes (Chikashige *et al.*, 1994) perhaps in conjunction with either heterochromatin (Hawley and Theurkauf, 1993) or recombination-independent processes (Davis and Smith, 2003). Once chromosomes have correctly aligned they can undergo efficient homologous recombination.

1.12 The mechanism of homologous recombination: adaptations for meiosis

Meiotic-specific recombination takes place at a frequency ~100-1000 fold higher than in mitotically dividing cells. Of the three main types of chromosomal recombination used by eukaryotes, namely non-homologous end joining (NHEJ), single-strand annealing and homologous recombination, homologous recombination predominates during prophase of meiosis I. Several models have been proposed for how eukaryotic homologous recombination may take place. Although none of these models alone can fully explain every aspect of eukaryotic homologous recombination (Hunter and Kleckner, 2001; Osman *et al.*, 2003; Paques and Haber, 1999) the current most favoured model is called the double strand break repair (DSBR) model (Sun *et al.*, 1991; Szostak *et al.*, 1983).

In the DSBR model DSBs are formed in a DNA duplex. Thereafter the newly created DNA ends of the DSB undergo a 5′-3′ exonucleolytic resection leading to formation of single-stranded free 3′-OH DNA tails which are then able to invade a homologous DNA duplex. In *S. cerevisiae*, the Spo11 protein (Bergerat *et al.*, 1997) catalyses the creation of meiosis-specific DSBs (Keeney *et al.*, 1997). Spo11 shares structural domain homologies with a member of a novel type II topoisomerase present in the archaebacteria *Methanococcus jannaschi* (Nichols *et al.*, 1999) and has homologues in *S. pombe* (*rec12*), *Drosophila melanogaster* (McKim and HayashiHagihara, 1998), mouse (Keeney *et al.*, 1999), humans (Romanienko and CameriniOtero, 1999) and many other eukaryotes (Shannon *et al.*, 1999). Hence the catalysed formation of DSBs by Spo11 homologues is most likely an evolutionary conserved step in the initiation of meiotic recombination (Celerin *et al.*, 2000). In *S. cerevisiae*, in addition to Spo11 at least 10 other gene products are required for DSB formation. In *S. pombe* DSB formation is also suppressed in mutants other than *Rec12* (Cervantes *et al.*, 2000). Thus the catalysis of DSB formation most likely takes place in the context of specifically configured chromosomal structure. In *S. cerevisiae* this assembled structure includes both axial element components (Mao-Draayer *et al.*, 1996) and the Rad50-Mrell-Xrs2 complex (Furuse *et al.*, 1998). The Rad50-Mrell-Xrs2 complex (Johzuka and Ogawa, 1995) seems to have distinct functions. The first of these being the alteration of local chromatin structure possibly to facilitate DSB

formation (Furuse *et al.*, 1998; Ohta *et al.*, 1998), then an exonucleolytic 5′- 3′ resection (Nairz and Klein, 1997) of the blunt ends, in similar fashion to that as performed by SbcC and SbcD, the Rad50 and Mrell homologues of *E. coli* (Sharples and Leach, 1995). The requirement for Rad50 and Xrs2 in meiotic DSB repair is not absolute (Malkova *et al.*, 1996) whereas a third gene, *COM1/SAE2*, is specifically required for meiotic DSB resection (McKee and Kleckner, 1997; Prinz *et al.*, 1997). The Com1/Sae2 protein could mediate either the removal of the 5′ attached Spo11, irreversibly committing DSBs to the recombinational repair pathway protein, or perhaps modulate DSB resection by the Rad50/Mre11/Xrs2 complex. In either case DSB resection creates a free 3′-OH single strand that is then capable of invading a homologous duplex and initiating new DNA synthesis. In the DSBR model, the next stage of homologous recombination involves formation of a double Holliday junction (HJ) (Schwacha and Kleckner, 1995; Schwacha and Kleckner, 1997). In basic terms the free 3′ single strand created by DSB resection invades an homologous chromatid and initiates both new DNA synthesis and a strand exchange event, involving amongst others, the RecA like members of the *RAD52* epistasis group such as the homologues *RAD51, RAD55, RAD57* (Thacker, 1999). Individual members of the *RAD52* epistasis group have been implicated in the choice of whether a HJ is formed between sister-chromatids or, as is predominately the case in meiosis, between the chromatids of homologues (Schwacha and Kleckner, 1994).

1.13 Intersister recombination

Under *in vitro* conditions Rad51 polymerises onto single-stranded DNA and mediates a strand exchange reaction which requires ATP and the heterotrimeric single-stranded DNA-binding factor replication protein A (RPA) (Shinohara and Ogawa, 1995). Evidence for interactions between Rad51, Rad52, Rad55 and Rad57 (Gasior *et al.*, 1998) supports a mechanism in which Rad52 assisted by a Rad55-Rad57 complex displaces RPA bound to ssDNA, which then allows Rad51 (New *et al.*, 1998) to stimulate complementary single-strand exchange (Mortensen *et al.*, 1996). The Rad54 protein then interacts with this

exchange and directs it towards intersister recombination (Shinohara *et al.*, 1997b; Zenvirth *et al.*, 1997).

1.14 Interhomologue recombination

DMC1 (Bishop *et al.*, 1992) like *RAD51* is another member of the *RAD52* epistasis group. Both *DMC1* and *RAD51* mutations have similar meiotic recombination and sporulation defects including hyper-resection of DSBs and delayed joint molecule (JM) formation (Schwacha and Kleckner, 1997; Shinohara and Ogawa, 1995). However, the proteins with which both *DMC1* and *RAD51* associate appear to define two mutually exclusive groups. One key protein that interacts with Rad51 (Jiang *et al.*, 1996) but not Dmc1 (Dresser *et al.*, 1997) is Rad54. The Rad54 protein belongs to the *SWI2/SNF2* family of chromatin remodelling enzymes (Shinohara and Ogawa, 1995). *RAD54* homologues have been identified in *S. pombe* (Muris *et al.*, 1996), mouse and humans (Kanaar *et al.*, 1996) and in *S. cerevisiae* itself, *RDH54/TID1* (Klein, 1997). *S. cerevisiae rdh54* mutants have pronounced meiotic defects and *rad54 rdh54* double mutants are totally meiotically impaired, accumulating DSBs with more resected ends and producing fewer physical recombinants than does either single mutant (Shinohara *et al.*, 1997a). This finding combined with the fact that the Rdh54 protein binds Dmc1 but not Rad51 (Dresser *et al.*, 1997) and that *RAD54* promotes a *DMC1*-independent DSB repair pathway (Bishop *et al.*, 1999) indicates that Rdh54 bound Dmc1 is not normally involved in inter-sister recombination. Taken together with the fact that Rad54 and not Tid1 mediates sister chromatid-based DNA repair (Arbel *et al.*, 1999) this suggests that Rdh54/Tid1 may create specifically Dmc1 configured single-stranded DNA. Hence, Dmc1 may be specifically loaded onto one strand of the processed DSB, so forming a novel asymmetric single end invasion (SEI) recombination intermediate which may promote interhomologue strand exchange (Hunter and Kleckner, 2001). Evidence to support this is provided by the fact that the formation of inter-homologue recombination intermediates requires Red1 (Schwacha and Kleckner, 1997) a protein which modulates Dmc1 loading (Blat *et al.*, 2002).

Figure 1.3
The "Double Strand Break Repair" model of homologous recombination.

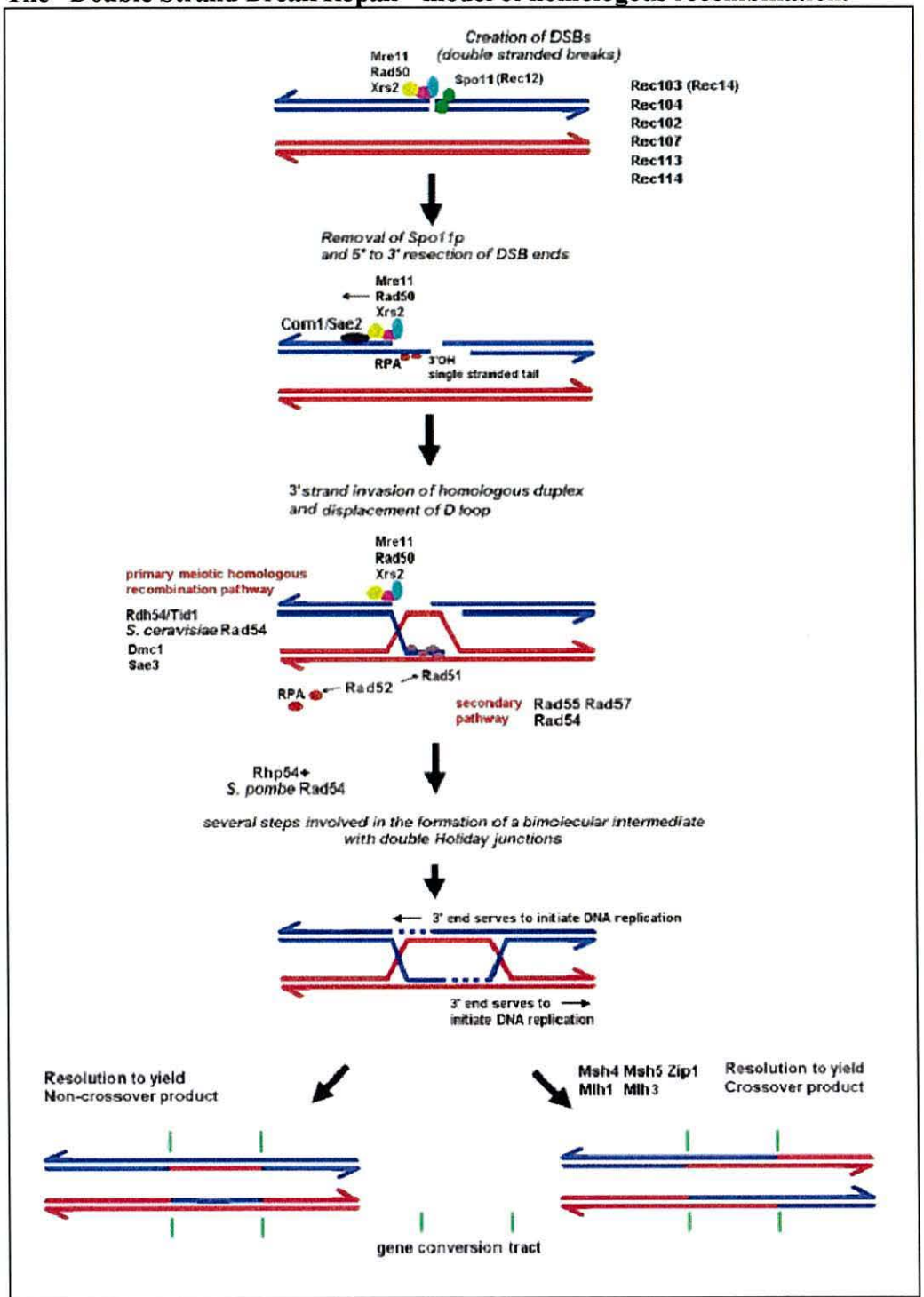


Figure 1.4 shows a diagrammatic representation of proteins required for homologous recombination in *S. cerevisiae* and *S. pombe*.
Figure 1.4 is adapted from Roeder, (1997). Appropriate references can be found in sections 1.20-1.22 and (Roeder, 1997)

1.15 The Holliday junction and its role in meiotic recombination

The resolution of recombination is as important as its initiation. In the previous section I discussed the creation and processing of meiotic DSBs. In the DSBR resolution of a HJ can result in gene conversion with or without a crossover event (Figure 1.5). Although there is strong evidence to suggest this process takes place *in vivo* (Petes *et al.*, 1991) a recent study has shown that the interhomologue and inter-sister pathways may not share a common recombination pathway and be differentiated at a very early stage possibly before the formation of HJ (Allers and Lichten, 2001), reviewed in Paques and Haber, (1999). With this caveat in mind I will now discuss the methods of HJ resolution.

In *E. coli* HJs can be resolved by RuvC and RusA (Sharples *et al.*, 1999). Moreover, depending on the way in which these proteins cut the cruciform HJ structure, resolution can result in either gene conversion or crossing over (Figure 1.5). These findings prompted a long search for eukaryotic homologues of RuvC or RusA. Although this search was unsuccessful, recent studies have identified at a novel class of eukaryotic proteins that can act as HJ resolvases (Boddy *et al.*, 2001; Chen *et al.*, 2001; Ciccia *et al.*, 2003).

1.16 The Mus81-Eme1 complex and Holliday junction resolution

The use of *in vitro* studies and genetic evidence suggests the best candidate for a eukaryotic meiotic HJ resolvase complex may be formed by homologues of the *S. pombe* Mus81-Eme1 family (Boddy *et al.*, 2001). *S. pombe mus81* and/or *eme1* mutants exhibit severe meiotic lethality (Boddy *et al.*, 2001). This lethality depends on the activity of *Rec12* and can be suppressed by expressing the HJ resolvase RusA, implying a link to the formation and resolution of meiotic HJ structures (Boddy *et al.*, 2001). Although this is strong evidence to suggest Mus81-Eme1 homologues resolve HJs formed during DSBR, further evidence suggests that *in vivo* the level, timing and method of HJ resolution do not match those expected for this role (De Los Santos *et al.*, 2003; Osman *et al.*, 2003; Whitby *et al.*, 2003). Further study of the *in vivo* functions of Mus81-Eme1 homologues is required to establish whether they are *in vivo* DSBR HJ resolvases or perhaps define a new meiotic recombination pathway distinct from DSBR.

Figure 1.4
Holliday junction resolution

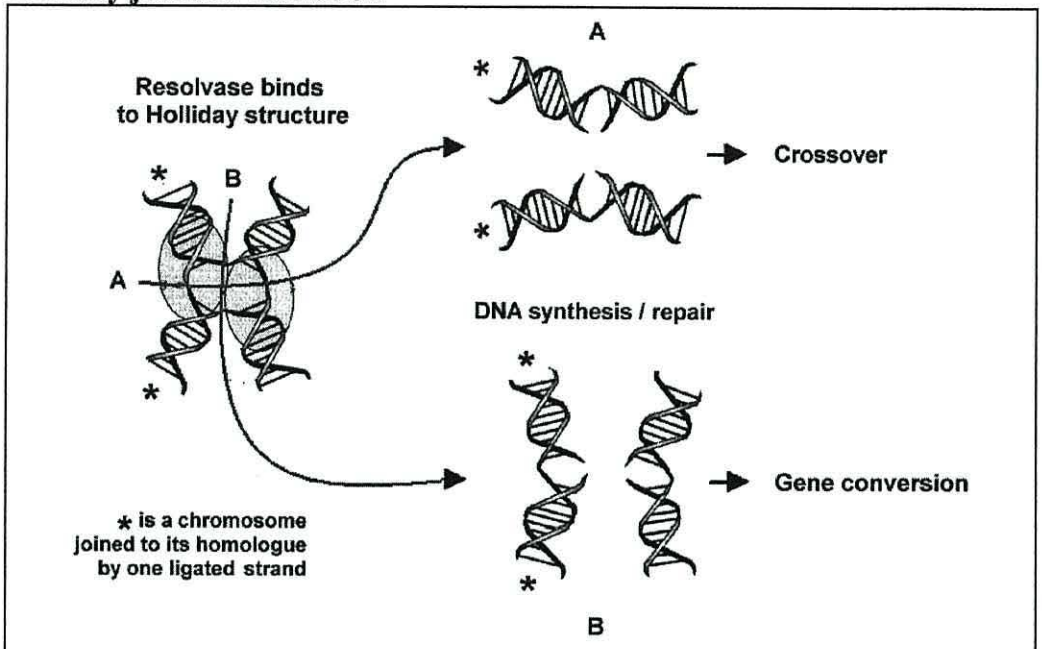


Figure 1.5 shows a schematic representation of possible outcomes of resolution of a Holliday junction.
Pathway A. represents resolution to create a crossover event
(Note a gene conversion event may also occur during this pathway)
Pathway B. represents resolution to create a non crossover event
See text for known prokaryotic proteins involved in Holliday junction and potential eukaryotic Holliday junction resolving proteins.
See text for further possibilities of alternative pathways that can lead to crossover or gene conversion events.

1.17 Recombination hotspots

Although the frequency of recombination between any two points on a chromosome is roughly proportional to the physical distance between them, certain chromosomal regions experience higher or lower recombination levels than the physical distance between them would imply. Regions with recombination frequencies lower than the genome average, for example the yeast *mat* locus, centromeric and telomeric regions, are said to be recombination "coldspots" (Egel, 1984; Grewal and Klar, 1997; Lambie and Roeder, 1988; Nakaseko *et al.*, 1986). Regions that exhibit a recombination frequency higher than the genomic average are termed "hotspots". Hotspots exist in prokaryotes and eukaryotes. As many of the advances in our knowledge of hotspot recombination have taken place through the study of recombination in bacteria, I will firstly outline prokaryotic hotspot recombination. I will then discuss the aspects of meiotic-specific hotspot recombination with special emphasis on the factors that play a role in activation of the *S. pombe M26* and CRE hotspots.

1.18 Prokaryotic hotspot recombination

In *E. coli*, homologous recombination occurs at elevated frequencies at or near specific octameric 5′-GCTGGTGG-3′ sequences (Smith, Kunes *et al.* 1981) termed Chi (χ) sites (Henderson 1975). In addition to *E. coli*, similarly functioning sequences have also been found in other bacteria (Chedin *et al.*, 2000; el Karoui *et al.*, 1998; Sourice *et al.*, 1998). In the *E. coli* genome there are 4–8-fold more Chi octamers than would be predicted to occur by chance and about two thirds of these Chi sequences are orientated so that Chi points back towards *oriC*, the *E. coli* replication origin (Kuzminov *et al.*, 1994). This none random distribution of Chi-like sequences is also apparent in other prokaryotes (Chedin *et al.*, 2000; el Karoui *et al.*, 1998; Sourice *et al.*, 1998). As a result it has been suggested that Chi sites are used in a recombination-dependent process, possibly in the reinitiation of collapsed replication forks (Kuzminov *et al.*, 1994). Chi sequences function in unison with a heterotrimeric protein complex consisting of the RecB, C and D proteins (Smith, 1990) to create an orientation-dependent hotspot. RecBCD is capable of numerous enzymatic activities and is essential for

99% of the recombination events occurring at DSBs in *E. coli* (Kowalczykowski *et al.*, 1994). *In vitro* studies show RecBCD functions as both a nuclease and a highly processive helicase (Roman *et al.*, 1992; Roman and Kowalczykowski, 1989a; Roman and Kowalczykowski, 1989b). The RecBCD complex favours blunt ended dsDNA substrates, which it unwinds in a 5′-to- 3′ direction (Taylor 1980) relative to its point of entry, and degrades preferentially on the 3′-strand (Dixon and Kowalczykowski, 1991; Dixon and Kowalczykowski, 1993; Taylor and Smith, 1995). Although this degradation can vary with experimental conditions (Taylor and Smith, 1995). RecBCD unwinding and differential nuclease activity produces a characteristic loop-tail or twin-loop structure (Taylor, 1980). When RecBCD reaches the 5′-GCTGGTGG-3′ Chi sequence its nuclease activity is adjusted, "normal" 3′-to-5′ nuclease activity is inhibited and a 5′-to-3′ activity is stimulated (Anderson and Kowalczykowski, 1997a; Bianco and Kowalczykowski, 1997; Dixon and Kowalczykowski, 1991; Dixon and Kowalczykowski, 1993; Stahl *et al.*, 1990). Furthermore, although potential helicase activity remains unaltered, RecBCD can no longer respond to another Chi sequence (Spies *et al.*, 2003). Therefore a single Chi octamer acts as a molecular switch to RecBCD-Chi recombinational activity.

As a result of this switch ssDNA is generated, with the Chi sequence at its 3′ terminus. Normally this ssDNA would create a substrate for the loading of either the RecA, or SSB protein. However, RecBCD specifically directs the loading of RecA onto the Chi-containing ssDNA (Anderson and Kowalczykowski, 1997b; Dixon and Kowalczykowski, 1991; Dixon and Kowalczykowski, 1993) creating a recombinogenic, RecA-coated 3′ single-stranded DNA tail, that is capable of invading an homologous duplex. This favoured RecA loading has been suggested to be a significant event in the hotspot activity of the RecBCD-Chi complex (Arnold and Kowalczykowski, 2000). Recent studies suggest that these observed *in vitro* activities may *in vivo* take the form of a specific pause at a correctly orientated Chi sequence and then uncoupling one of the motor subunits of the holoenzyme (Spies *et al.*, 2003). The subunits of RecBCD have known and implied activities. It has been suggested that RecC acts as a Chi detector (Arnold *et al.*, 1998). The RecD and RecB subunits

are respectively 5′-to-3′ and 3′-to-5′ fast and slow processing helicases (Dillingham *et al.*, 2003; Taylor and Smith, 2003). The RecBC enzyme (without the RecD subunit) is a recombination-proficient helicase with little or no nuclease activity (Korangy and Julin, 1993). Hence, it is possible that *in vivo,* a single RecBCD complexes may unidirectionally unwind the dsDNA (Taylor, 1980) towards the 3′ side of a Chi sequence (Ponticelli 1985; Taylor, Schultz *et al.*). Spies and co-workers (2003) then propose that a RecC initiated pausing at Chi, of the fast RecD helicase may then allow the slow RecB helicase (Taylor and Smith, 2003) to catch up. It would then be possible for RecD or other nuclease activity to create a single-stranded nick in the DNA duplex, four to six nucleotides to the 3′ side of the Chi octamer (Taylor, Schultz *et al.* 1985). Finally, RecBCD would then once more unwind the duplex to form the single-stranded free 3′-OH DNA recombinogenic tail (Ponticelli 1985; Taylor, Schultz *et al.* 1985; Cheng 1987).

The studies into the aspects of the Chi recombination hotspot have greatly enhanced our knowledge of recombination. However, bacteria do not undergo meiosis and eukaryotic chromosomes are structurally very different from the bacterial chromosome. Therefore many of the aspects of meiotic hotspots can only be investigated in eukaryotic model organisms.

1.19 Eukaryotic meiotic hotspots

Eukaryotic recombination hotspots can function purely during mitosis (Goldman and Gutz, 1974; Voelkel-Meiman *et al.*, 1987) in both mitosis and meiosis (Treco and Arnheim, 1986) or purely during meiosis (Ponticelli *et al.*, 1988; Szankasi *et al.*, 1988). In meiosis, it is estimated that most homologous recombination is initiated at recombination hotspots (Baudat and Nicolas, 1997; Lichten and Goldman, 1995; Szostak *et al.*, 1983). Transcription factors can activate (Fox *et al.*, 2000; Kon *et al.*, 1997; White *et al.*, 1993; White *et al.*, 1991) and modulate (Abdullah and Borts, 2001) the level of recombination of certain recombination hotspots. Moreover, a forced interaction between a transcription factor and the meiotic recombination machinery is all that is required to create a potential recombination hotspot (Pecina *et al.*, 2002). These data combined with the fact that in *S. cerevisiae*, meiotic recombination hotspots are predominately

located within promoter regions (Baudat and Nicolas, 1997) raises the possibility of a relationship between transcriptional activity and recombination (Nicolas, 1998). However, studies in *S. cerevisiae* (White *et al.*, 1992; White *et al.*, 1993) and *S. pombe* (Kon *et al.*, 1997; Kon *et al.*, 1998) suggest this is most likely not the case. Instead, a number of studies have highlighted a link between transcription factor dependent modification of chromatin structure and modulation of hotspot activity (Fox *et al.*, 2000; Hirota *et al.*, 2003; Mizuno *et al.*, 1997; Mizuno *et al.*, 2001; Wu and Lichten, 1994; Yamada *et al.*, 2004).

The remodelling of local chromatin is thought to increase the ease of access of the DNA to recombination initiating factors. Termed the "chromatin transition", local chromatin remodelling is found at meiosis-specific hotspots and visualised as an increased sensitivity of specific chromatin regions to *micrococcal* nuclease (MNase) or DNase 1 activity (Ohta *et al.*, 1994; Wu and Lichten, 1994; Mizuno *et al.*, 1997). In *S. cerevisiae*, pre-meiotic DNA replication is required for this chromatin transition (Murakami *et al.*, 2003). These facts suggest a need for a defined local chromatin structure to ensure hotspot activation. Indeed, in *S. pombe* potential gross alterations to local chromatin can lead to a failure to establish the expected hotspot activity of an *M26* sequence in translocated sequences (Virgin *et al.*, 1995), whilst the creation of an *M26* sequence via specific point mutations, which potentially minimise chromatin disruption, can create active hotspots (Fox *et al.*, 1997; Fox *et al.*, 2000). The aspects of chromatin and its relationship to hotspot activity are discussed below.

1.20 Chromatin

The DNA of eukaryotic chromosomes is compacted into highly organized structurally distinct domains. These domains can be classified into two main categories termed heterochromatin and euchromatin. Characterized by their level of compaction and DNA accessibility, heterochromatic domains are tightly packaged and relatively silent in both transcription and recombination whilst euchromatin domains are far less compacted, contain transcriptionally active regions and are the primary location for recombinogenic hotspots (Blat *et al.*, 2002). The basic unit of chromatin is the nucleosome. Nucleosomes consist of

1.65 turns (146 bp) of DNA wrapped around an octameric protein complex (Luger *et al.*, 1997; Richmond and Davey, 2003). The octameric complex itself is formed from two tetramers each composed of one H2A, H2B, H3 and H4 proteins, which are members of the highly evolutionary conserved histone protein family (Arents *et al.*, 1991).

Recently a further classification system of chromatin structure has been proposed based upon the 3D architecture of primary, secondary and tertiary nucleosome interactions (Woodcock and Dimitrov, 2001). The primary interaction is defined by the "classical" nucleosome phasing pattern often described as a 10nm filament of nucleosome "beads" threaded by DNA "string" (Kornberg, 1974; Olins and Olins, 1974). The proposed secondary chromatin structure is based upon the folding of the primary 10nm string into a 30nm chromatin fibre through the use of histone H1 like proteins (Finch and Klug, 1976; Thoma *et al.*, 1979) and then the sequence dependent organisation of 30nm fibres into 3D structures via the action of other chromatin regulatory proteins, an example of which is Tup1 (Ducker and Simpson, 2000). Finally, the tertiary chromatin structure level is proposed to be composed of complex and potentially long range interactions between secondary structure elements controlled via proteins that bind to specific transcription regulatory elements (Fraser and Grosveld, 1998).

One of most important aspects of chromatin formation is how histones undergo covalent attachment and removal of a range of moieties, which in turn can modulate DNA-nucleosome and nucleosome-nucleosome interactions (Wolffe and Hayes, 1999). The N-termini of all four core histones and the C-terminus of H2A are the primary sites for these covalent modifications (reviewed in Wolffe and Hayes, 1999). It is proposed that the covalent modification of histones most likely regulates chromatin structure in two ways, firstly by altering the electrostatic interaction between DNA and histones and secondly, by acting as a signal for the targeting of histone binding proteins. These signals have been referred to as the "histone code" (Strahl and Allis, 2000). Recent studies suggest that the histone code modulates many DNA-associated processes, such as transcription (Wolffe, 2001), replication, and DNA recombination and repair (Verger and Crossley, 2004).

1.21 Chromatin remodelling and meiotic hotspot activation

Meiosis-specific hotspot recombination is initiated by the formation of DSBs (Keeney *et al.*, 1997; Sun *et al.*, 1991) and the relative intensities of DSBs corresponds well with the level of recombination (Bullard *et al.*, 1996; Steiner *et al.*, 2002). Studies using both sequence-specific and non-sequence-specific hotspots have highlighted a correlation between the process of meiotic-specific chromatin remodelling and recombination hotspot activation (Fox *et al.*, 2000; Mizuno *et al.*, 1997; Ohta *et al.*, 1998; Ohta *et al.*, 1994; Wu and Lichten, 1994). Meiotic-specific chromatin remodelling has been shown to be dependent on the action of protein complexes required for the creation of DSBs (Ohta *et al.*, 1998; Pecina *et al.*, 2002) and transcription regulation (Fox *et al.*, 2000; Mizuno *et al.*, 1997; Yamada *et al.*, 2004). These and other studies have lead to a classification of meiotic hotspots based on what factors they require for their activation and chromatin modulation (Petes, 2001). Alpha (α) hotspots are proposed to require the direct binding of transcription factors and the activity of chromatin remodelling complexes such as histone acetyl transferase (HAT) and histone deactylase complexes (HDAC). Beta (β) hotspots require only the activity of chromatin remodelling complexes. Finally, gamma (χ) hotspots, which contain a high G + C base composition, may be activated by either the stalling of DNA replication forks or the binding of an unknown protein(s) with high G + C binding affinity that can stabilise chromatin remodelling complexes. However, whilst it seems that meiotic-specific remodelled and other relatively nucleosome free chromatin configurations are capable of creating potential recombination hotspots (Kirkpatrick *et al.*, 1999; Xu and Kleckner, 1995) they do not always do so. One theory proposed to explain this observation is a possible competition between potential hotspot-capable chromatin and a limited number of DSB producing complexes (Borde *et al.*, 2000; Ohta *et al.*, 1999; Wu and Lichten, 1995). Another caveat to the correlation between chromatin remodelling and hotspot activity also exists. Although strong evidence for the necessity for specific chromatin remodelling at hotspot sequences is provided by the fact that deletion of Snf22, a Swi2/Snf2-like potential chromatin remodeller, results in an almost total loss of

ade6-M26 hotspot activation (Yamada *et al.*, 2004) and modification of higher order chromatin structure undoubtedly effects hotspot activity (Ponticelli and Smith, 1992; Virgin *et al.*, 1995), not all meiotic-specific nuclease hypersensitivity sites correspond well to DSB sites (Cervantes *et al.*, 2000; Fan and Petes, 1996).

1.22 The *S. pombe M26* and CRE recombination hotspots

In *S. pombe*, the *ade6-M26* allele can increase homologous recombination up to 15-fold when compared to its *ade6-M375* control allele (Gutz *et al.*, 1971). The hotspot activity of the *ade6-M26* allele is due to the presence of a seven base pair 5′-ATGACGT-3′ sequence (Schuchert *et al.*, 1991) located at codon 45 within the *ade6* open reading frame (Ponticelli *et al.*, 1988; Szankasi *et al.*, 1988). Termed the *M26* heptamer this sequence is one of the most intensely studied meiotic-specific hotspot sequences. The *M26* heptamer is capable of forming a position and orientation-independent hotspot (Fox *et al.*, 1997). In addition to the *M26* heptamer a closely related 5'-NTGACGT(C/A)-3' sequence which matches the binding sequence of the cyclic AMP response element (CRE) consensus of higher eukaryotes is also capable of forming a meiotic recombination hotspot (Fox *et al.*, 2000).

Through the use of a biochemical approach, Wahls and Smith (1994) isolated two trans-acting proteins that bind to the *ade6-M26* sequence (Wahls and Smith, 1994). These proteins were subsequently identified as the transcription factors Atf1 and Pcr1/Gad7 (Kon *et al.*, 1997). The Atf1 (Kanoh *et al.*, 1996) and Pcr1 (Watanabe and Yamamoto, 1996) transcription factors are related to the cyclic AMP response element binding (CREB) proteins. Both Atf1 and Pcr1 are required for activation of the *ade6-M26* hotspot and at least one CRE hotspot (Kon *et al.*, 1997; Fox *et al.*, 2000). In addition to Atf1 and Pcr1, the MAP kinase pathway that modulates their activity is also required for *M26* and CRE hotspot activity (Fox *et al.*, 2000; Kon *et al.*, 1998; Mizuno *et al.*, 2001).

It has recently been shown that both Atf1 and Pcr1/Gad7 are most likely directly required for *ade6-M26*-specific chromatin remodelling, possibly in conjunction with the *S. pombe* histone acetyl transferase (HAT) homologue, Gcn5

(Yamada *et al.*, 2004). Deletion of *gcn5* results in a significant delay and an approximate 50% reduction in hotspot-specific chromatin remodelling, DSB formation and subsequent recombination frequencies, pointing to a close correlation between these processes (Yamada *et al.*, 2004). Meiotic-specific chromatin remodelling at the *ade6-M26* heptamer can also be repressed by the *S. pombe* Tup1 homologues (Hirota *et al.*, 2003), Tup11 and Tup12. The *S. cerevisiae* Tup1 protein is a global corepressor of transcription (Redd *et al.*, 1997; Varanasi *et al.*, 1996) which can bind to histones (Edmondson *et al.*, 1996), and HDACs (Watson *et al.*, 2000). The Tup11/Tup12 repression of chromatin remodelling is antagonised by the Rts2 transcription factor (Hirota *et al.*, 2003) linking *ade6-M26* hotspot activation to the PKA signalling pathway (see section 1.5). However, what effect these aspects of *ade6-M26* chromatin remodelling have on *ade6-M26* hotspot activity has not yet been assessed.

In summary, there is now strong evidence that one limiting step in activation of *M26* and CRE hotspots is the requirement for a specific remodelling of the local chromatin context in which an *M26* or CRE sequence is embedded (Mizuno *et al.*, 1997; Mizuno *et al.*, 2001; Yamada *et al.*, 2004). In addition, as Tup1 has also been linked to the creation of specific inhibitory chromatin architectures (Ducker and Simpson, 2000) and higher order chromatin configuration has also been shown to affect *ade6-M26* hotspot activity (Ponticelli and Smith, 1992; Virgin *et al.*, 1995) this also implicates a requirement for a chromatin remodelling at regions other than just at *M26* CRE sequences.

1.23 Chromosome disjunction in meiosis

In most organisms the correct disjunction of chromosomes at meiosis requires at least three interacting processes. Firstly each pair of homologous chromosomes must be connected by at least one recombination event (chiasmata, which is resolved as a crossover). Secondly in meiosis I the sister centromeres of each homologue must act as a unit and attach to microtubules emanating from a single pole (monopolarity) whilst in meiosis II they must act individually and attach to microtubules emanating from opposite poles (bipolarity). Finally, in

meiosis I a loss of SCC must take place only between homologues and not between sister chromatids; whilst in meiosis II, all SCC must be removed. How and where recombination is initiated, regulated and resolved has been discussed in previous sections. In the following sections I will discuss how monopolarity, bipolarity and the differential loss of SCC may be achieved and their importance to the meiotic process.

1.24 Monopolar centromere orientation in meiosis I.

Mitotic cohesins are crucial for bipolar centromere attachments whilst meiotic cohesins are required for monopolar attachments (Tanaka *et al.*, 2000b; Yokobayashi *et al.*, 2003). It has recently been proposed that in *S. cerevisiae* mitotic cohesins may enforce bipolar kinetochore attachments via a tension sensing mechanism that involves the AuroraB mitotic kinase homologue (Kimura *et al.*, 1997), *IPL1*(Chan and Botstein, 1993), kinetochore proteins (Kaitna *et al.*, 2000) and cohesin (Sonoda *et al.*, 2001). Ipl1 is required in the spindle-checkpoint which is activated by sister chromatids that are not under tension and hence is a possible kinetochore tension sensor (Tanaka *et al.*, 2002). In *S. cerevisiae* after a mitotic division *ipl1* mutants preferentially retain mono-polar kinetochore attachments to the SPB inherited from the previous division (Tanaka *et al.*, 2002). It has been suggested that Ipl1 acts to correct this mono-polar attachment by destabilizing microtubule-kinetochore interactions via the phosphorylation of Dam1 subunit of the Dam-Duo kinetochore complex (Cheeseman *et al.*, 2002), so enabling the resetting of the kinetochores. Other *S. cerevisiae* mutants for example *mam1* mutants also fail to correctly orient their centromeres in meiosis I (Toth *et al.*, 2000). The requirement of Mam1 and other proteins to "enforce" monopolar attachments in meiosis I has lead to the proposal that bipolar mitosis like attachments may be the default pathway for *S. cerevisiae* (Petronczki *et al.*, 2003).

In *S. pombe* Rec8-dependent chromatin formation is directly implicated in the monopolar orientation of centromeres that enables a correct meiosis I disjunction (Molnar *et al.*, 2003; Watanabe *et al.*, 2001; Yokobayashi *et al.*, 2003). It has previously been assumed that all centromeric Rec8 was located in

the same fashion. However, a study by Nonaka and co-workers (2002) suggests this may not be the case. Pericentric Rec8 located to the outer centromere is not required for monopolar attachments (Kitajima *et al.*, 2003b). Therefore a possible method for differentiating meiosis I from mitosis (meiosis II-like) kinetochore arrangements in *S. pombe* could involve the removal of Rec8 from the inner centromere to allow the default mitotic mechanism to segregate sister chromatids at the meiosis II division. As *S. pombe* centromeric structure resembles that of higher eukaryotes (Chikashige *et al.*, 1989) this may be a conserved process.

1.25 The removal of SCC and chromosome segregation

Recent studies in mitosis have revealed some of the proteins involved in the removal of cohesion (Figure 1.5). In *S. cerevisiae*, separation of chromatids at the metaphase to anaphase transition in mitosis is due to the ubiquitin–mediated proteolytic degradation of the securin, Pds1 by the anaphase promoting complex (APC) (Cohen-Fix *et al.*, 1996). Pds1 is part of the Pds1-Espl (securin-separin) complex (Ciosk *et al.*, 1998). Proteolysis of Pds1 results in release of separin inhibition and destruction of Scc1, resulting in a loss of cohesion between sister chromatids at anaphase A, in the metaphase anaphase transition (Uhlmann *et al.*, 1999). Completion of segregation then takes place in anaphase B via the "pulling" action of microtubules which are attached to the kinetochore proteins of each sister chromatids centromere (Rieder and Salmon, 1998). Homologues for both Pds1 (Cut2) and Espl (Cut1) exist in *S. pombe* (Funabiki *et al.*, 1996a). However, these homologues have limited amino acid homology (Ciosk *et al.*, 1998) and differ substantially in their range of functions (Yanagida, 2000). An extreme example is that Pds1 is not essential for cell viability (Cohen-Fix *et al.*, 1996) whereas Cut2 is (Funabiki *et al.*, 1996a). However, the recent identification of human and *Xenopus* anaphase-inhibitory proteins that have Pdsl like properties, i.e. bind to Espl and are degraded by APC^{CDC20}, suggested that the budding yeast model may also function during mitosis in both *S. pombe* and animal cells (Zou *et al.*, 1999). This indeed seems to be the case. Furthermore, it appears a similar system of cohesin destruction is also utilised in removal of meiotic SCC. Espl is a CD clan cysteine endopeptidase that targets the phosphorylated form of Scc1

(Uhlmann *et al.*, 2000) and cleaves one of the two SxExGRR sites within Scc1. These Esp1 consensus sequences are also present in both mitotic and meiotic cohesins of *S. pombe* and *S. cerevisiae* (Uhlmann *et al.*, 1999). Indeed in both yeasts separase cleaves Rec8 (Buonomo *et al.*, 2000; Kitajima *et al.*, 2003a).

Meiosis I chromosome segregation requires a loss of arm cohesion to allow the resolution of chiasmata, but requires that centromeric cohesion is maintained. This fact leaves us with the problem of how a separase-dependent cleavage process could differentiate between arm and centromeric cohesion. One possible method would be to alter the relative timing of proteolytic degradation of cohesin subunits at arms and centromeres.

1.26 Differentiating between centromeric and arm cohesion

Meiosis I and meiosis II are achieved by two processes, retention of cohesion between centromeres and loss of cohesion on chromosome arms. In theory arm cohesion stabilises crossovers. This stabilisation enables the joined homologue pair to resist the separating forces exerted on it caused by the monopolar orientated sister kinetochore attaching to microtubule arrays emanating from opposite poles of the cell (Moore and Orr-Weaver, 1998). Correct reductional disjunction of homologues at meiosis I is then achieved by the subsequent release of arm cohesion between sister chromatids and retention of centromeric cohesion. The release of arm cohesion may allow any crossovers present to resolve by simply migrating off the chromatid arms (Moore and Orr-Weaver, 1998), whilst retention of centromeric cohesion would prevent sister chromatids from separating (LeBlanc *et al.*, 1999; Watanabe and Nurse, 1999).

During meiosis I in *S. pombe*, both the known mitotic and meiotic cohesins vary their partners and are spatially displaced. In *S. cerevisiae* the only meiotic-specific cohesin is Rec8 and this appears to be present within all meiotic cohesion complexes (Klein *et al.*, 1999).

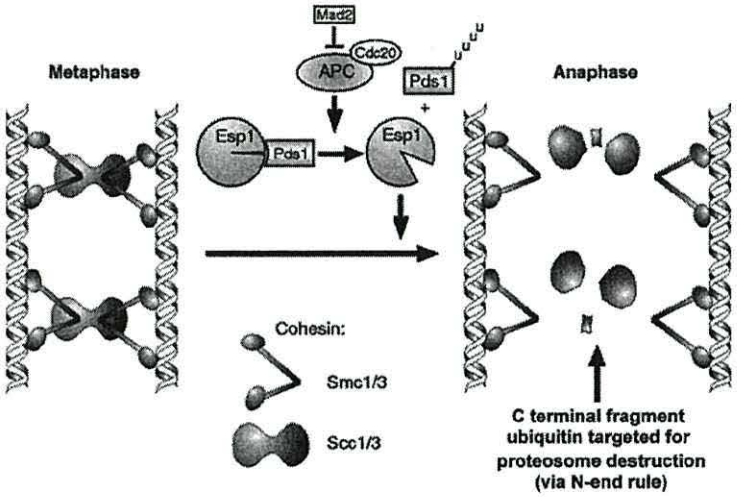
In *S. pombe*, Rad21 maintains cohesion mainly towards telomeric regions. It appears Rec11 is vital for arm cohesion (Kitajima *et al.*, 2003b) whilst Rec8 is primarily responsible for maintaining centromeric cohesion (Kitajima *et al.*, 2003b; Watanabe and Nurse, 1999).

Rad21 (Birkenbihl and Subramani, 1995) contains two near matches for separin (Esp1) mediated cleavage motifs, located in a similar region of the protein to those found in Scc1 (Uhlmann *et al.*, 1999). In both yeasts Rec8 predominately replaces Rad21/Scc1 during meiosis, and its cleavage is crucial for separating sister chromatids during meiosis in *S. cerevisiae* (Buonomo *et al.*, 2000) and *S. pombe* Rec8 (Kitajima *et al.*, 2003a).

The Drosophila melanogaster Mei-S332 protein has an important role in maintaining sister chromatid cohesion at centromeric regions in mitosis and in meiosis until meiosis II (Moore *et al.*, 1998). During meiosis, MeiS-332 binds to centromeric specific heterochromatin during metaphase of meiosis I, and then persists there until sister centromeres separate during anaphase of meiosis II (Kerrebrock *et al.*, 1992). It is not known whether MeiS-332 interacts directly with cohesins and it is not a recognised cohesin. Unlike cohesins, MeiS-332 assembles into a multimeric protein complexes that localise to centromeric regions during prometaphase rather than in S-phase (Tang *et al.*, 1998). However, whilst *MEI-S332* maintains centromeric cohesion (LeBlanc *et al.*, 1999; Moore *et al.*, 1998) and fits the proposed role of "centromeric cohesion protection" it is not meiotic-specific in this process (LeBlanc *et al.*, 1999). Perhaps its role is to maintain kinetochores (Kerrebrock *et al.*, 1992) and its "centromere protecting action" is a by-product of this process. This is an interesting possibility as the Bub1 protein kinase in *S. pombe* is also not meiotic-specific but is required for maintaining centromere cohesion in meiosis I (Bernard *et al.*, 2001).

Both flies and *S. pombe* have highly heterochromatic centromeres, and in *S. pombe* mitotic centromeric cohesin-mediated cohesion is established via a pathway that is distinct from arm cohesion (Nonaka *et al.*, 2002). Hence, one possible explanation for the differential loss of arm and centromere cohesion in meiosis I could be the role of this heterochromatic established cohesion.

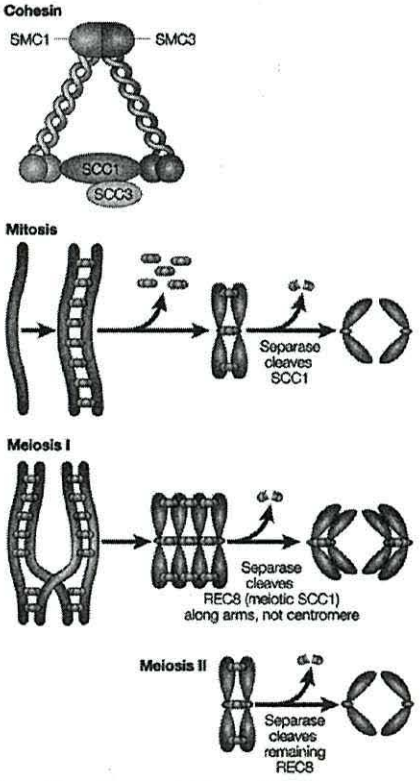
Figure 1.5
A model for the removal of chromatid cohesion in mitosis and meiosis.



Adapted from Uhlmann *et al.*, (1999)

Figure 1.5 is adapted from (Uhlmann *et al.*, 1999)
See Table 1.2 for homologues of *S. cerevisiae* cohesins.
In *S. pombe* Pds1 is Cut2 (Funabiki *et al.*, 1996a) Esp1 is Cut1 (Funabiki *et al.*, 1996a)
Cdc20 and APC are part of the anaphase promoting complex
Mad2 is a mitotic checkpoint sensing protein
UUUU attached to free Pds1 are ubiquitin residues

Figure 1.6
The differential removal of chromatid cohesion in mitosis and meiosis.



Adapted from Hagstrom and Meyer, (2003)

(Hagstrom and Meyer, 2003)

1.27 Comparison of Rec10 and Red1

In *S. cerevisiae* or *S. pombe*, many of the meiotic processes I have discussed in the previous sections require the Red1 or Rec10 proteins. In this study we demonstrate that the *rec20-144* mutant is an allele of *rec10*$^{+}$ and that the Rec10 protein shares structural and limited sequence homology with Red1 (Lorenz *et al.*, 2004). Therefore in the following section I will compare and contrast some of the functions(s) of the Rec10 and Red1 proteins.

Immunofluorescence staining reveals Red1 localises to meiotic chromosomes (Smith and Roeder, 1997) in a discontinuous pattern not usually seen in other established lateral element proteins observed via electron microscopy (von Wettstein *et al.*, 1984). This discontinuous pattern is lost in *zip1* mutants or via *RED1* over expression, indicating Red1 may be physically confined to these discontinuous regions (Smith and Roeder, 1997). Red1 localisation has been further resolved using ChiP analysis of HA-tagged Red1 revealing Red1 has a broad localisation pattern with peaks predominating at sites at which cohesins bind (Blat *et al.*, 2002). Red1 localization to axial elements does not depend upon meiotic recombination (Smith and Roeder, 1997). Furthermore, as *red1* mutants form only fragments of axial elements (early precursors to SC) and totally fail to assemble SC (Bailis and Roeder, 1998; Rockmill and Roeder, 1990; Smith and Roeder, 1997), it has been suggested that one function of Red1 may be to initiate nucleation of axial elements (Smith and Roeder, 1997) possibly emanating from initial sites of cohesion. In *S. cerevisiae* Rec8 is required for Red1 localisation to AEs (Klein *et al.*, 1999). This fact makes it unlikely that Red1 is solely responsible for any axial element nucleation process but may perform this process in conjunction with Rec8.

In *S. pombe*, Rec10 localises to chromosomes in patterns that correspond virtually identically to those observed via silver staining of the linear elements (Lorenz *et al.*, 2004; Molnar *et al.*, 2003). Rec10 is also required for LE formation and the localisation of Rec10 to LEs does not depend upon recombination (Lorenz *et al.*, 2004). Co-localisation of Rec8 and Rec10 also occurs within LEs (Lorenz *et al.*, 2004). Remnants of LE do form in a *rec8*$^{+}$ mutant (Molnar *et al.*, 2003) and Rec10 does localise to these remnants (Lorenz *et al.*, 2004), although whether

these LEs are functional is unknown. As mutations in *rec8*$^+$ have been shown to be epistatic to mutations in *rec10*$^+$ (Krawchuk *et al.*, 1999), it may be possible that Rec8 and Rec10 co-operate in the formation of functional LEs.

Loss of Red1 function results in a loss of SCC (Bailis and Roeder, 1998), a decrease in homologue pairing efficiency (Nag *et al.*, 1995), reductions in inter-homologue recombination and a locus-specific intragenic recombination defect (Rockmill and Roeder, 1990). The reason for the locus-specific intragenic recombination reduction in *red1* mutants is unclear although it appears not to be linked to proximity to centromeres (Rockmill and Roeder, 1990). In the *rec10-155* mutant, SCC appears to be unaffected at the loci tested to date whilst homologue pairing efficiency is reduced (Molnar *et al.*, 2003). In the *rec10-109* mutant inter-homologue and intergenic recombination are reduced in a regional-dependent manner (DeVeaux and Smith, 1994; Ponticelli and Smith, 1989). This reduction in recombination appears to be linked to the proximity of a locus to centromeric regions (DeVeaux and Smith, 1994; Krawchuk and Wahls, 1999). However, as the *rec20-144* mutant displays reductions in recombination that are more evenly distributed along chromosomes (J. L. Wells and R. J. McFarlane, personal communication), this phenotype may be specific to the *rec10-109* mutant. To clarify whether SCC depends upon a function(s) of Rec10 and the extent of Rec10 involvement in homologue pairing and the genome wide recombination reduction a *rec10*$^+$ null strain is required.

1.28 Concluding remarks

Chromosome segregation in meiosis is achieved by numerous interacting processes. Firstly the modulation of meiotic S-phase allows meiotic-specific proteins to organise the correct mono-polar orientation of kinetochores, the maintenance of centromere SCC and ensure interhomologue recombination. The combined actions of these processes results in the faithful meiosis I chromosome disjunction of homologues. Finally, the loss of meiotic specific-chromosomal structures and differential loss of SCC enables the "default" mitotic chromosome segregation pattern to faithfully segregate sister chromatids in meiosis II. It can therefore be seen one of the key aspects to the meiotic process is meiotic-specific recombination. The targeting of this meiotic recombination to certain regions of

recombination enables the creation of a "shuffled" genome whilst excluding the dangers of a more random recombination. Hence, the study of proteins that create and regulate these meiotic recombination "hotspots" is of immense benefit to our understanding of how meiosis and evolution are regulated. In the following chapters we will demonstrate that the *S. pombe* protein Rec10 plays a significant role in the regulation of hotspot recombination in *S. pombe*.

Chapter 2

Materials and methods

2.1 Meiotic crosses protocol

Cultures were grown in yeast extract liquid (YE, see section 2.9 for ingredients) supplemented with adenine (200 mg/l) to a density of approximately 2×10^7 cells/ml. Equal volumes of each culture were mixed in microfuge tubes, pulse centrifuged and aspirated. Cell pellets were washed with 1ml of dH_2O and finally resuspended in 20 µl dH_2O. Suspensions were spotted onto fully supplemented synthetic sporulation media (SPA, see section 2.9 for ingredients) plates and incubated at the required temperature for 3-4 days (4-5 days for room temperature crosses). After incubation, sporulating cells were scraped into a microfuge tube containing 1 ml of a 0.6% β-glucuronidase® (Sigma)/dH_2O solution and incubated for 16 hrs at 25°C. After incubation, spores were harvested and resuspended in 30% ethanol and incubated at room temperature for not longer than 5 minutes. Suspensions were then centrifuged, aspirated dry and cell pellets were re-suspended in 1 ml dH_2O.

2.2 Determination of meiotic recombination rates

To determine total viable spore numbers, 100µl aliquots from serial dilutions of spore suspensions were plated onto YE plates. After 3 days incubation at 34°C (*rec20-144* crosses) or 4 days incubation at 30°C (*rec10-155*) at least 2 plates, each with >50 colonies where then counted and their average used to determine each viable spore total. Ade^+ recombinant totals were determined as follows. 100µl aliquots from serial dilutions of each spore suspension were plated onto YE+guanine plates containing 20mg/ml of guanine dissolved in 0.35M NaOH/dH_2O, and media pH then adjusted to pH 6.5 with 1M HCl. The addition of guanine to YE agar inhibits growth of non recombinant *ade*$^-$ spores (Cummins, 1967). After 3 days incubation at 34°C or 4 days incubation at 30°C (*rec10-155*) colonies where counted and recombination frequencies determined as Ade^+ prototrophs/10^6 viable spores. In most cases 2 plates, each with >50 colonies were

used to determine each Ade^+ total. If however, less than 10 Ade^+ colonies were present on individual plates derived from the neat spore suspension, neat suspensions were aspirated and re-suspended into an approximately 100µl volume and then plated onto a single YE+guanine plate.

To determine total viable spore numbers, 100µl aliquots from serial dilutions of spore suspensions were plated onto Edinburgh minimal medium agar plates (EMM2, see section 2.9 for ingredients) supplemented with adenine and uracil to a final concentration of 225mg/l. After 3 days incubation at 34°C at least 2 plates, each with >50 colonies where then counted and their average used to determine the viable spore total. Ura^+ recombinant totals were determined as follows. 100µl aliquots from serial dilutions of spore suspensions were spread onto EMM2 plates supplemented with adenine to a final concentration of 225mg/l. After 3 days incubation at 34°C or 4 days incubation at 30°C (*rec10-155*) colonies where counted and recombination frequencies determined as Ura^+ prototrophs/10^6 viable spores. In most cases 2 plates, each with >50 colonies were used to determine each Ura^+ total. If however, less than 10 Ura^+ colonies were present on individual plates derived from the neat spore suspension, neat suspensions were aspirated and re-suspended into an approximately 100µl volume and then plated onto a single EEM2+adenine plate.

2.3 Determination of hotspot values and statistical analysis

Individual experiments consisting of three to four independent matings of a test allele against both hotspot and non hotspot control alleles were performed under identical mating conditions in both *rec*$^+$ and *rec*$^-$ backgrounds. Recombination rates for hotspot and control alleles were then derived as in section 2.2. Hotspot values were calculated as the ratio of the recombination rate of a hotspot allele divided by that of its control allele. All possible hotspot values were then derived from each individual experiment.

Example calculation for single experiment of three independent matings:

Recombination rates obtained for hotspot allele against test allele = A, B, C (n=3)

Recombination rates obtained for control allele against test allele = D, E, F (n=3)

Total hotspots calculated = 9: (A/D, A/E, A/F; B/D, B/E, B/F; C/D, C/E, C/F).

The mean hotspot value, standard deviation and Student t-test p significance values were then calculated by a comparison of all *rec*$^+$ and *rec*$^-$ hotspot values generated from at least two individual experiments*.

*The *atf1* experiment which consisted of a single experiment.

2.4. Preparation of chromosomal DNA (for PCR)

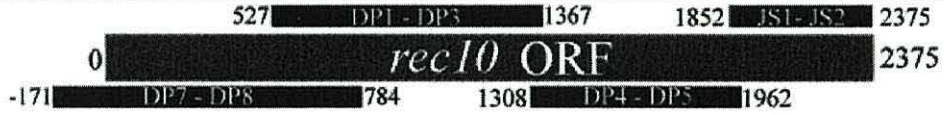
5ml cultures were grown to saturation in YEL+ adenine (200mg/ml) then harvested by centrifugation at 5000rpm for 1 minute in a bench top Microfuge. Cell pellets then transferred to screw-cap microcentrifuge tubes and washed with 1ml of ddH_2O, re-centrifuged, and aspirated to an approximately 0.5 ml of ddH_2O. To each sample was then added; 0.2 ml of (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and 0.2 ml of phenol:chloroform:isoamyl alcohol (25 : 24 : 1) and 0.3 g of acid-washed glass beads. Tubes were then vortex for 3 - 4 minutes and then spun in a bench top Microfuge for 5 minutes at 15000rpm. The aqueous layer was then transferred to fresh 1.5ml microfuge tubes and 1 ml of 100% ethanol was added. Tubes were then mixed by inversion, and spun for 2 min at 15000rpm. Pellets were then aspirated and resuspended in 0.05 ml of TE (pH 7.6-8.0), and stored at 4°C until use.

2.5. PCR sequencing

1 µl volumes of chromosomal DNA solutions prepared via method 4, Preparing Chromosomal DNA (for PCR), were mixed with 46 µl of MegaMix blue® PCR master mix and 1.5 µl each, of appropriate forward and reverse primers of 10 pM primer/ ddH_2O concentrations. Sequencing reactions were then carried out using the following PCR program. One denaturation cycle of 1min at 95°C followed by 25 cycles of 94°C for 1 minute / 55°C for 1 minute and 72°C for 1-2 minutes and a final extension cycle of 72°C for 4mins. 10µl aliquots were then removed from each reaction and checked for product size and approximate concentration on 0.8% agarose, 0.3 µg/l ethidium bromide gels. PCR reactions were then purified using the Roche® PCR product purification kit. Appropriate

quantities of PCR product (amounts specified by MWG biotech Germany), were then precipitated and sent to MWG Biotech Germany for sequencing.

Figure 2.1
Positions, sequences and primers[a] used for sequencing *rec20-144*



Primer designation	Actual Sequence	Position relative to start codon
DP7	CCGTACCTTATCGATGGC	-171
DP8	CAATATTTGAGCCCGACG	784
DP1	TAATTGGTCAACGCTTGC	527
DP3	GTTCCGTCTTTGCTAGC	1367
DP4	GCAAATTGAGGATCTCG	1308
DP5	ATTAGGCGGGCTTGAGC	1962
JS1	ACTAGATGGTACCTGCTC	1852
JS2[b]	...TGACAGTTTGATGTTTAAGT	

[a] All primers were manufactured and supplied by MWG biotech Germany
[b] Primer JS2 is an 80 bp primer, the last 20 bp of the 5′-3′ sequence are given.

2.6 *S. pombe* transformation via electroporation

200 ml cell cultures of *S. pombe* cells were grown to a density of 1 x 10^7 ml^{-1} (OD595 = 0.5) in supplemented Mitchens minimal (MB) or nitrogen base (NB) media. 50ml samples where then chilled on ice for 20 minutes before being harvested by centrifugation at 3000 rpm for 5 min at room temperature. Each sample was then washed three times in ice-cold 1M sorbitol before being resuspended in ice-cold 1M sorbitol to a density of 1 x 10^9 cells/ml^{-1}. Samples were then mixed in pre-chilled Eppendorfs containing 1μg DNA in 3μl of T.E buffer (pH 7.6) then immediately electroporated in pre-chilled Equibio ECU-102® cuvettes using settings of 2.25 kV, 201 Ohm, 25μF. Immediately after pulsing 1 ml of ice-cold 1M sorbitol was added to the cuvette, and the sample transferred to pre-chilled Eppendorfs. Samples where then washed and resuspended in 1ml of ice-cold 1M sorbitol and 50μl aliquots were spread onto EMM2 plates with or without required supplements and incubated for 6 days at 30°C.

2.7 The pRMSPL-1 library screening methods

1) The microtitre plate spotting protocol

Individual transformed colonies of relatively uniform size (medium to large) were picked with sterile flat toothpicks and dispersed into individual sterile microtitre wells each containing 100μ1 of a 0.6% β-glucuronidase (Sigma)/sterile dH_2O solution. Microtitre trays were then covered and incubated for 16 hr at 25°C. After which an 8-channel pipette was then used to mix and pipette approximately 2μl volumes from each well onto YEA plates. After incubation, Ade^+ recombinant (white) papillae frequencies were compared to positive and negative controls.

2) The NB (nitrogen base) replica platting protocol

Transformed colonies present on EMM2 selective plates were replica plated onto SPA mating plates NBA and incubated for 3 days at 30°C. Subsequently, SPA plates were replica plated onto NB plates containing 10mg/l adenine and incubated for a further 3 days at 30°C. After incubation, Ade^+

recombinant (white) papillae frequencies were compared to positive and negative controls.

2.8 Microscope analysis of meiotic crosses

Heterothallic crosses of *rec20-144* strains were constructed as per meiotic crosses. Asci were then fixed by scraping each mating into Eppendorfs containing 1ml of an 8% formaldehyde (w/v), 5% DMSO (v/v) in dH_2O solution. Samples were then mounted onto poly-L-lysine (Sigma) treated coverslips using 5µl of mounting media (100% glycerol/1mg/ml paraphenylene diamine) before being stained with 5µl of a 1µg/ml DAPI solution. Photography was under U.V. or white light using a Nikon type 120 fluorescent microscope® and digital camera.

2.9 Media

When minimal media was used appropriate amino acid supplements were added to a final concentration of 225mg/l. For liquid media the agar was omitted.

SPA	**500ml**
Glucose	5g
KH_2PO_4	0.5g
Agar	15g
Vitamins (x1000)	0.5ml

YE	**1 litre**
Yeast extract	5 g
Glucose	30g
Agar	14 g

EMM2	**1 litre**
potassium hydrogen phtalate	3g
Na_2HPO_4	2.2g
NH_4Cl	5g
Glucose	20g
Vitamins (x1000)	1 ml
Minerals (x10,000)	0.1 ml
Salts (x50)	20ml
Agar	14g

SALTS x50	**500ml**
$MgCl_2.6H_2O$	26.25g
$CaCl_2.2H_2O$	0.3675g
KCl	25g
Na_2SO_4	1g

NITROGEN BASE NB	**1 litre**
NB	1.7g
$(NH_4)_2SO_4$	5g
Glucose	5g
Agar	10g

VITAMINS x1000	**500ml**
Pantothenic acid	0.5g
Nicotinic acid	5g
myo-inositol	5g
biotin	5mg

MINERALS x10,000	**500ml**
boric acid	2.5g
$MnSO_4$	2g
$ZnSO_4.7H_2O$	2g
$FeCl_2.6H_2O$	1g
KI	0.5g
Molybdic acid	0.2g
$CuSO_4$	0.2g
Citric acid	5g

after autoclaving add a few drops of 1:1:2 chlorobenzene/dichloroethane/chlorobutane

Chapter 3

Characterisation of *rec20-144*

3.1 Introduction

In the late 1980s Smith and co-workers developed a genetic screen for meiotic recombination mutants (Ponticelli and Smith, 1989). The screening method relied upon a reduction in plasmid by chromosome recombination between a plasmid containing an *ade6-469* marker allele and the chromosomal meiotic recombination hotspot allele, *ade6-M26* (Gutz, 1971). In total, this screening method isolated 39 recessive mutant alleles from 17 "*rec*" complementation groups (Ponticelli and Smith, 1989; DeVeaux *et al.*, 1992). In addition to a *rec* gene classification, each mutant allele was also placed into one of three classes based upon the extent to which it lowered the level of *ade6-M26* intragenic recombination in two-factor chromosome-by-chromosome recombination assays (Ponticelli and Smith, 1989; DeVeaux *et al.*, 1992). Class I *rec* mutant alleles lowered recombination levels by approximately 100–1000-fold, class II mutants by 10–100-fold and class III mutants by 3–10-fold (Table 3.1 shows a summary *rec* genes cloned to date). The number and distribution of mutant alleles isolated from classes I and II suggests that this screening method is saturated for those classes of *rec* mutations. However, the class III complementation groups were comprised of single alleles; this indicates that this screening method does allow for identification of mutants with 3–10-fold reductions in intragenic recombination at *ade6-M26*, but is unlikely to be saturated for this class of mutant (DeVeaux *et al.*, 1992).

Prior to this study the *rec9-104* mutant (DeVeaux *et al.*, 1992) was the only other class III mutant isolated by Smith and co-workers for which the corresponding gene had been identified. *rec9* is allelic to *hus2* (Enoch *et al.*, 1992) a gene also known as *rqh1/rad12* (Davey *et al.*, 1998; Murray *et al.*, 1997). Rqh1 is a member of a putative DNA helicase family which includes the *E. coli* RecQ, *S. cerevisiae SGS1* and human *BLM* (Ellis *et al.*, 1995) and *WRN* (Yu *et al.*, 1996) proteins. Other screening methods have isolated a further six mutant alleles which cause 3–10-fold reductions in *ade6* meiotic intragenic recombination. In addition to their roles in meiotic recombination these mutants are required for mating type

switching, *swi5* (Gutz and Schmidt 1985) meiotic nuclear movement *dhc1*, *dlc1* (Miki *et al.*, 2002; Yamamoto *et al.*, 1999); nuclear architecture organisation, *kms1* (Shimanuki *et al.*, 1997), homologous chromosome pairing, *meu13* (Nabeshima *et al.*, 2001) and telomere maintenance, *taz1* (Cooper *et al.*, 1998)

In this chapter we utilise two-factor chromosome-by-chromosome recombination assays and suppression with $rec10^{+}$ plasmids to demonstrate that the *rec20-144* allele is a novel allele of the $rec10^{+}$ gene. Utilising bioinformatic analysis, we identify Rec10 has structural homology to the *S. cerevisiae* protein, Red1, a protein required for axial element and synaptonemal complex formation (Rockmill and Roeder, 1990) and a limited sequence homology to the *C. elegans* Xnp1 protein, a homologue of human ATRX protein (Picketts *et al.*, 1996). We employ Immunofluorescence to investigate Rec10 localisation during meiosis and show that Rec10 localises to linear elements (L.E), structures thought to be analogous to *S. cerevisiae* axial elements. Finally, we begin to characterise meiotic chromosome segregation errors that occur in *rec20-144* mutant strains and assess their effects on spore viability.

Table 3.1
Homologues and known roles of *rec* genes originally isolated by Smith and co-workers (Ponticelli and Smith, 1989; DeVeaux *et al.*, 1992).

Gene name and class	Proposed requirement(s)	Eukaryotic Homologues	References
Class I			
rec6$^+$	Meiotic DSB formation		(Lin and Smith, 1994)
rec7$^+$	Meiotic DSB formation	*REC114* *S. cerevisiae*	(Cervantes *et al.*, 2000; Lin *et al.*, 1992; Molnar *et al.*, 2001b)
rec8$^+$	**Cohesin** Sister chromatid cohesion, linear element formation, homologue disjunction in meiosis I (monopolarity) Meiotic DSB formation	*REC8* *S. cerevisiae* Human Mouse *Drosophila* *A. thalina* *C. elegans*	(Eijpe *et al.*, 2003; Klein *et al.*, 1999; Molnar *et al.*, 1995; Molnar *et al.*, 2003; Parisi *et al.*, 1999; Watanabe and Nurse, 1999)
rec12$^+$	**Endonuclease** Meiotic DSB formation	*SPO11* *S. cerevisiae* Human Mouse *Drosophila* *A. thalina* *C. elegans*	(Bergerat *et al.*, 1997)
rec14$^+$	Meiotic DSB formation	*REC103* *S. cerevisiae*	(Evans *et al.*, 1997; Gardiner *et al.*, 1997; Molnar *et al.*, 2003)
rec15$^+$	Early Meiosis I events,		(Molnar *et al.*, 2001a)
Class II			
rec10$^+$	Linear element formation Meiotic DSB formation		(Molnar *et al.*, 2003)
rec11$^+$	**Cohesin** Sister chromatid cohesion, Linear element formation	*psc3* *S. pombe* *SCC3* *S. cerevisiae* SA3 humans	(Kitajima *et al.*, 2003b; Li *et al.*, 1997; Molnar *et al.*, 2003)
rec16$^+$ (*rep1*$^+$)	**Transcription factor activator** Meiosis-specific Linear element formation		(Ding and Smith, 1998; Li and Smith, 1997; Molnar *et al.*, 2003; Sugiyama *et al.*, 1994)
Class III			
rec9$^+$ (*hus2*$^+$/*rqh1*$^+$ /*rad12*$^+$)	**Helicase** **DNA damage recovery**	*SGS1* *S. cerevisiae* Human	(Davey *et al.*, 1998; Laursen *et al.*, 2003; Murray *et al.*, 1997; Stewart *et al.*, 1997)

3.2 Results

3.3 The attempted isolation of *rec20-144* suppressing clones

The screen employed by Smith and co-workers isolated *rec* mutants based upon a reduction in plasmid by chromosome recombination between the chromosomal *ade6-M26* gene and a plasmid containing an *ade6*-marker allele, *ade6-469* (Ponticelli and Smith, 1989). Prior to this study, the use of a cloning strategy based on the use of *S. pombe* genomic libraries in conjunction with *ade6*-marker allele containing plasmids had successfully been used to isolate suppressing clones of *rec10-109* (Lin and Smith 1995) and *rec11-111* (Li *et al.*, 1997). As the *rec20-144* mutant reduces plasmid by chromosome recombination to a level approximately equivalent to that observed in *rec10-109* and *rec11-111* mutants (Ponticelli and Smith, 1989), a similar approach was employed to attempt to isolate of *rec20-144* suppressing clones (see Figure 3.1 for experimental outline).

3.4 The pRMSPL-1 genomic library based screen

The *S. pombe* genomic library pRMSPL-1 (R. J. McFarlane) had previously been used to a *rec10-109* suppressor (R. J. McFarlane, personal communication). We therefore employed the pRMSPL-1 library to transform the *rec20-144* strain BP82 (*ade6-M26 rec20-144 ura4-294* h^{90}; see section 2.6 for protocol). Individual transformed colonies were then assayed for restoration of rec^+ levels of plasmid-by-chromosome recombination. To aid identification of rec^+ clones both positive and negative controls were created by transforming BP151 (*ade6-M26* rec^+ *ura4-294* h^{90}) and BP82 respectively, with plasmid pRM6, the vector on which the pRMSPL-1 library was based. These controls were then analysed in unison with each BP82 pRMSPL-1 transformation. Figure 3.2 shows a typical result of the pRMSPL-1 screen. In total, approximately 6900 pRMSPL-1 transformants were assessed for suppression of the *rec20-144* plasmid-by-chromosome recombination deficiency. Of these 52 possible candidates were selected for re-testing. However, all 52 candidates proved to be false positives.

Figure 3.1
Outline of the pRMSPL-1 library screening method

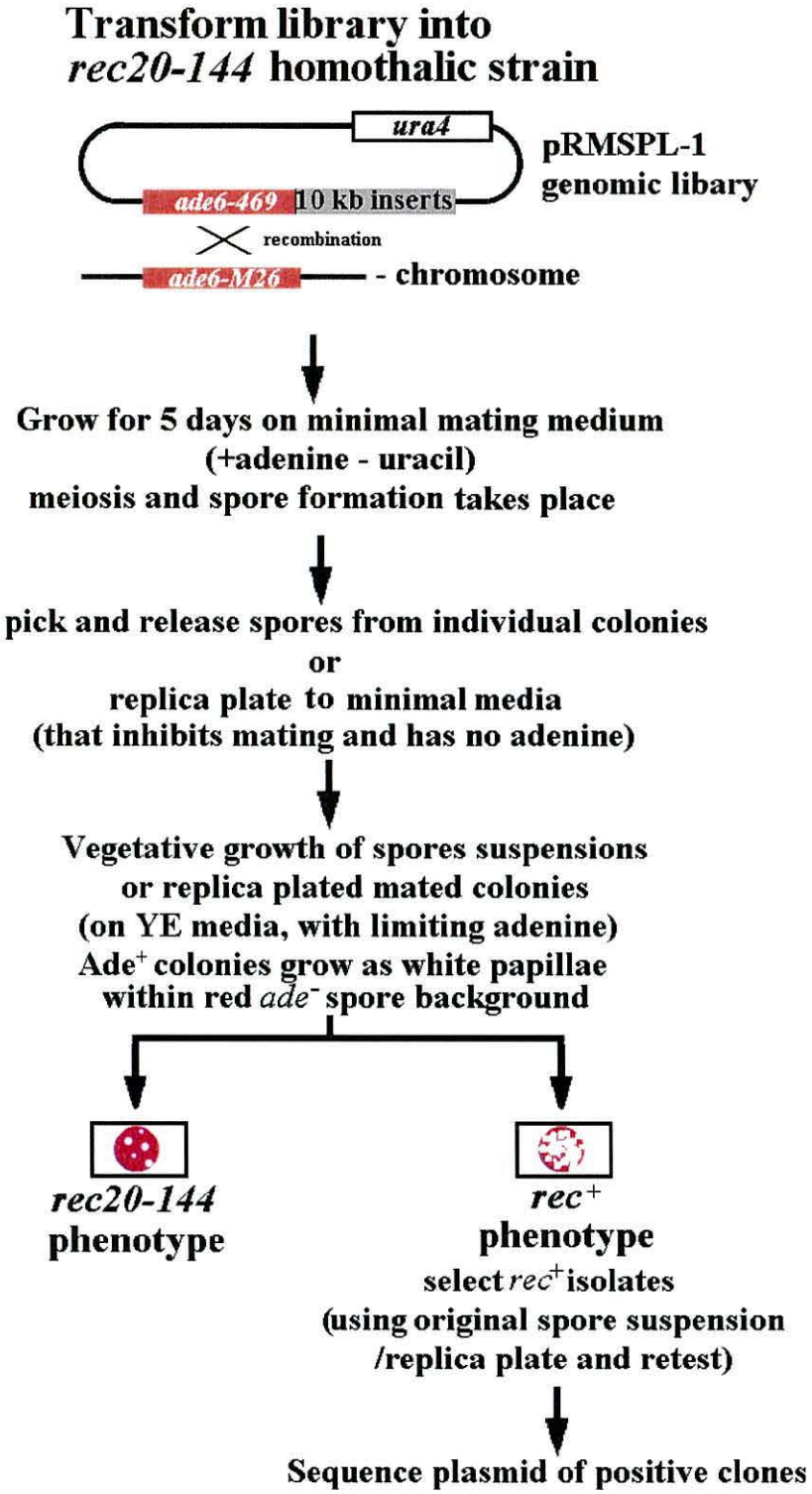


Figure 3.2
Example of screen plate from a typical pRMSPL-1 genomic library transformation.

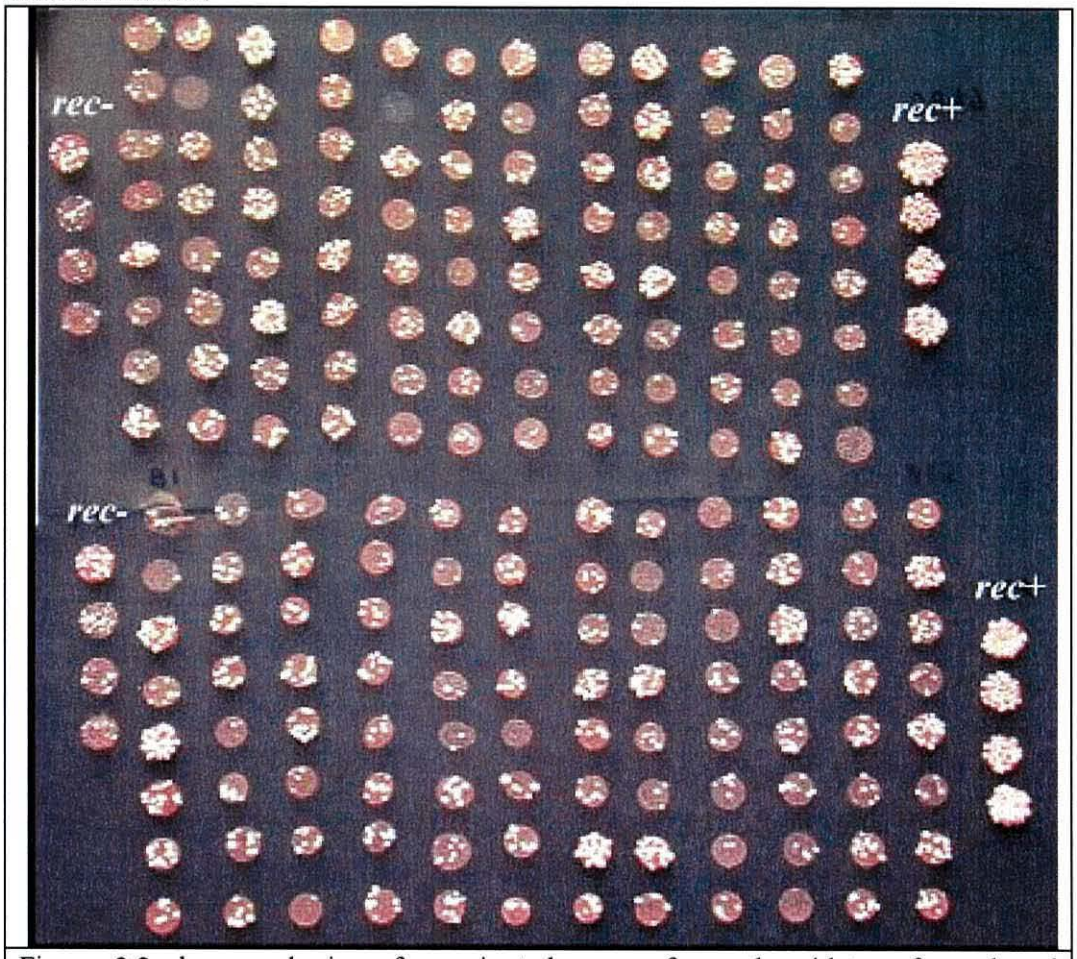


Figure 3.2 shows colonies of germinated spores from plasmid transformed and mated *rec20-144* mutants and control strains grown on YE media containing limiting adenine.
Non recombinant (*ade*$^-$) spores grow as red colonies recombinant Ade$^+$ spores grow as white colonies.
Detection of *rec20-144* suppressing plasmid clones was based upon restoration of *rec*$^+$ levels of white Ade$^+$ spores generated from plasmid-by-chromosome recombination.
rec$^+$ and *rec20-144* controls contain the pRMSPL-1 control plasmid, pRM6
Candidate colonies show wide ranges of proportions of white Ade$^+$ spores per spot. From this plate 5 candidates were classed as having *rec*$^+$ levels of Ade$^+$ spores.

3.5 The *leu2* linkage

The original chromosome linkage analysis of *rec20-144* performed by DeVeaux and co-workers (1992) assigned the *rec20-144* mutation to chromosome I. During strain constructions, a further loose genetic linkage was noticed between *rec20-144* and the *leu2* gene on chromosome I (R. J. McFarlane, D. Pryce data not shown). Due to this observation it was decided to utilise an additional cloning strategy. This strategy involved choosing likely candidate genes located in an approximately 250 kb region flanking the *leu2* gene (Figure 3.3 shows this 250 kb region in detail), up to and including the meiotic recombination gene, *rec10*$^{+}$ (Lin and Smith, 1995). Available mutants of these genes would be used to perform an *ade6-M26* intragenic complementation analysis against the *rec20-144* mutant. In a preliminary investigation of this 250 kb region, based on their putative and known function(s), the *translin* and *rec10*$^{+}$ genes were selected as the first potential candidates for investigation.

Figure 3.3

A) Map of candidate genes from 250 kb region flanking the *leu2* gene.

Red bars are Sanger annotated cosmids. Genes of possible interest are listed below their cosmid

B) List of known functions of possible *rec20-144* candidate genes

B)

Gene	Description of phenotypes	References:
moe1	Eukaryotic translation initiation factor and microtubule destabilizing protein.	(Chen *et al.*, 1999)
ssm4	Microtubule-associated coiled-coil protein involved in meiotic development	(Yamashita *et al*,. 1997)
mei2	essential for premeiotic DNA synthesis and meiosis I	(Watanabe and Yamamoto 1994)
nuc2	nuclear scaffold-like protein blocks spindle elongation in mitotic anaphase	(Hirano *et al.*, 1988)
cut8	Required for normal progression of anaphase.	(Samejima and Yanagida 1994)
YDFB	Hypothetical protein with zinc finger domain	(Wood *et al.*, 2002)
rad26	DNA damage and S-phase mitotic checkpoint	(Khodairy *et al.*, 1994)
wos2	Cell cycle regulatory protein interacts with Cdc2p in the control of the M-G1 transition.	(Munoz *et al.*, 1995)
cbh1	*CENP-B* homologue nucleates centromeric heterochromatin by promoting heterochromatin-specific histone tail modifications	(Murakami *et al.*, 1996)
translin	Homolgue of the translin family DNA-binding proteins. Members specifically recognise consensus sequences at breakpoint junctions in chromosomal translocations. Binds single-stranded DNA ends generated by staggered breaks occurring at recombination hot spots.	(Aoki *et al.*, 1997)
NNF1	Elongated microtubules fragmented nucleolous	(Shan *et al.*, 1997)
rfc2	Probable activator 1 subunit 2 (Replication factor C subunit 2) (Replication factor C2). Required for elongation of primed DNA templates.	(Wood *et al.*, 2002)
nuf2	Spindle pole body-associated protein required for nuclear division, spindle pole body separation and spindle elongation, leading to chromosome segregation.	(Nabetani *et al.*, 2001)
cut7	Novel potential mitotic motor protein.	(Hagan and Yanagida 1990)
rec10	Meiotic recombination protein	(Lin and Smith 1995)

3.6 The *rec20-144* mutant is not in a *translinΔ* complementation group

In response to DNA-damaging reagents, the mouse Translin homologue has been shown to undergo active transport to the nucleus (Kasai *et al.*, 1997) indicating a possible role in DNA repair. In humans and mouse, Translin binding sites have been found at a human male meiotic recombination hotspot and Translin itself has been shown to bind to single-stranded DNA consensus sequences at chromosomal breakpoints found at lymphoid malignancies and solid tumours (Aoki *et al.*, 1995; Badge *et al.*, 2000; Chalk *et al.*, 1997; Kasai *et al.*, 1997). These observations and the fact that prior to this study the *S. pombe translin* homologue had not previously been studied, lead us to investigate whether the *rec20-144* allele was an allele of the *S. pombe translin* gene. To enable a *rec20-144* to *translin* complementation analysis, the technique used by Bahler and co workers (1998) was employed to construct a *translinΔ* strain. A *translinΔ ade6-M26* double mutant was then created. Random spore analysis was then performed on a series of zygotic meiotic crosses, performed at 25°C, involving *translinΔ* and *rec20-144* strains. Results showed that, compared to control crosses, heterozygotic *rec20-144* and *translinΔ* crosses a produced an average 1.75-fold, non significant reduction in the level of *ade6-M26* intragenic recombination. Furthermore, the actual mean recombination value obtained from the set of heterozygous *rec20-144 translinΔ* crosses was approximately 10-fold higher than that attained from homozygous *rec20-144* crosses. The results of this analysis lead us to conclude that *translin* and *rec20-144* are not members of the same recombination complementation group (Table 3.2).

Table 3.2
The *rec20-144* allele is not a member of a *translinΔ* complementation group.

Parental strains		Mean recombination frequency	Reduction
ade6-M26 h$^+$	*ade6-L52 h*$^-$	(Ade$^+$ recombinants/10^6 viable spores)	in recombination
rec$^+$	*translin*$^+$	4370 n=2 (6130, 2609)	-
rec20-144	*rec20-144*	269 n=2 (308, 230)	16.2
translinΔ	*rec20-144*	2500 n=2 (3000, 2000)	1.75[a]

In a zygotic heterozygous cross the *ade6-M26* intragenic recombination defect of the *rec20-144* mutant is complemented by a *translinΔ* mutation.
[a]Due to the small sample number, the level of significance of the 1.75-fold reduction in intragenic cannot be assessed.
Figures in brackets are actual recombination values from independent repeats used to derive mean recombination values.

3.7 The *rec20-144* mutant is a novel *rec10*$^+$ allele

Prior to this study, DeVeaux and co-workers (1992) had assigned the *rec20-144* allele into a unique *rec20* complementation group. This separation of the *rec20-144* mutant allele into a complementation group distinct from the *rec10*$^+$ gene had been carried out using the *rec10-109* mutant (Ponticelli and Smith, 1989). A possibility therefore remained that *rec20-144* mutant may reside in a *rec10*$^+$ complementation group, that did not include the *rec10-109* allele, and therefore *rec20-144* and *rec10-109* are complementary alleles. An *ade6* intragenic complementation analysis was therefore carried out, at a mating temperature of 30°C, between *rec20-144* mutants and two *rec10*$^+$ mutants, *rec10-155* (Lin and Smith, 1995) and *rec10-109* (Ponticelli and Smith, 1989). In heterozygous *rec20-144 rec10-109* crosses, a partial but significant suppression of the *rec20-144 ade6* intragenic recombination deficiency was observed, whereas in heterozygous *rec20-144* by *rec10-155* crosses the mean recombination frequency was not significantly different from that obtained from homozygous *rec10-155* crosses (Table 3.3). These results indicate that the *rec20-144* allele partially complements the *rec10-109* allele and is in the same complementation group as *rec10-155* (Lin and Smith, 1995). As the *rec20-144* mutation is also significantly suppressed by two *rec10*$^+$ containing plasmids (Table 3.4), we concluded that the *rec20-144* allele is a novel mutant allele of *rec10*$^+$.

Table 3.3
The *rec20-144* allele is a member of the *rec10-155* complementation group.

Parental strains crossed *ade6-M26 h*$^+$ *ade6-L52 h*$^-$		Recombination frequency (Ade$^+$ recombinants/10^6 viable spores)	
rec	*rec*	Mean	Experimental values
+	+	5537	7442, 5667, 5531, 3506
+	*rec20-144*	3481	3481
+	*rec10-155*	2266[a]	2266
rec20-144	*rec20-144*	130	133, 127
rec10-109	*rec20-144*	968[b]	1011, 925
rec10-109	*rec10-109*	16	21, 18, 10
rec10-155	*rec20-144*	26[c]	61, 20, 20, 19, 11
rec10-155	*rec10-155*	24[c]	61, 20, 18, 11, 11
rec20-144	*rec20-144*	130	133, 127

Table 3.3 shows the results of random spore analysis of two-factor meiotic recombination assays performed at 30°C, using *rec*$^+$, *rec20-144*, *rec10-109* and *rec10-155* strains.
[a]In heterozygous *rec*$^+$ by *rec10-155* (Lin and Smith, 1995) crosses, the mean recombination value is approximately half of homozygous *rec*$^+$ *ade6-M26* crosses (This reduction is consistently recorded, J. L. Wells, personal communication).
[b]Compared to the mean homozygous *rec20-144* recombination value, the *rec10-109* mutant significantly suppresses the *rec20-144* mutant ($p<0.01$) but recombination is significantly reduced relative to heterozygous *rec*$^+$, *rec20-144* crosses ($p<0.01$)
[c]The *rec20-144* mutation is fully recessive to the *rec10-155* mutation (Lin and Smith 1995).

Table 3.4
The *rec20-144* mutant is suppressed by *rec10*$^+$ containing plasmids

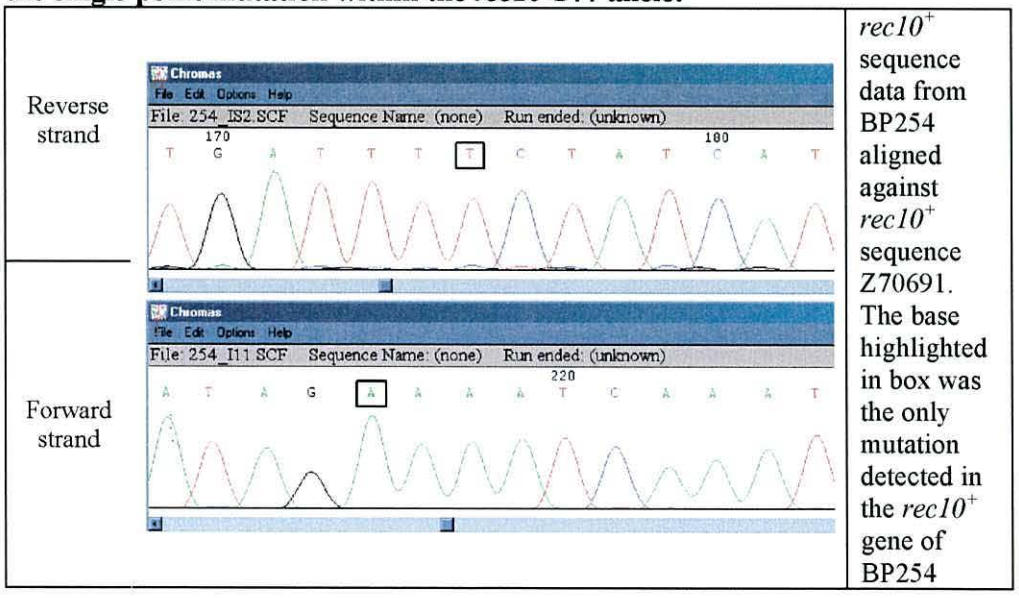
Parental strains crossed *ade6-M26 h*$^+$	*ade6-L52 h*$^-$		Recombination frequency (Ade$^+$ recombinants/10^6 viable spores)	
Rec	*Rec*	Plasmid	Mean	Experimental values
+	*rec20-144*	pFY20[a]	4281	7176, 3934, 1733
rec20-144	*rec20-144*	pFY20	341	391, 290
rec20-144	*rec20-144*	pSP2[a]	87	149, 126, 65, 59, 38
rec10-155	*rec20-144*	pFY20	20	20
rec20-144	*rec20-144*	pJS3	5090	7042, 4295, 3934
rec20-144	*rec20-144*	pYL176	2691	4219, 4183, 3327, 1186, 542
rec10-155	*rec20-144*	pJS3	2423	2658, 2188

The *rec20-144* mutation is suppressed by the two *rec10*$^+$ containing plasmids, pJS3 and pYL176 (Lin and Smith, 1995).
[a]Control plasmids for pJS3 and pYL176, respectively

3.8 Sequencing of the *rec20-144* mutation

The complementation analysis of the *rec20-144* mutation had identified *rec20-144* as an allele of *rec10*$^+$ (Tables 3.3 and 3.4). In order to confirm this and to ascertain the nature of the *rec20-144* mutation within the *rec10*$^+$ gene, a series of PCR primers were designed based on the *rec10*$^+$ sequence. High fidelity PCR amplification was then performed on genomic DNA extracts obtained from the *rec20-144* mutant strain, BP254 (*ade6-M26 rec20-144 h*$^+$). Sequencing reactions were then carried out on these PCR fragments by MWG biotech Ltd and the resulting sequence data files were aligned against the *S. pombe* genomic database sequence for *rec10*$^+$ using the alignment programme ClustalX (version 1.8). Analysis of these alignments identified a single G to A (transcribed strand) transversion mutation located at position 2180, relative to the A of the presumed *rec10*$^+$ ATG start codon (Figure 3.4). Confirmation that this was the only mutation within the *rec10*$^+$ gene was ensured as both strands were sequenced at least twice and the region containing the *rec20-144* mutation was re-sequenced in all further *rec20-144* strains created for genetic analysis. Translation of the *rec20-144* allele indicates that the 2180 transversion mutation results in a single Glycine to Glutamate mis-sense substitution at amino acid position 727 within the predicted Rec10 protein sequence (Figure 3.5).

Figure 3.4
Sequence data obtained for BP254 (*ade6-M26 rec20-144 h*$^+$) showing the single point mutation within the *rec20-144* allele.



rec10$^+$ sequence data from BP254 aligned against *rec10*$^+$ sequence Z70691. The base highlighted in box was the only mutation detected in the *rec10*$^+$ gene of BP254

3.9 Bioinformatic analysis of Rec10

Prior to this study no homologues of the Rec10 protein had been identified, nor had any significant functional domains been noted. We analysed the entire Rec10 protein sequence accession number Q09823 using a range of bioinformatic programmes (Figure 3.5). An independent bioinformatic analysis was also carried out by our collaborators Maria Novatchkova and Frank Eisenhaber in the Bioinformatics Group of the Research Institute of Molecular Pathology, Vienna. These analyses exposed the fact that the central region of the Rec10 protein exhibits a 22% level of homology with the *C. elegans* protein XNP-1 and possesses a nuclear localisation motif within a lysine rich containing sequence as do the *S. cerevisiae* and *Kluyvermyces lactis* Red1 proteins. It should be noted though that a relatively high concentration of lysine residues can lead to an increase in a predicted level of homology. However, using a conserved region of C-terminal amino acid sequence derived from *K. lactis* and *S. cerevisiae* Red1, a further degree of similarity between Rec10 and *S. cerevisiae* and *K. lactis* Red1 proteins is also detected; this, in combination with structural similarities between Red1 and Rec10, suggest that Red1 and Rec10 are functionally related (Lorenz *et al.*, 2004).

The *C. elegans* XNP-1 protein is a homologue of the human ATRX protein, mutations in which cause X-linked alpha-thalassemia/mental retardation associated syndromes (Gibbons and Higgs, 2000). The predicted ATRX protein has been classified as a putative transcription factor with helicase/ATPase functions similar to the SNF2 protein family, members of which are known to be involved in DNA recombination and repair (Picketts *et al.*, 1996). Although the bioinformatic analysis of Rec10 demonstrates that regions of the Rec10 protein have only distant homology to ATRX and Red1 we have begun to support these analyses.

Loss of Red1 function severely reduces axial element and synaptonemal complex formation (Rockmill and Roeder, 1990). *S. pombe* linear elements are proposed to be analogous structures to *S. cerevisiae* axial elements. In *rec10-155* mutants linear elements are absent (Molnar *et al.*, 2003). This fact in combination with the Rec10 homology to Red1 lead us (in collaboration with Alexander

Lorenz and Joseph Loidl, University of Vienna) to employ immunofluorescence to investigate Rec10 localisation during meiosis.

The Rec10 protein sequence Q09823 was analysed by Eurogentec S. A. for regions which would allow production of specific antibodies. This analysis revealed two peptide sequences which were used for production of N-terminal and C-terminal affinity purified polyclonal antibodies (residues 32-45 and 670-684 respectively; see figure 3.6). In collaboration with Alexander Lorenz and Joseph Loidl (University of Veinna), the C-terminal antibody was used to detect the presence and localisation of Rec10 in nuclear spreads produced via diploid azygotic meiosis (Lorenz *et al.*, 2004 and Figure 3.7 overleaf). This localisation detected Rec10 in structures that corresponded closely to silver staining structures identified as linear elements (Bahler *et al.*, 1993). The localisation pattern of Rec10 also resembled the pattern of Red1 when detect in *S. cerevisiae* meiotic nuclei spreads.

Nuclear spreads were also performed on *rec10-115*, *rec10-109* and *rec20-144* mutants. In *rec10-115* mutants no linear element structures are detectable (Lorenz *et al.*, 2004). This result is in agreement with Molnar *et al.*, (2003) in their analysis of meiotic nuclear spreads, where no silver staining LEs could be observed. In *rec10-109* mutants linear elements are almost totally absent with only a few short Rec10 positive staining lines observed (Lorenz *et al.*, 2004) whilst in *rec20-144* mutants they are significantly disrupted (Pryce *et al.*, 2004, in press).

These data show that linear element formation is either totally lost or severely inhibited in the *rec10*$^{+}$ mutants tested in this study. Hence, we have begun to support the bioinformatic analysis by demonstrating that Rec10 forms similar meiosis-specific structures to those formed by Red1 and that Rec10 co-localises with Hop1, a protein known to interact with Red1 in *S. cerevisiae*.

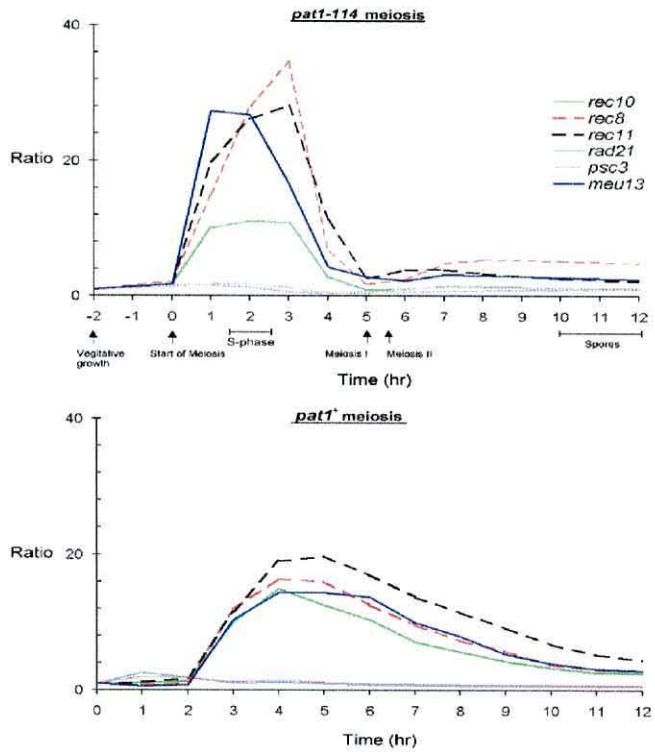
Figure 3.5
Bioinformatic analysis of predicted Rec10 protein

Blast homology data was obtained by standard protein-protein blast search. Predicted PEST sequences, nuclear localisation sequences and tyrosine kinase phosphorylation site motifs were obtained via web analysis packages using the EMBnet Austia PEST find program and the web analysis packages repectively:
http://www.at.embnet.org/embnet/tools/bio/PESTfind/
http://cubic.bioc.columbia.edu/predictprotein/
The putative Rec10-155 protein sequence is derived from the removal of the last 103 C-terminal Rec10 amino acids predicted to be lost via insertion of the *S. cerevisiae* LEU2 marker gene into the XhoI site located at position 2064 within the ***rec10*** ORF (Lin and Smith, 1995). Finally, predicted protein sequences for Rec10-109 and Rec20-144 were derived from translation of DNA sequencing information obtained from strains BP44 (***ade6-M26 rec10-109 h$^+$***) and BP82 (***ade6-M26 rec20-144 h$^+$***).

3.10 Transcription patterns of the *rec10*$^+$ gene.

The availability of an extensive meiotic transcriptomic data permits a comparison of *rec10*$^+$ transcript levels relative to other know meiotic genes (Mata *et al.*, 2002). In our study, *rec10*$^+$ transcript levels were compared to *meu13*, a gene required for recombination, chromosome pairing (Nabeshima *et al.*, 2001) and normal linear element formation (Molnar *et al.*, 2003), together with the meiotic cohesin genes, *rec8*$^+$ and *rec11*$^+$ and their mitotic homologues *rad21*$^+$ and *psc3*$^+$. This analysis showed that in a *pat1-114* induced meiosis, the level of *rec10*$^+$ transcription is approximately half that of in a *pat1*$^+$ diploid meiosis (Figure 3.6). The *pat-144* allele is a temperature sensitive mutant of the Pat1 protein, a protein kinase that controls entry into meiosis in *S. pombe* (Nurse, 1985). These data could indicate that Pat1 may play a role in regulation of *rec10*$^+$ transcription.

Figure 3.6
Comparison of meiotic transcription levels of *rec10*$^+$.



Plot produced from data exported from *S. pombe* Gene Expression Viewer http://www.sanger.ac.uk/PostGenomics/S_pombe/
Meiotic transcription levels of *rec10*$^+$ were plotted against known meiotic and mitotic cohesins and *meu13*$^+$, during *pat1-114* and *pat1*$^+$ diploid meiosis (Mata *et al.*, 2002).

Figure 3.7
Meiotic localisation of Rec10 to linear elements in meiosis
(Lorenz *et al.*, 2004).

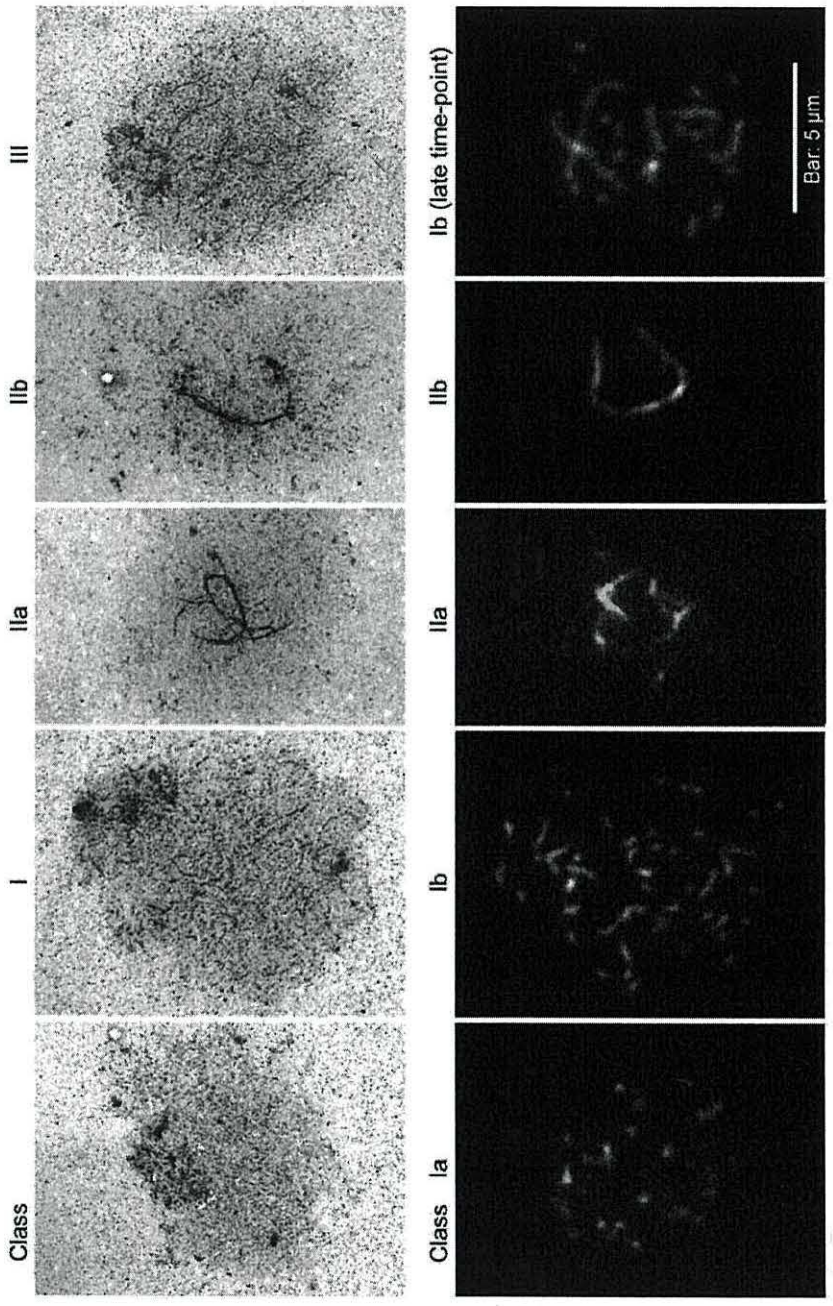


Figure 3.6
Comparison of LE morphologies in silver stained electron microscope (EM) (upper row) and immunostained Rec10 (lower row).
The classification of LEs is modified from (Bähler *et al.*, 1993).
Class Ia nuclei containing numerous (up to ~50) very short pieces of LEs were not described previously as they appear unconspicuous in the EM.
Shown is an empty nucleus which by its shape and size may correspond to the same stage as the nucleus underneath.
The remaining classes are easily detected both by immunostaining and EM

3.11 Analysis of spore viabilities and meiotic chromosome segregation errors in *rec20-144* mutants.

The viability of spores produced from homozygous *rec10-109* crosses carried out at a mating temperature of 25°C, is reduced to 46% of that of control *rec*$^+$ crosses (Ponticelli and Smith, 1989). In homozygous *rec20-144* crosses, the reduction in intragenic recombination is less significant than that which occurs in homozygous *rec10-109* crosses (DeVeaux *et al.*, 1998, Ponticelli and Smith, 1989). Spore viabilities of *rec20-144* mutant crosses were therefore assessed to determine if a link exists between loss of spore viability and the level of reduction of *ade6* intragenic recombination in different *rec10*$^+$ mutants. A mating temperature of 33°C was chosen as this is where the maximum *rec20-144*-dependent reduction in *ade6* intragenic recombination is observed (Table 4.1). Interestingly, this analysis showed that, in a *rec*$^+$ background, there is a slight but significant drop in the viability of spores produced from *ade6-M26* (66%) compared to *ade6-M375* (75%) crosses ($p=0.03$). The same comparison of viabilities carried out in *rec20-144* mutant crosses shows a greater and highly significant drop in viability, 69% and 37% respectively ($p>0.01$, Figure 3.8). This indicates that during meiosis at 33°C, the presence of the *ade6-M26* allele has a negative effect on spore viability in both *rec*$^+$ and *rec20-144* backgrounds (Figure 3.8). As the *rec20-144* mutation causes a greater significant decrease in *ade6-M26* than *ade6-M375* intragenic recombination, this may link the extent of reduced recombination to a loss in spore viability (Table 3.5). However, it does not explain why the highly recombinogenic *rec*$^+$ *ade6-M26* crosses produce proportionally less viable spores than the less recombinogenic *rec*$^+$ *ade6-M375* crosses. One possible model is the increased loss in viability of spores from *ade6-M26* compared to *ade6-M375* crosses, may be due to a failure to process the proportionally larger number of recombination events initiated at *ade6-M26*. In *rec20-144 ade6-M26* double mutants, this phenomenon maybe increased and act alone or in unison with a reduction in recombination initiation. The implications of the observation that the *rec20-144* mutation causes a greater decrease in *ade6-M26* than *ade6-M375* intragenic recombination leading to a decrese in hotspot activity in *rec10*$^+$ mutants is explored in the following chapters.

The use of the DNA stain DAPI has been successfully employed to observe the location of chromatin bodies and chromosome disjunction during mitosis (Funabiki *et al.*, 1996a; Funabiki *et al.*, 1996b; Hirano, 1986; Samejima and Yanagida, 1994). A similar approach using DAPI staining was undertaken to observe meiotic chromosome mis-segregation events in both in rec^{+} and *rec20-144* strains. Crosses of *ade6-M26* and *ade6-M375* against the *ade6-L52* marker allele were carried out at 25°C in both rec^{+} and *rec20-144* strains. The resulting tetrads were then stained with DAPI and observed via DIC light and fluorescent microscopy to determine if any possible chromosome mis-segregation (DAPI) and mutant tetrad morphologies (DIC) were apparent (Figure 3.10). The distribution and intensity of DAPI staining material in tetrads observed suggests that both meiosis I and meiosis II mis-segregation may be occurring during homozygous *rec20-144* meiosis (Figure 3.10). To begin an initial quantification of the distribution of mis-segregation errors in *rec20-144* mutants, DIC Light microscopy was used to count and classify tetrads from a random sample of 500 tetrads obtained from *ade6-M375*-by-*ade6-L52* crosses carried out at 25°C in both rec^{+} and *rec20-144* backgrounds. The results showed that whilst 96% of tetrads derived from rec^{+} crosses were normal in appearance, in homozygous *rec20-144* crosses, only 73% appeared normal and 27% had unusual spore morphologies. The largest class of these unusual tetrads was designated class I, and as *S. pombe* forms a linear tetrad (Lindner 1893), is indicative of a possible meiosis I mis-segregation phenotype (Figure 3.10).

Figure 3.8
Comparison of relative spore viabilities of the *ade6-M26* and *ade6-M375* strains in *rec20-144* and *rec*$^+$ backgrounds.

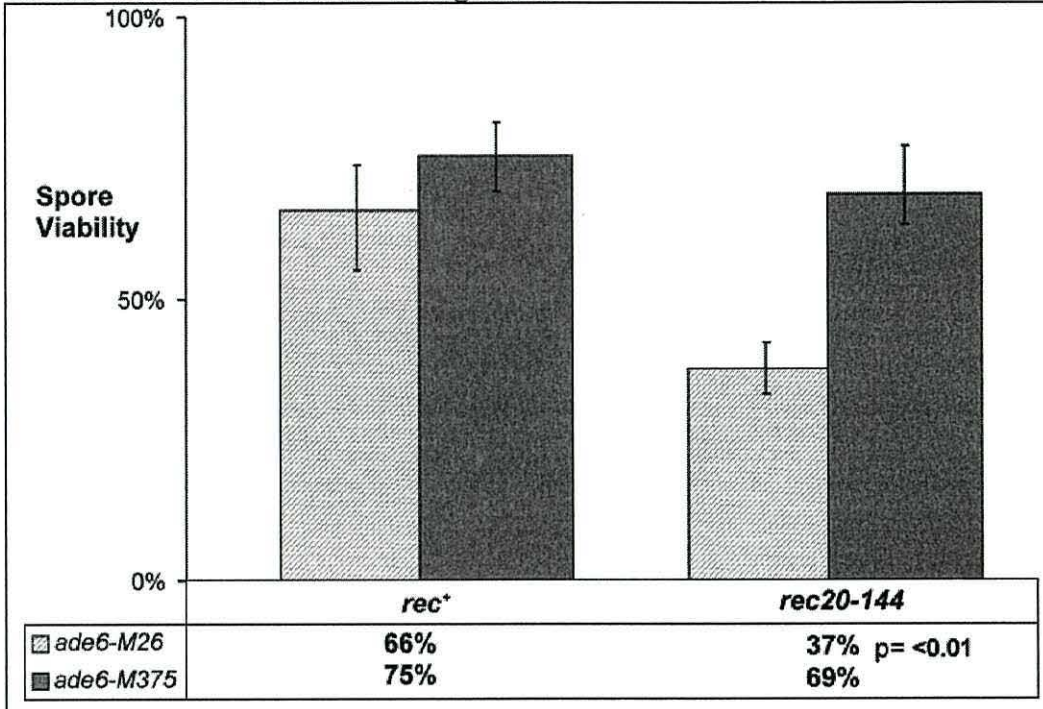


Figure 3.8 shows the comparative spore viabilities and recombination data obtained via random spore analysis of two-factor meiotic recombination assays, performed at a mating temperature of 33°C, using the *ade6-M26* and *ade6-M375* alleles against the *ade6-L52* marker allele in both a *rec*$^+$ and *rec20-144* mutant backgrounds.
Mean spore viability values are shown as % values and obtained from an average of 8 samples taken from two individual repeats (n=2).
Error bars are ranges derived from maximum and minimum spore viability values.
In an *ade6-M375* background the *rec20-144* mutation causes no significant change in spore viability (p=0.06).
In an *ade6-M26* background the *rec20-144* mutation causes a highly significant change in spore viability (p>0.01).
p values derived via Student's t-test.
Mean recombination data obtained during this analysis is presented below

ade6 allele	Mean recombination values Ade$^+$ /10^6 viable spores[a]		Fold reduction in recombination[b, c]
	rec$^+$	*rec20-144*	
ade6-M26	5078 (39)	30 (6)	169
ade6-M375	200 (37)	11 (1)	18

Figures in parentheses are 1 standard deviation
[a] See section 2.2 for protocol used to determine Ade$^+$ recombinants/viable spore.
[b] The reduction in recombination is derived by dividing the mean recombination values of *rec*$^+$ strains by *rec20-144* mutants
[c] The difference in reduction in recombination between the *ade6-M26* and *ade6-M375* is highly significant in both cases (p<0.01). This would implicate *rec20-144* mutants have a reduction in *ade6-M26* hotspot activity.
p values derived via Student's t-test.

Figure 3.9
Examples of *rec20-144* dependent tetrad morphologies produced during heterothallic *ade6-M375*-by-*ade6-L52* crosses.

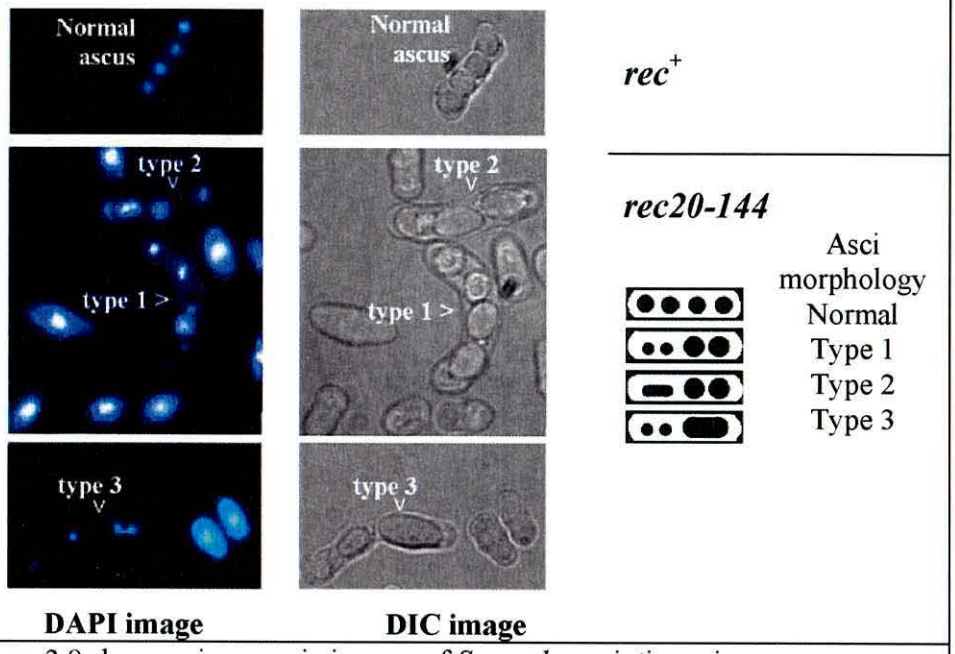
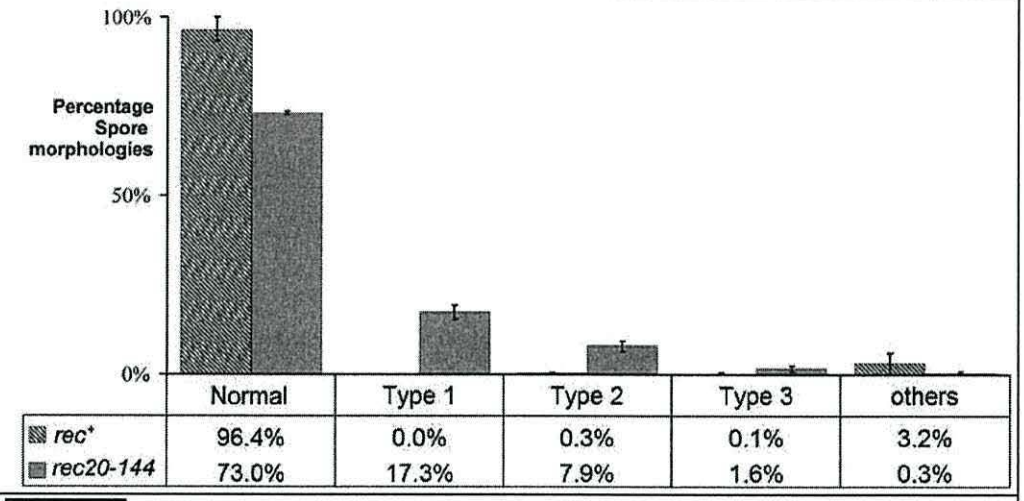
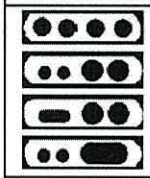


Figure 3.9 shows microscopic images of *S. pombe* meiotic asci
Spores were stained with DAPI and viewed under fluorescent light to observe spore DNA content.
DIC microscopy was then used to visualise spore morphology and classify spores into 3 types (see section 3.19 for discussion)

Figure 3.10
Quantification of spore morphologies observed in homozygous *rec*$^+$ and *rec20-144* crosses between the *ade6-M375* and *ade6-L52* alleles



	Normal	Type 1	Type 2	Type 3	others
rec$^+$	96.4%	0.0%	0.3%	0.1%	3.2%
rec20-144	73.0%	17.3%	7.9%	1.6%	0.3%



Normal
Type 1
Type 2
Type 3

Tetrad morphology classes, based on DIC microscopic analysis.

Figure 3.10 shows a table representing the percentage of spore morphologies obtained from homozygous *rec*$^+$ and *rec20-144* crosses between the *ade6-M375* and *ade6-L52* alleles.
Spores were observed under via light microscopy and only spores still contained within intact asci were counted.
Classification of spores is discussed in section 3.19

3.12 Discussion

3.13 Cloning of *rec20-144*

We have demonstrated that the *ade6* intragenic recombination defect observed in *rec20-144* mutants is suppressible by a *rec10*$^+$ plasmid (Table 3.4). The *rec10*$^+$ ORF is located on chromosome I in a region flanked by *his1* and a hypothetical *b-zip1* gene, c25G10.03, (Wood *et al.*, 2002). The pRMSPL-1 library contains partial *Sau*3AI digested genomic fragments of approximately 4.6 kb, inserted with approximately 89% efficiency into the pRM6 vector (R. J. McFarlane, personal communication). If we assume the minimum fragment length required to suppress *rec20-144* includes the DNA regions up to the respective start and stop codons of the genes that flank the *rec10*$^+$ ORF, as these may contain essential regulatory elements, a 3.922 kb fragment is needed. As this 3.9 kb region is within the approximately 4.6 kb average insert size of the pRMSPL-1 library, one *rec10*$^+$ clone should have been isolated per approximately 3044 transformants. This number is calculated by multiplying the insertion frequency (100/89) by the size of the *S. pombe* genome 12,462,637 bp (Wood *et al.*, 2002) divided by the fragment size, 4600 bp.

Prior to this study the pRMSPL-1 library had been successfully used to isolate two *rec10-109* suppressing clones out of a total of approximately 4000 transformants, a number consistent with the expected frequency (R J. McFarlane personal communication). We therefore predicted that the ease of selection of *rec20-144* suppressing clones should have been at least equivalent to that of the *rec10-109* clone isolation. However, no *rec20-144* suppressing clones were isolated in our screen. As the pRMSPL-1 library had undergone re-amplification prior to its use in this study one possible explanation for this result is that *rec10*$^+$ has become under represented within the pRMSPL-1 sample of used for this screen. Whatever the reason was for the failure of the pRMSPL-1 screen subsequent events precluded the necessity to refine a library-based screen.

3.14 *rec20-144* is a novel allele of *rec10*$^+$

Two independently created *rec10*$^+$ plasmids suppress the *rec20-144* intragenic recombination phenotype (Table 3.4). This result is not due to

multicopy suppression as the *rec20-144* intragenic recombination phenotype is not complemented by the *rec10-155* allele in a heterozygous *rec20-144*-by-*rec10-155* cross (Table 3.3). We therefore sequenced the *rec20-144* allele and found a single mis-sense mutation in the *rec10*$^{+}$ ORF. Together these data provide conclusive evidence that *rec20-144* is an allele of *rec10*$^{+}$.

In the original investigation of recombination in *rec20-144* mutants DeVeaux and co-workers (1992) noted that the *rec10-109* mutant fully suppressed the *rec20-144* mutant. In our analysis however the *rec10-109* allele did not fully suppress the *rec20-144* allele (Table 3.3). A possible explanation for this is that we have demonstrated that *rec20-144* mutation causes a temperature sensitive reduction in intragenic recombination at *ade6-M26* (Table 4.1). As our analysis was performed at 30°C and the recombination assays performed by DeVeaux and co-workers (1992) were carried out at room temperature it is possible that the partial *rec10-109* suppression observed in this study is due to the t.s. nature of the *rec20-144* mutant. However, as the recombination frequency ranges of our crosses overlap, the partial reduction may also be due to experimental variation.

In homozygous *rec20-144* crosses the reduction in *ade6* intragenic recombination phenotype is significantly less than that of homozygous *rec10-155* or *rec10-109* crosses (Table 3.3, Ponticelli *et al.*, 1989). However, whilst the *rec10-109* allele partially suppresses the *rec20-144 ade6* intragenic recombination phenotype, *rec10-155* allele is dominant in this respect. This dominance is unaffected by either mating type or which *ade6* allele is associated with which *rec10*$^{+}$ mutant allele (Table 3.1, and data not shown). The relevance of these observations remains unclear. As the *rec20-144* and *rec10-155* mutations are C terminal, whilst the *rec10-109* mutations are N-terminal this may indicate possible distinct roles for the Rec10 protein N and C termini.

3.15 Bioinformatic analysis

3.16 Rec10 protein homologies

Prior to this study, bioinformatical analysis using the Rec10 protein sequence had failed to identify any protein(s) or domains with significant homologies to Rec10. However, our bioinformatic analysis has revealed limited

homologies to two proteins, *C. elegans* XNP1 and *S. cerevisiae* Red1 (Lorenz *et al.*, 2004).

3.17 Rec10 and XNP1/ATRX

The central region of the predicted Rec10 amino acid sequence exhibits 22% homology to the *C. elegans* XNP-1 (Figure 3.6). The XNP-1 protein shares homology with the human ATRX protein, mutations in which cause X-linked alpha-thalassemia mental retardation associated syndromes (Gibbons and Higgs, 2000). The predicted ATRX protein has been classified as a putative transcription factor, with helicase/ATPase functions similar to the SNF2 protein family, a protein family known to be involved in DNA recombination and repair (Picketts *et al.*, 1996). Whilst there is no direct significant amino acid homology between human ATRX and Rec10, the distant homology to a SNF2-related protein may still be of significance.

During interphase and mitosis, both human and mouse ATRX associate with pericentric regions and heterochromatin (McDowell *et al.*, 1999). In addition phosphorylated ATRX has also been shown to localise to condensed chromatin in association with the human heterochromatin protein 1-alpha (HP1α) (Berube *et al.*, 2000). Furthermore, ISWI, also known as SNF2h, is part of a human chromatin remodelling complex which may actively load the mitotic cohesin hRad21 onto chromosomes (Hakimi *et al.*, 2002). In *S. pombe*, during mitosis (Nonaka *et al.*, 2002) and meiosis (Kitajima *et al.*, 2003b) recruitment of cohesins to the outer heterochromatic centromeric regions is dependent upon the *S. pombe* HP1 homologue, Swi6. Furthermore, the *rec10*$^{+}$ mutant, *rec10-109* (Ponticelli and Smith, 1989) causes a reduction in intragenic recombination that is most pronounced close to the centromeres of all three *S. pombe* chromosomes, a phenotype also observed with mutants of the meiotic cohesins, *rec11*$^{+}$ and *rec8*$^{+}$ (DeVeaux and Smith, 1994; Krawchuk and Wahls, 1999). Finally, a recent study has shown that the *S. pombe* SNF2 homologue, *snf22*$^{+}$, is essential for the activation of the *ade6-M26* hotspot (Yamada *et al.*, 2004). In summary, although it is unknown if ATRX performs any function(s) in meiosis, there are plausible

links between the location and functions of ATRX during mitosis and those of Rec10 during meiosis.

3.18 Rec10 and Red1

In *S. cerevisiae* loss of Red1 function severely reduces axial element (AE) and prevents SC formation (Rockmill and Roeder, 1990). Red1 mutants also have reduced homologue pairing (Nag *et al.*, 1995) and a loss of sister chromatid cohesion (Bailis and Roeder, 1998). In *rec10-155* mutants, linear elements, possibly the *S. pombe* analogues of axial elements, do not form (Molnar *et al.*, 2003). This results in a partial reduction in homologue pairing efficiency at all loci so far tested, but no significant loss of sister chromatid cohesion (Molnar *et al.*, 2003). Immunofluorescence staining reveals Red1 localises to meiotic chromosomes in patterns that closely resemble those of Rec10 (Figure 3.7 this study; Smith and Roeder 1997). These data show, that in two highly diverged eukaryotes, Red1 and Rec10 share similar localisation patterns on meiotic chromosomes and are both required for the formation of meiotic-specific structures implicated in chromosome pairing and recombination.

3.19 Spore viability and tetrad morphologies in *rec20-144* mutants

Mutations that effect meiotic recombination can result in non-disjunction (NDJ) of chromosomes in meiosis I and meiosis II. These NDJ events can result in the production of either inviable or impaired meiotic progeny. Although *S. pombe* possesses an achiasmatic chromosomal segregation system that can produce a percentage of viable spores in the absence of recombination (Davis and Smith, 2003), in the *rec10*$^{+}$ mutants, *rec10-109* (Ponticelli and Smith, 1989) and *rec20-144* (this study), spore viability levels are significantly reduced.

Previous studies have shown that in crosses involving the *ade6-M26* hotspot allele in a *rec10-109* background, spore viability is reduced to approximate 50% of *rec*$^{+}$ crosses (Ponticelli *et al.*, 1989). This figure is close to that obtained for the *rec20-144*-dependent reduction in spore viability in strains carrying the *ade6-M26* allele but not the *ade6-M375* allele (Figure 3.8). One reason for the reduction in spore viability in *rec20-144* mutants could be genome

wide decrease in crossover formation leading to a failure to disjoin chromosomes in meiosis I. However, the reduction in spore viability in *ade6-M26 rec20-144* double mutants is significantly greater than that recorded for *ade6-M375 rec20-144* double mutants (Figure 3.8). In *rec20-144* mutants the reduction in *ade6* intragenic recombination is greater within the *ade6-M26* allele than the *ade6-M375* allele. However, the reduction in intragenic and intergenic recombination at all other loci so far tested is generally more equivalent (D. W. Pryce, R. J. McFarlane, unpublished data, J. L. Wells, personal communication). It therefore seems unlikely that in *rec20-144* mutants a greater reduction in recombination within the *ade6* gene alone could cause the significant difference between *ade6-M26* and *ade6-M375* spore viability.

During a haploid *pat1-114* induced meiosis Steiner and co-workers (2002) noted that in a *rec10*$^{+}$ mutant there was still a detectable *M26*-specific increase in DSB formation relative to *ade6-M375* strains. Hence, it is likely that there are more recombinogenic DSBs formed in *rec20-144 ade6-M26* double mutants than in *rec20-144 ade6-M375* double mutants. Recent studies have shown that some meiotic DSBs are destined to result in interhomologue recombination and crossovers (Hunter and Kleckner, 2001). Red1 is required in meiosis to enforce this interhomologue recombination pathway (Schwacha and Kleckner, 1997). In humans, *Drosophila* and yeast, meiosis I NDJ has been shown to be associated with a reduction in pericentric (close to centromeres) crossovers (Ross *et al.*, 1992; MacDonald *et al.*, 1994; Moore *et al.*, 1994; Sherman *et al.*, 1994; Hassold *et al.*, 1995). Furthermore, in mitosis and meiosis failure to remove inter-sister chromatid catenations results in abnormal chromosomal segregation patterns (Goodwin *et al.*, 1999; Watt *et al.*, 1995). These events may have a combined effect in *S. pombe* meiosis. *S. pombe* forms linear tetrads (Lindner 1893). Our study of spore morphology and the more extensive study performed by Krawchuk and co-workers (1999) shows approximately 11-17% of the abnormal tetrads produced from *rec10*$^{+}$ mutant crosses display a morphology that is indicative of a meiosis I NDJ. In addition, Krawchuk and co-workers (1999) also noted 20% of viable *rec10-109* diploids experienced a meiosis II type disjunction. These results seem to indicate that the majority of abnormal tetrads result from MI NDJ,

although a small percentage of MII non-disjunction also occurs. Hence, two possible events may cause the greater reduction in spore viability in *ade6-M26 rec20-144* double mutants. Firstly, crossover recombination events may be formed, but fail to correctly resolve. Secondly, the enforcement of interhomologue recombination may be lost, or at least reduced, leading to an increase in intersister recombination. These defects could result in a failure to correctly disjoin chromosomes in both meiosis I and meiosis II.

3.20 Summary of main findings

- The *rec20-144* mutant is not an allele of a *rec20* gene, but is a novel allele of the meiotic recombination gene, *rec10*$^{+}$ (Tables 3.3 and 3.4, Figure 3.5).
- The Rec10 protein has limited but significant sequence and structural homologies to *C. elegans* XNP1, a homologue of human ATRX, a putative transcription factor, with helicase/ATPase functions similar to the SNF2 chromatin remodelling protein family and *S. cerevisiae* Red1, a protein required for axial element and synaptonemal complex formation.
- In chromosome spreads of *S. pombe* meiotic nuclei, Rec10 localises to linear elements, meiotic specific structures thought to be analogues of *S. cerevisiae* axial elements (Figure 3.7).
- The meiotic localisation patterns of the Rec10 protein and the *rec10* mutant recombination phenotypes indicate that Rec10 may perform similar functions to its sequence and structural homologues, *C. elegans* XNP1 and *S. cerevisiae* Red1.
- During zygotic crosses in homozygous *rec20-144* mutants performed at 33°C, spore viability is significant reduced in matings involving the *ade6-M26* allele but not the *ade6-M375* allele. In the same zygotic crosses, the level of *ade6-M26* intragenic recombination is reduced to a greater extent than *ade6-M375* intragenic recombination (Figure 3.8). This results in a reduction in *ade6-M26* hotspot activity and implicates Rec10 in the control of hotspot activation.

3.21 Conclusions

The *rec20-144* allele is a novel mutant allele of the *rec10*$^{+}$ gene. Specific-immunostaining shows Rec10 localises to linear elements. In *rec10*$^{+}$ mutant backgrounds, linear elements are absent or disrupted to varying degrees indicating Rec10 is most likely required for linear element formation. The Rec10 protein has structural and sequence homologies to Red1 and XNP1 that may link possible further functions of Rec10 to the establishment of meiotic interhomologue recombination bias (Red1, Schwacha and Kleckner, 1997, Blat *et al.*, 2002) and chromatin remodelling (*snf22,* Yamada *et al.*, 2004). In *rec20-144* mutants, the reduction in the level of intragenic recombination frequency and spore viabilities is greater in *ade6-M26* hotspot than *ade6-M375* non hotspot strains indicating possible distinct functions in the processing of hotspot and non hotspot recombination. A study into the function(s) of Rec10 in *ade6-M26* hotspot activation should be under taken.

Chapter 4

Rec10 is required for full activation of the *ade6-M26* meiotic recombination hotspot

4.1 Introduction

In general, during meiosis, the frequency of recombination between any two points on a chromosome is roughly proportional to the physical distance between them. However, certain chromosomal regions experience either higher or lower recombination levels than the physical distance between them would suggest. Chromosomal regions with recombination frequencies lower than the genome average, for example in yeast the *mat* locus, centromeres and telomeric regions are recombination "coldspots" (Egel, 1984; Nakaseko *et al.*, 1986; Lambie and Roeder, 1988; Grewal and Klar, 1997). In contrast, regions that exhibit a recombination frequency higher than the genomic average are termed "hotspots". Hotspots occur in both prokaryotes and eukaryotes (see section 1.18 to 1.21).

In *S. pombe* the *ade6-M26* allele contains an artificially created meiosis-specific hotspot that can increase homologous recombination by approximately 15-fold when compared to its *ade6-M375* control allele (Gutz *et al.*, 1971). The creation, through specific point mutation(s), of the *M26* heptameric sequence at other locations within either the *ade6* or *ura4* genes or an *M26* related CRE sequence within the *ade6* gene, results in the formation of a meiotic recombination hotspot (Fox *et al.*, 1997; Fox *et al.*, 2000). These facts suggest that both *M26* and CRE sequences may have the potential to act as meiotic recombination hotspots at other locations with the *S. pombe* genome and hence may play a significant role in directing the locations of meiotic-specific recombination events (see sections 1.22 for a more complete overview of *M26* and CRE recombination hotspots).

To date few factors are known to be involved in the regulation of *ade6-M26* hotspot activity and only deletion of the *gcn5* gene has been shown to partially reduce the extent of *ade6-M26* recombination hotspot activation (Yamada *et al.*, 2004). In the genetic investigations of the *rec* mutants isolated by Smith and co-workers (1989), none of the mutant alleles tested was noted as having a significant effect on hotspot activity (Ponticelli and Smith, 1989;

DeVeaux *et al.*, 1992). However, analysis of the data presented in Ponitcelli and Smith (1989) indicates that two recombination deficient mutants, the *rec10-109* and the *rec11-111* mutants, may retain approximately 50% of the level of rec^+ *ade6-M26* hotspot activity.

In this chapter we investigate the role of Rec10 in regulation of the *ade6-M26* hotspot. We show that the presence of the *rec20-144* mutation creates a temperature sensitive reduction in meiotic recombination at the *ade6* locus. Furthermore, at 25°C, 30°C and 33°C the extent of the reduction in *ade6-M26* recombination is not equivalent to that observed in the none hotspot *ade6-M375* control and as a result, *rec20-144* mutants fail to fully activate the *ade6-M26* recombination hotspot. In addition we show that the insertional inactivated $rec10^+$ mutant, *rec10-155* (Lin and Smith, 1995), also fails to fully activate the *ade6-M26* recombination hotspot. These observations indicate that as well as a role in "basal" meiotic recombination (Ponticelli *et al.*, 1989; DeVeaux *et al.*, 1992; this study) and linear element formation (Molnar *et al.*, 2003; Lorenz *et al.*, 2004) some function of the Rec10 protein is required for full activation of a meiotic-specific recombination hotspot. Finally, we demonstrate that *ade6-M26* hotspot recombination appears to undergo temperature sensitive stimulation and suggest that Rec10 maybe required for this process.

4.2 Results

4.3 The effect of *rec20-144* on *ade6* intragenic recombination.

DeVeaux and co-workers (1992) originally classified the *rec20-144* allele as a meiotic recombination deficient mutant which lowers intragenic recombination levels at the *ade6* locus by approximately 3-10-fold. In that series of experiments meiosis was performed at room temperature (DeVeaux *et al.*, 1992). Experiments performed prior to this study had shown that the *rec20-144* mutation resulted in a further decrease in *ade6-M26* intragenic recombination to levels >3-10-fold when meiosis was performed at temperatures ≥25°C (personal communication R. J. McFarlane). We therefore decided to carry out a thorough analysis of the effect of temperature on intragenic recombination at the *ade6* locus in *rec20-144* mutant strains. Four mating temperatures were chosen for a series of heterothallic meiotic crosses involving the *ade6-M26* hotspot and the *ade6-M375* non-hotspot alleles (Gutz, 1971) against an *ade6-L52* test allele, in both rec^+ and *rec20-144* backgrounds. Random spore analysis of the progeny of these crosses demonstrated *rec20-144* strains exhibit an approximately 8-fold reduction in recombination at 20°C (Table 4.1). This reduction is within the range previously reported by DeVeaux and co-workers (1992), who carried out crosses at room temperature. At higher mating temperatures, the *rec20-144* mutation further reduces *ade6-M26* intragenic recombination. At 25°C and 30°C, relative to rec^+ controls, the *rec20-144* mutation decreases *ade6-M26* intragenic recombination by approximately 17.5-fold and 46-fold respectively, whilst at 33°C, the maximum temperature at which *S. pombe* cells efficiently mate, *ade6-M26* intragenic recombination is further reduced by approximately 78.5-fold (Table 4.1).

DeVeaux and co-workers (1992) demonstrated that at room temperature the *rec20-144* mutation lowers *ade6-M375* non-hotspot recombination to a similar extent as *ade6-M26* hotspot recombination. Prior to this study the affect the *rec20-144* mutation may have on *ade6-M375* non-hotspot recombination at 25°C, 30°C or 33°C had not been determined (personal communication R. J. McFarlane). Therefore a series of meiotic crosses were performed at those

temperatures to measure the intragenic recombination frequencies between the *ade6-M375* and *ade6-L52* alleles. Random spore analysis of these progeny showed that when meiosis was performed at 25°C, 30°C and 33°C the *rec20-144* mutant displays a mild temperature sensitive decrease in *ade6-M375* recombination, although, at each temperature, the reduction in recombination was less pronounced than that observed in the equivalent *ade6-M26* experiment. This effect was greatest at 33°C where, in a *rec20-144* background, *ade6-M375*-by-*ade6-L52* recombination was reduced by only 19-fold compared to the 78.5-fold reduction seen in *ade6-M26*-by-*ade6-L52* recombination (Table 4.1).

The *ade6-M26* hotspot activity is determined as the ratio of the number of Ade^+ prototrophs obtained during *ade6-M26* recombination divided by the number of Ade^+ prototrophs obtained during non-hotspot *ade6-M375* recombination. When a comparison of the levels of rec^+ vs. *rec20-144* hotspot activities is made at 20°C, 25°C, 30°C and 33°C a progressive reduction in the fraction of rec^+ hotspot activity is apparent (hotspot ratios were calculated from the average recombination frequencies shown in Table 4.1). For example, at 20°C, the mean rec^+ hotspot activity is 6.2 whereas mean *rec20-144* activity is 5.2, approximately 85% of the rec^+ value. At 25°C, mean rec^+ hotspot activity is 12.7, whilst the mean *rec20-144* activity is 5.6, only 44% of the rec^+ value. The fraction of rec^+ hotspot activity achieved in *rec20-144* mutants is decreased further at 30°C, where mean rec^+ and *rec20-144* hotspot activities are 17.9 and 5.6 respectively. Hence, *rec20-144* mutants achieve only 31% of the rec^+ value at 30°C. Finally, at 33°C, rec^+ hotspot activity is 16 and *rec20-144* activity 3.9 only 24% of the rec^+ value.

In *rec20-144* mutants hotspot activities are substantial decreased relative to rec^+ hotspot activities. To allow an accurate statistical determination of the effect of mating temperature on the loss of *ade6-M26* hotspot activity in the $rec10^+$ mutants, *rec20-144* and *rec10-155* (Lin and Smith, 1995) a further series of experiments were performed under identical mating conditions.

4.4 Activation of the *ade6-M26* hotspot is significantly reduced in *rec10*$^+$ mutants.

The point mutation within the *rec10*$^+$ gene that creates the *rec20-144* allele (Figure 3.5) may result in the creation of a Rec10 mutant protein with a unique defect in *ade6-M26* hotspot activation. To further test this possibility, the levels of *ade6-M26* hotspot activity attained with the *rec20-144* mutant and the insertionally inactivated *rec10*$^+$ mutant, *rec10-155* (Lin and Smith, 1995), were compared. Hotspot activity in the *rec20-144* mutant was determined at 20°C, 25°C, 30°C and 33°C, whilst hotspot activity in the *rec10-155* mutant was determined at 25°C and 30°C (Figure 4.1 A. and B. respectively). An analysis of hotspot activation was not performed at 33°C in *rec10-155* strains as meiosis performed at this temperature in *rec10-155* mutants resulted in insufficient Ade$^+$ recombinants to allow a reliable determination of hotspot activity.

Figure 4.1 shows that at 25°C and 30°C hotspot activity is significantly suppressed ($p<0.01$) in both *rec20-144* and *rec10-155* mutants. This indicates that the *rec20-144* allele is not unique and that Rec10 is required for full *rec*$^+$ levels of hotspot activation when meiosis is performed at temperatures ≥25°C (Figure 4.1 A and B). The actual reduction in the level of *ade6* intragenic recombination obtained from *rec10-155* mutant strains is significantly greater than the reduction obtained from *rec20-144* mutant crosses. For example, at 30°C, *ade6-M26* intragenic recombination in the *rec10-155* mutant is reduced 705-fold compared to *rec*$^+$ levels, whilst *rec20-144* strains are only reduced 46-fold (Tables 4.1 and 4.2). However, when matings are performed at 25°C the percentage of *rec*$^+$ hotspot activity retained in both the *rec10-155* and *rec20-144* mutants is very similar, at an approximate average of 55% and 48% respectively. These data suggest the point mutation of the *rec20-144* allele results in a loss of function, which, in respect of *ade6-M26* hotspot activation, is as severe as the C-term truncation of the *rec10*$^+$ ORF in the *rec10-155* allele.

4.5 The *ade6-M26* hotspot undergoes a temperature-dependent stimulation of recombination.

An analysis of *rec*$^+$ *ade6-M26* intragenic recombination reveals a temperature-dependent decrease in Ade$^+$ recombinant spore production (Table 4.1). A calculation of the number Ade$^+$ spores/10^6 viable spores produced at 20°C and 25°C shows that this 5°C increase in mating temperature results in a significant 1.8-fold increase in the number of Ade$^+$ recombinant spores produced ($p< 0.01$, Table 4.1). A similar calculation of the number Ade$^+$ spores/10^6 viable spores produced at 25°C and 30°C shows that this 5°C increase in mating temperature results in a further 1.4-fold significant increase in the number of Ade$^+$/10^6 viable spores produced ($p< 0.01$), and a 2.5-fold increase from that of 20°C ($p< 0.01$). In both the above examples no significant increases are seen in *ade6-M375* non-hotspot recombination (Table 4.1, Figure 4.2). A further 3°C increase in mating temperature, from 30°C to 33°C, results in significant 2.3-fold and 2.1-fold decreases in *ade6-M26* and *ade6-M375* intragenic recombination respectively (figure 4.2). These almost equivalent fold-drops in recombination may be due to the meiotic recombination process reaching a thermal limit.

Table 4.1
The effect of mating temperature on *ade6-M26* and *ade6-M375* recombination in *rec*$^+$ and *rec20-144* mutant strains.

Temperature °C	*ade6* allele	Mean recombination frequency Ade$^+$ /10^6 viable spores[a]				Fold reduction in recombination[b]
		rec$^+$		*rec20-144*		
20	*M26*	2320 (960)	n=14	268 (204)	n=14	8.7
	M375	373 (155)	n=14	51 (29)	n=14	7.3
25	*M26*	4192 (1408)	n=25	240 (195)	n=25	17.5
	M375	330 (75)	n=25	43 (27)	n=25	7.7
30	*M26*	5705 (1692)	n=19	124 (80)	n=19	46.0
	M375	318 (86)	n=19	22 (12)	n=19	14.5
33	*M26*	2432 (1396)	n=41	31 (20)	n=35	78.5
	M375	152 (93)	n=41	8 (5)	n=35	19.0

Table 4.1 shows the results of random spore analysis obtained from two-factor meiotic recombination assays using the *ade6-M26* and *ade6-M375* alleles against the *ade6-L52* marker allele in both a *rec*$^+$ and *rec20-144* mutant backgrounds.
(Figures in parentheses are 1 standard deviation).
[a] See section 2.2 for protocol used to determine Ade$^+$ recombinants/viable spore.
[b] The *rec20-144*-dependent reduction in recombination is derived by dividing mean *rec*$^+$ Ade$^+$ values by mean *rec20-144* Ade$^+$ values. Reductions in recombination were highly significant at all temperatures with p values of < 0.01 in all cases derived via Student's t-test.

Table 4.2
The effect of mating temperature on *ade6-M26* and *ade6-M375* recombination in *rec*$^+$ and *rec10-155* mutant strains.

Temperature °C	*ade6* allele	Mean recombination frequency Ade$^+$ /10^6 viable spores[a]				Fold reduction in recombination[b]
		rec$^+$		*rec10-155*		
25	*M26*	4987 (1332)	n=8	9.3 (3)	n=8	536
	M375	337 (102)	n=13	1.2 (0.5)	n=13	281
30	*M26*	6560 (1718)	n=7	9.3 (4)	n=7	705
	M375	311 (111)	n=10	1.2 (0.4)	n=10	259

Table 4.2 shows the results of random spore analysis obtained from two-factor meiotic recombination assays using the *ade6-M26* and *ade6-M375* alleles against an *ade6-L52* marker allele in both a *rec*$^+$ and *rec10-155* mutant backgrounds.
Figures in parentheses are 1 standard deviation.
[a] See section 2.2 for protocol used to determine Ade$^+$ recombinants/viable spore.
[b] The *rec10-155*-dependent reduction in recombination is derived by dividing mean *rec*$^+$ Ade$^+$ values by mean *rec10-155* Ade$^+$ values. Reductions in recombination were highly significant at all temperatures with p values of < 0.01 in all cases derived via Student's t-test.

Figure 4.1
Rec10 is required for full activity of the *ade6-M26* hotspot.

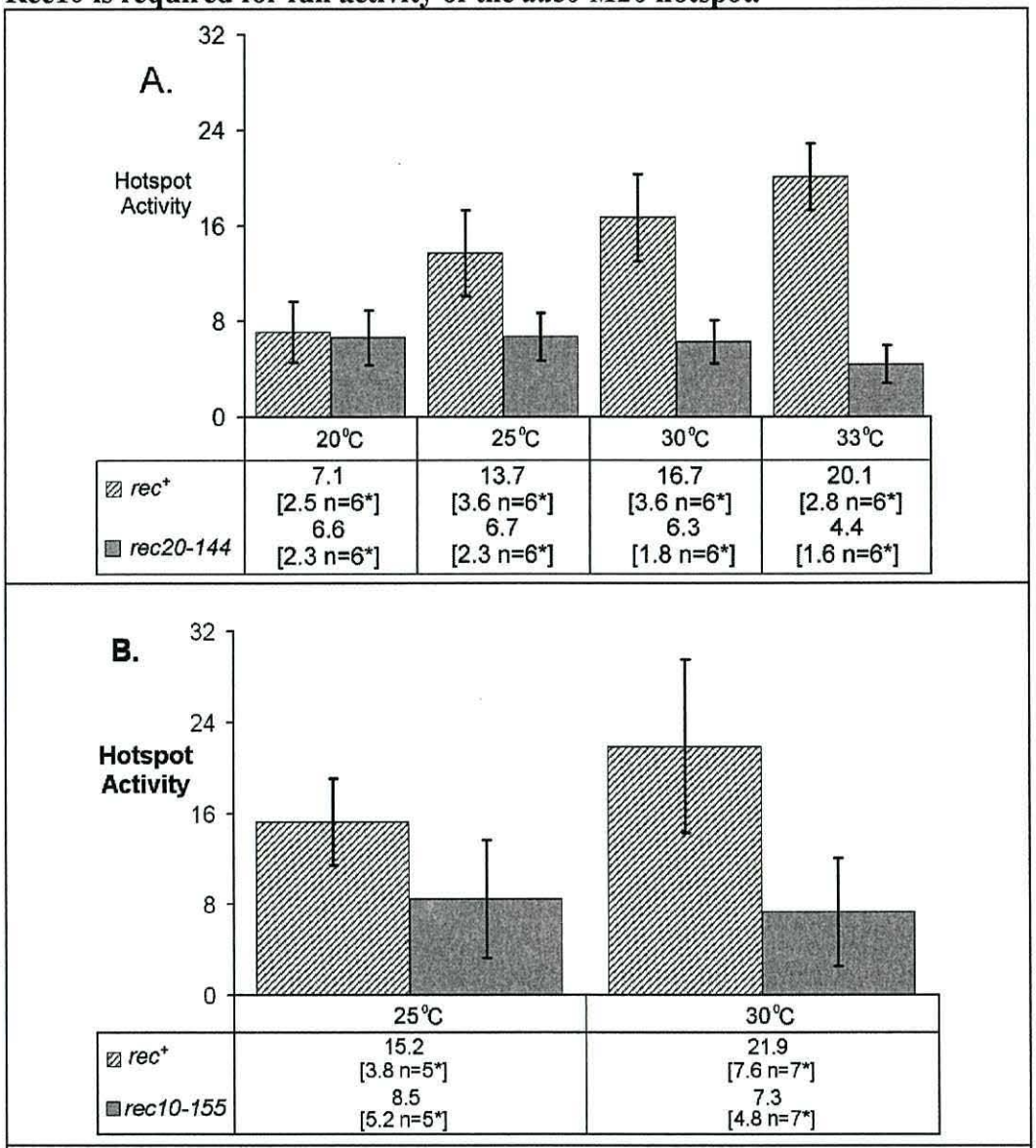
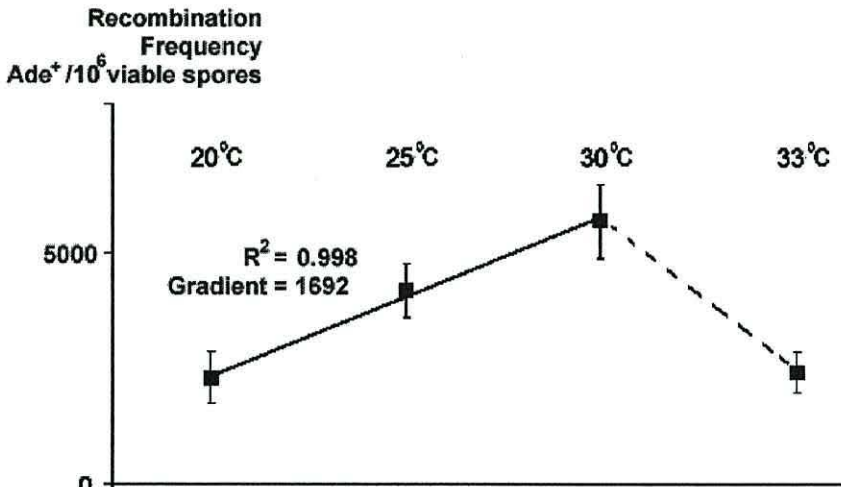


Figure 4.1 At mating temperatures >20°C *ade6-M26* hotspot activation is significantly suppressed in two different *rec10*$^+$ mutants.
A. In the *rec20-144* mutant background hotspot activity is significantly suppressed when matings are performed at temperatures 25°C, 30°C and 33°C ($p<0.01$) but not at 20°C ($p=0.13$).
B. In a *rec10-155* mutant background, at both 25°C and 30°C, hotspot activity is significantly suppressed ($p<0.01$).
*n= number of independent repeats used to determine mean hotspot activities.
p values for each temperature were determined via Student's t-test comparison of all *rec*$^+$ and *rec*$^-$ hotspot values generated from two experiments.
Figures in brackets and error bars are 1 standard deviation.
(See material and methods sections 2.2-2.3 for full explanations of hotspot value determinations and statistical analysis).

Figure 4.2
Mating temperature has a significant effect on *ade6-M26* intragenic recombination.



	Fold change in recombination			
ade6-M26	0	+ 1.8 (p<0.01)	+ 2.5 (p<0.01)	- 2.3 (p<0.01)
ade6-M375	---------- no significant increase or trend ----------			- 2.1 (p<0.01)

Figure 4.2 shows the effect of mating temperature on *ade6-M26* intragenic recombination

From 20°C to 30°C, each 5°C increase in mating temperature results in a significant increase in *ade6-M26* recombination but no significant change in *ade6-M375* recombination. An increase in mating temperature from 30°C-to-33°C, results in significant almost equivalent-fold reductions in both *ade6-M26* and *ade6-M375* recombination.

Fold changes in recombination are calculated from the mean recombination figures given in table 4.1. (e.g. from 20°C-30°C, *ade6-M26* recombination increases from 2320-5705 Ade^+ recombinants/10^6 viable spores = 2.5-fold increase. Fold-decrease in recombination is calculated from 30°C and 33°C mean recombination figures).

Significance p values were determined via Student's t-test with a paired comparison of at least 14 individual repeats from each mating temperature.

Error bars are derived from standard deviation figures given in table 4.1.

4.6 Discussion

In this chapter we have shown the *rec20-144* mutation creates a temperature sensitive defect in *ade6* intragenic recombination which causes a greater reduction in *ade6-M26* hotspot recombination than *ade6-M375* non-hotspot intragenic recombination (Table 4.1). As a result, when zygotic matings are performed at 25°C, 30°C and 33°C, the activity of the *ade6-M26* hotspot in *rec20-144* mutants is significant reduced relative to that of rec^+ strains (Figure 4.1. A). In addition, when heterothallic matings are performed at 25°C and 30°C using the insertionally inactivated $rec10^+$ mutant, *rec10-155* (Lin and Smith, 1995), a significant reduction in *ade6-M26* hotspot activity also occurs, with both the actual mean *rec10-155* hotspot values and the fraction of rec^+ hotspot activity attained, being very similar to those of the *rec20-144* mutant (Figure 4.1).

A statistical analysis of the recombination data presented in table 4.1 shows that, from 20°C to 30°C, each 5°C increase in mating temperature produces a significant increase in *ade6-M26* intragenic recombination but no significant change in *ade6-M375* intragenic recombination (Figure 4.2). This indicates the increase in hotspot activity, caused by raising the mating temperature, is due solely to the increase in *ade6-M26* intragenic recombination. This analysis also suggests that, from 20°C to 30°C, *M26* hotspot activity may be a composite process involving a "basic" activation of *ade6-M26* recombination together with a temperature-dependent stimulation over and above this basic activation.

From 20°C to 30°C, the *rec20-144* mutation causes significant reductions in both *ade6-M26* and *ade6-M375* recombination (Table 4.1). However the actual hotspot ratios obtained at 20°C, 25°C and 30°C in the *rec20-144* mutant show no significant change in their mean activities ($p>0.2$, Figure 4.1A). As we have shown in a rec^+ background the increase in hotspot activity, caused by raising the mating temperature, is due solely to the increase in *ade6-M26* hotspot recombination, the fact that hotspot activity ratios obtained at 20°C, 25°C and 30°C in *rec20-144* mutants do not significantly increase suggests that fully functional Rec10 may be required for a possible temperature-dependent element of *ade6-M26* hotspot activation. This trend does not continue to 33°C, as a slight

significant drop is observed ($p<0.01$), although this may be due to meiosis reaching a thermal limit.

4.7 Summary of main findings.

- The *rec20-144* mutation causes a temperature sensitive reduction in *ade6* intragenic recombination which at mating temperatures ≥25°C, is greater in *ade6-M26* strains than *ade6-M375* strains (Table 4.1).
- In a rec^+ strains, the *ade6-M26* allele undergoes a temperature-dependent stimulation of intragenic recombination which is solely responsible for a temperature-dependent stimulation of hotspot activity (Figures 4.1 and 4.2).
- In $rec10^+$ mutants, the *ade6-M26* hotspot does not appear to undergo thermal stimulation as at mating temperatures of 20°C, 25°C and 30°C (*rec20-144* mutants) or 25°C and 30°C (*rec10-155* mutants) the level of *ade6-M26* hotspot activation is relatively constant.

4.8 Conclusions

The effect of temperature on the level of intragenic recombination and hotspot activity in both rec^+ and $rec10^+$ mutants should be assayed in other *ade6* alleles that contain *M26* sequences.

Chapter 5

The *M26* sequence orientation and hotspot activity in *rec10*$^+$ mutants.

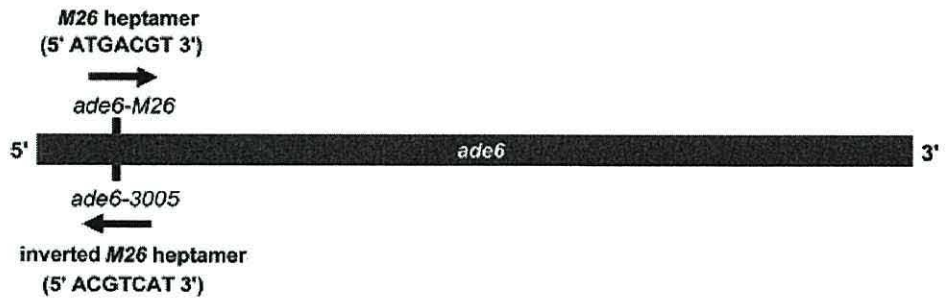
5.1 Introduction

In the prokaryote *Escherichia coli* homologous recombination occurs at elevated frequencies at or near a specific octameric 5′-GCTGGTGG-3′ sequences (Smith *et al.*, 1981) termed Chi sites (Henderson, 1975). An important feature of Chi, and Chi-like recombination hotspots, is their dependency on two factors namely the orientation of the Chi sequence, relative to the *E. coli* chromosome replication origin, *oriC*, and the presence of a heterotrimeric RecBCD enzyme complex (Cheng, 1987; Ponticelli, 1985; Taylor *et al.*, 1985).

To test whether the activity of a eukaryotic hotspot was also dependent upon the orientation of a hotspot sequence, Smith and co-workers created a number of *M26* heptameric sequences within the *S. pombe ade6* and *ura4* genes (Fox *et al.*, 1997). Analysis of the hotspot activities of these sequences demonstrated that the *M26* heptamer operates as a meiotic recombination hotspot in an orientation-independent manner (Fox *et al.*, 1997).

In chapter 4 we determined that full activation of the *ade6-M26* hotspot requires a function(s) of Rec10 that is lost in the *rec20-144* and *rec10-155* mutants. In this chapter we aim to establish if a function(s) of Rec10 determines the orientation-independent activation of an *M26* heptameric sequence. The *ade6-3005* allele contains an *M26* heptameric sequence that is inverted relative to the *M26* heptamer within *ade6-M26*, is fully active in a *rec*$^+$ background, and maps to the same seven base region within the *ade6* gene as the *ade6-M26* heptamer (Ponticelli *et al.*, 1988, Fox *et al.*, 1997; Figure 5.1). We therefore assayed the level of *ade6-3005* hotspot activity in *rec10*$^+$ mutants.

Figure 5.1
Location and relative orientation of the *M26* sequences within the *ade6* gene that create the *ade6-M26* and *ade6-3005* hotspot allele.



The *M26* heptamer sequence is shown in brackets as the 5'-3' sequence relative to transcription of the *ade6* open reading frame (transcription is left to right).
Control allele for the *ade6-3005* hotspot is the *ade6-3006* allele (Fox *et al.*, 1997)

5.2 Results

5.3 The *ade6-3005* hotspot is fully activated in *rec10*$^+$ mutants.

To determine the level of activation of the *ade6-3005* hotspot in *rec10*$^+$ mutants, a series of zygotic crosses of the *ade6-L52* test allele against the *ade6-3005* hotspot and *ade6-3006* non-hotspot alleles (Fox *et al.*, 1997) were performed at 20°C, 25°C, 30°C and 33°C in *rec20-144* mutants and at 25°C and 30°C in *rec10-155* (Lin and Smith, 1995) mutants. Random spore analysis of the progeny of these crosses showed that, at 20°C, the *rec20-144* mutation causes an almost equivalent, approximately 3-4-fold reduction in both *ade6-3005* and *ade6-3006* intragenic recombination and hence does not significantly alter the level of *ade6-3005* hotspot activation (Table 5.1, Figure 5.2). This result is similar to that observed in the analysis of *ade6-M26* hotspot activity at 20°C in *rec20-144* mutants strains (Figure 4.1A). At mating temperatures of ≥25°C the *rec20-144* mutation further reduces the level of intragenic recombination of both the *ade6-3005* hotspot and *ade6-3006* none hotspot control alleles indicating that the *rec20-144* mutation causes a temperature sensitive defect in *ade6-3005* and *ade6-3006* as well as *ade6-M26* and *ade6-M375* intragenic recombination (Chapter 4, Table 5.1). However, in contrast to the differential effect mutation of the *rec10*$^+$ gene has on intragenic recombination levels of the *ade6-M26* hotspot and *ade6-M375* none hotspot control alleles (Table 4.1), there is no significant difference between the levels of reduction of intragenic recombination of the *ade6-3005* hotspot or

ade6-3006 none hotspot control alleles (Table 5.1 and 5.2). As a direct result, there is no significant reduction in *ade6-3005* hotspot activity in *rec20-144* or *rec10-155* mutants (Figure 5.2). It should be noted that in *rec20-144* mutants a slight, but significant, reduction in hotspot activity is noted at 30°C when strains are grown prior to meiosis in YE growth media containing no supplementary adenine. Interestingly, this low adenine concentration YE media has no significant effect on the levels of *ade6-3005* or *ade6-3006* intragenic recombination in *rec10*$^{+}$ strains. The apparent adenine-dependent alteration in the requirement for Rec10 for full hotspot activation at 30°C is due to a significant increase in *ade6-3005* and decrease in *ade6-3006* intragenic recombination. In *rec20-144* mutants, changing the adenine concentration prior to meiosis has no significant effect on *ade6-M26* or *ade6-M375* intragenic recombination. These data suggests that, at a mating temperature of 30°C, activation of the *ade6-3005* hotspot may be dependent upon Rec10 function under certain nutritional states.

In the determination of hotspot activity in the *rec10-155* mutant, a wide range of values were obtained (Figure 5.2). Results from other experiments indicate that the determination of hotspot activation in the *rec10-155* mutant may be less reliable than in the *rec20-144* mutant (see results chapter 6). Hence, although the *ade6-3005* hotspot appears to undergo full activation in the *rec10-155* mutant this observation should be treated with some caution.

In summary, the function(s) of Rec10 which is lost in *rec20-144* mutant that is required for *ade6-M26* hotspot activation at mating temperatures ≥25°C is not required for activation of the *ade6-3005* hotspot under adenine rich conditions at any tested mating temperature.

Table 5.1
The effect of mating temperature on *ade6-3005* and *ade6-3006* intragenic recombination in *rec*$^+$ and *rec20-144* strains.

Temperature °C	*ade6* allele	Mean recombination frequency Ade$^+$ /10^6 viable spores[a]				Fold reduction in recombination[b]
		rec$^+$		*rec20-144*		
20	*3005*	4663 (1291)	n=7	1222 (661)	n=5	3.8
	3006	334 (119)	n=7	114 (33)	n=5	2.9
25	*3005*	6508 (1675)	n=12	1108 (264)	n=9	5.9
	3006	462 (65)	n=13	78 (37)	n=8	5.9
30	*3005*	7625 (2157)	n=13	338 (139)	n=12	22.6(18.2)*
	3006	412 (123)	n=12	30 (8)	n=11	13.7(24.5)*
33	*3005*	5276 (1258)	n=8	238 (60)	n=8	22.2
	3006	286 (85)	n=8	16 (7)	n=8	18.0

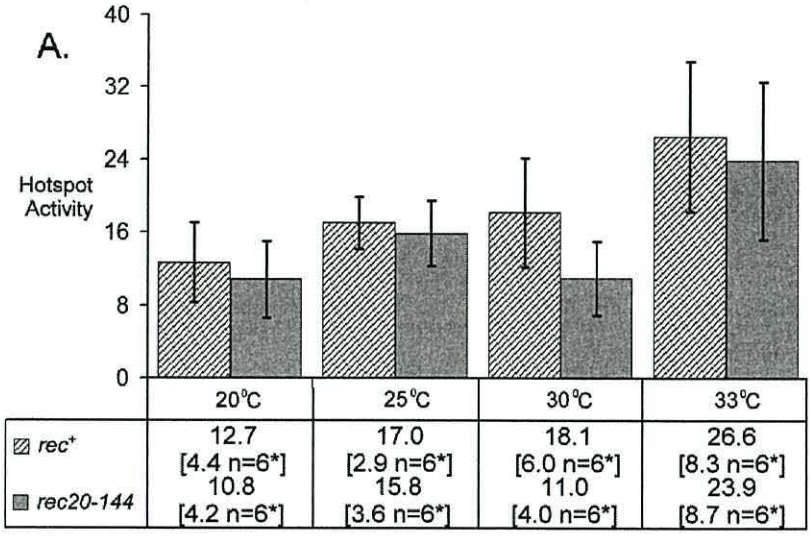
Table 4.1 shows the results of random spore analysis obtained from two-factor crosses using the *ade6-3005* and *ade6-3006* alleles against the *ade6-L52* marker allele in both a *rec*$^+$ and *rec20-144* backgrounds.
Figures in parentheses are 1 standard deviation.
[a] See section 2.2 for protocol used to determine Ade$^+$ recombinants/viable spore.
[b] The *rec20-144*-dependent reduction in recombination is derived by dividing mean *rec*$^+$ by mean *rec20-144* Ade$^+$/10^6 viable spore values. Reductions in recombination were highly significant at all temperatures with p values of < 0.01 in all cases derived via Student's t-test.
*Figures in brackets are the fold-reductions in recombination with addition of 250mg/l adenine to pre-meiosis YE growth media. The fold-changes are significant in both *ade6-3005* (increased p<0.01 n=3) and *ade6-3006* (reduced p<0.01 n=3) crosses.

Table 5.2
The effect of mating temperature on *ade6-3005* and *ade6-3006* intragenic recombination in *rec*$^+$ and *rec10-155* strains.

Temperature °C	*ade6* allele	Mean recombination frequency Ade$^+$ /10^6 viable spores[a]				Fold reduction in recombination[b]
		rec$^+$		*rec10-155*		
25	*3005*	6305 (1280)	n=5	15.6 (5)	n=6	403
	3006	349 (136)	n=5	1.1 (0.4)	n=6	313
30	*3005*	5633 (2034)	n=3	16.0 (5)	n=3	351
	3006	383 (72)	n=3	1.0 (0.4)	n=3	397

Table 5.2 shows the results of random spore analysis obtained from two-factor crosses using the *ade6-3005* and *ade6-3006* alleles against an *ade6-L52* marker allele in both a *rec*$^+$ and *rec10-155* backgrounds.
Figures in parentheses are 1 standard deviation.
[a] See section 2.2 for protocol used to determine Ade$^+$ recombinants/viable spore.
[b] The *rec20-144*-dependent reduction in recombination is derived by dividing mean *rec*$^+$ by mean *rec20-144* Ade$^+$/10^6 viable spore values. Recombination reductions were highly significant at all temperatures p values of < 0.01 in all cases derived via Student's t-test.

Figure 5.2
Rec10 is not required for full activity of the *ade6-3005* hotspot.



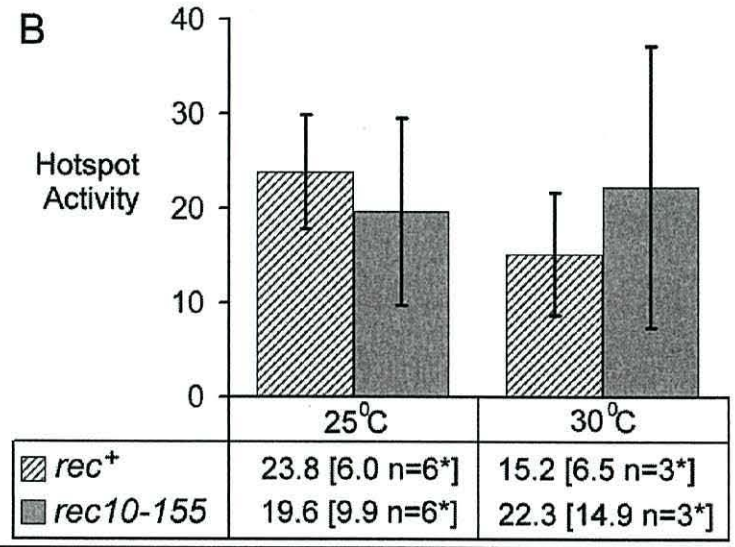


Figure 5.2 At mating temperatures of 20°C, 25°C, 30°C and 33°C *ade6-3005* hotspot activation is not significantly altered in the *rec10*$^+$ mutants, *rec20-144* and *rec10-155*.
A. In the *rec20-144* mutant background hotspot activity is not significantly altered when matings are performed at temperatures 20°C, 25°C, and 33°C ($p>0.05$).
Although, at 30°C, a significant reduction is hotspot activity is noted ($p<0.01$). This phenotype can be suppressed by growing *rec20-144* strains in YE growth media supplemented with 250mg/l adenine prior to mating (See results 5.3 for details).
B. In a *rec10-155* mutant background hotspot activity at 25°C and 30°C is not significantly suppressed ($p>0.05$). However, it should be noted that *ade6-3005* hotspot activity determined in *rec10-155* mutants gives a wide hotspot activity range.
*n= number of independent repeats used to determine mean hotspot activities.
p values for each temperature were determined via Student's t-test comparison of all *rec*$^+$ and *rec*$^-$ hotspot values generated in one or two independent experiments.
Figures in brackets and error bars are 1 standard deviation. (See material and methods sections 2.2-2.3 for full explanations of hotspot value) determinations and statistical analysis)

5.4 The *ade6-3005* hotspot is Atf1-dependent.

Prior to this study, it was unknown whether the inverted *M26* heptamer sequence in the *ade6-3005* allele requires Atf1•Pcr1 for its activation. A possibility therefore remained that the *ade6-3005* hotspot did not require Rec10 for full activation as it was activated by a different pathway from the *atf1*-dependent *ade6-M26* hotspot (Kon *et al.*, 1997; Mizuno *et al.*, 2001). To address this, the activity of the *ade6-3005* hotspot was compared in *atf1*$^{+}$ and *atf1*Δ mutants. Random spore analysis was performed on matings of the *ade6-L52* allele, against the *ade6-3005* hotspot and *ade6-3006* none-hotspot alleles, in both *atf1*$^{+}$ and *atf1*Δ strains. This analysis showed the *atf1*Δ mutation had no significant effect on *ade6-3006* non-hotspot intragenic recombination, whereas *ade6-3005* hotspot recombination was reduced to the level achieved by the *ade6-3006* non-hotspot allele. As a result, the *ade6-3005* hotspot shows complete inactivation in an *atf1*Δ background (Figure 5.3). This is identical to the level of inactivation of the *ade6-M26* hotspot in *atf1*Δ strains (Kon *et al.*, 1997).

Figure 5.3
The *ade6-3005* hotspot is Atf1-dependent.

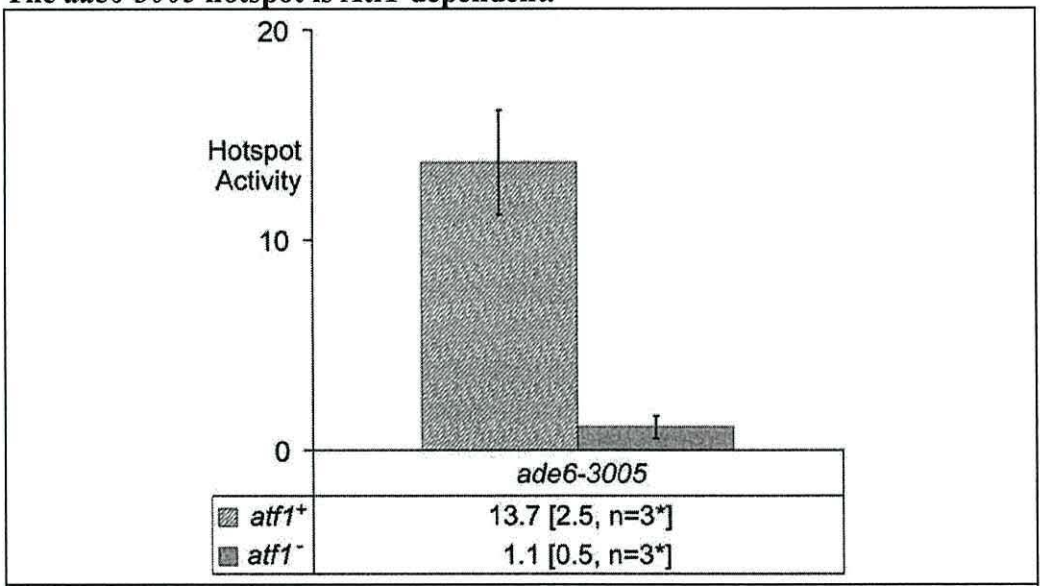


Figure 5.3 The *ade6-3005* hotspot requires Atf1 for hotspot activation.
At a mating temperature of 20°C, in *atf1*Δ mutants, there is no significant *ade6-3005* hotspot activity.
*n= number of independent repeats used to determine mean hotspot activities.
Figures in brackets and error bars are 1 standard deviation.
See material and methods sections 2.2-2.3 for full explanations of hotspot value determinations and statistical analysis.
Note: A value of 1 indicates no hotspot activity

5.5 The activity of the *ade6-M26* and *ade6-3005* hotspots are equally reduced in a sister chromatid cohesion mutant.

The *S. pombe* *rec11*$^+$ gene codes for a meiotic specific cohesin required for sister chromatid cohesion (Kitajima *et al.*, 2003b; Li *et al.*, 1997). In the *rec11*$^+$ mutant, *rec11-156* (Li *et al.*, 1997), sister chromatid cohesion along chromosome arms is severally impaired (Kitajima *et al.*, 2003b; Molnar *et al.*, 2003). A preliminary analysis of *ade6-M26* hotspot activity in the *rec11*$^+$ mutant, *rec11-111* (Ponticelli and Smith, 1989) shows that the level of hotspot activation achieved is similar to that of the *rec10*$^+$ mutants, *rec20-144* and *rec10-155* (Lin and Smith, 1997), (Figure 4.1). To determine if Rec11 is required for full levels of *ade6-3005* activity, a series of zygotic crosses were performed at 30°C, in both homozygous *rec*$^+$ and *rec11-156* backgrounds, between the *ade6-L52* marker allele and the *ade6-3005* hotspot and *ade6-3006* non hotspot control alleles. Random spore analysis of the number of Ade$^+$ recombinants obtained from these experiments showed that in homozygous *rec11-156* crosses, the hotspot activity of *ade6-3005* strains was significantly reduced; furthermore, the level of reduction was almost identical to that recorded for the *ade6-M26* hotspot (Figure 5.5). This result shows that Rec11 is required for full activation of both the *ade6-M26* and *ade6-3005* hotspot and a reduction or loss of sister chromatid cohesion equally affects the level of *ade6-M26* and *ade6-3005* hotspot activity. It should however be noted that the *rec11-156* allele is likely to be a null *rec11*$^+$ mutation; whereas *rec20-144* and *rec10-155* are not null alleles of *rec10*$^+$.

Figure 5.4
The *ade6-M26* and *ade6-3005* hotspots both require Rec11 for full activation.

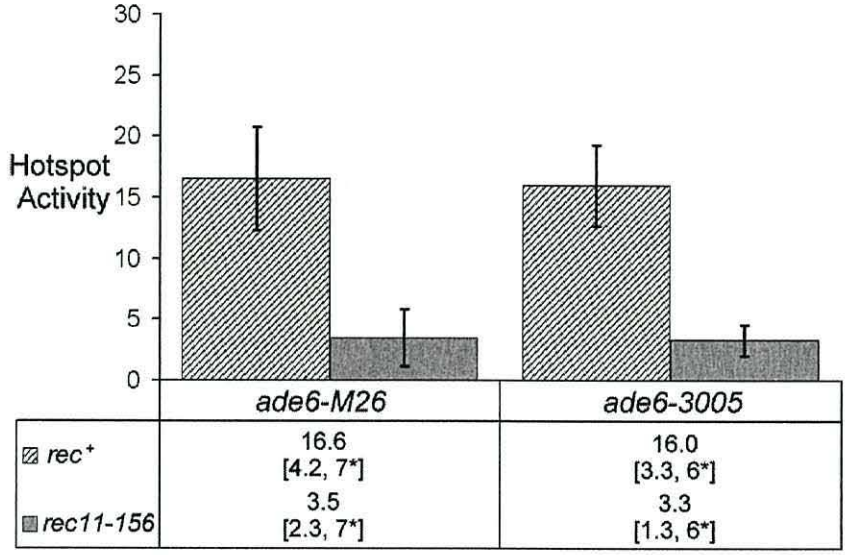


Figure 5.4
The *rec11-156* mutation causes a significant reduction in both *ade6-3005* and *ade6-M26* hotspot activity ($p < 0.01$ via Student's t-test).
There is no significant difference ($p > 0.5$) between *ade6-3005* and *ade6-M26* hotspot activity in either *rec*$^+$ or *rec11-156* strains (Li *et al.*, 1997).

Recombination data is given below

ade6 allele	Recombination frequency Ade$^+$ /10^6 viable spores				Fold reduction in recombination[a]
	rec$^+$		*rec11-156*		
3005	7039 (454)	n=6	11.4 (3.0)	n=6	617
3006	453 (84)	n=6	3.7 (1.3)	n=6	214
M26	5104 (990)	n=7	7.6 (2.6)	n=7	675
M375	317 (57)	n=7	2.8 (1.2)	n=7	115

Recombination figures were derived from random spore analysis obtained from two-factor meiotic recombination assays performed at 30°C using the *ade6-3005*, *ade6-3006*, *ade6-M26* and *ade6-M375* alleles against the *ade6-L52* marker allele in both *rec*$^+$ and *rec11-156* mutant backgrounds.
Figures in parentheses are 1 standard deviation.
[a] Reduction in recombination is derived by dividing mean *rec*$^+$ Ade$^+$ values by mean *rec11-156* Ade$^+$ values. Reductions in recombination are highly significant with ($p <$ 0.01 in all cases), derived via Student's t-test.
Note: Supplementary adenine was added to pre-meiotic growth media in all cases.

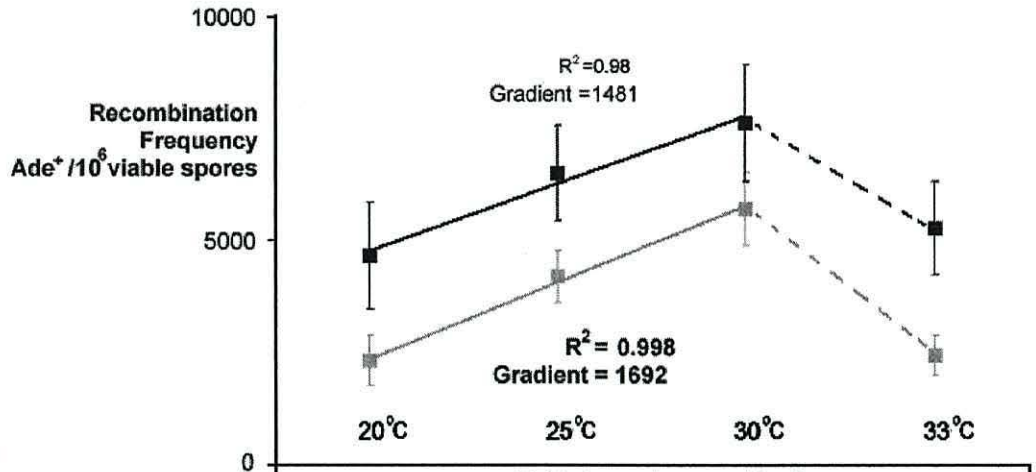
5.6 The *ade6-3005* hotspot undergoes a temperature-dependent stimulation of recombination.

In our study of *ade6-M26* intragenic recombination a temperature dependent-stimulation of intragenic recombination was noted (Chapter 4). An identical analysis of *rec*$^+$ *ade6-3005* intragenic recombination also reveals the *ade6-3005* allele undergoes a temperature-dependent increase in intragenic recombination (Table 5.1, Figure 5.5). A calculation of the number Ade$^+$ spores/10^6 viable spores produced at 20°C and 25°C shows that this 5°C increase in mating temperature results in a significant 1.4-fold increase in the number of Ade$^+$ recombinant spores produced (Figure 5.5). A similar calculation of the number Ade$^+$ spores/10^6 viable spores produced at 25°C and 30°C shows that this 5°C increase in mating temperature results in a further 1.1-fold, increase in the number of Ade$^+$/10^6 viable spores produced. Although this increase in intragenic recombination is not significant, the total increase from the level attained at 20°C is 1.6-fold and is highly significant (Figure 5.5). An analysis between 20°C and 30°C of the level of *ade6-3006* non-hotspot shows no similar significant increase in intragenic recombination (Table 5.1, figure and 5.5). A further 3°C increase in mating temperature, from 30°C to 33°C, results in significant 2.3-fold and 1.4-fold decreases in *ade6-M26* and *ade6-3005* intragenic recombination respectively (Figure 5.5). As almost equivalent fold-drops are also observed in the levels of intragenic recombination of the respective *ade6-M375* and *ade6-3006* non-hotspot control alleles, again we attribute this to the possibility that the meiotic recombination process may be close to a thermal limit.

The recombination stimulation gradients calculated for the *ade6-M26* and *ade6-3005* alleles define the increase in Ade$^+$ recombinant spore production per one degree increase in mating temperature. Figure 5.5 shows from 20°C to 30°C the stimulation of intragenic recombination of the *ade6-M26* allele is greater than that of the *ade6-3005* allele, indicating that mating temperature has a potentially greater stimulation on *ade6-M26* than *ade6-3005* hotspot activity. A further observation is that the *ade6-3005* allele appears inherently more recombinogenic than the *ade6-M26* allele as the maximum level of *ade6-M26* intragenic recombination, recorded at a mating temperature of 30°C, is not significantly

different from the minimum value recorded for *ade6-3005*, attained at a mating temperature of 20°C (Tables 4.1 and 5.1). An analysis of meiotic specific DSB formation of the *ade6-M26* and *ade6-3005* alleles performed by Smith and co-workers, (2001), showed a strong correlation between the levels of DSBs formed and intragenic recombination and the level of DSB formation within *ade6-3005* is higher than that attained by *ade6-M26* allele. This analysis, performed at 33°C, appears to provide good supporting evidence that the *ade6-3005* allele is possibly more recombinogenic than *ade6-M26* due to the production of more recombination initiating DSBs.

Figure 5.5
Mating temperature has a significant effect on the levels of *ade6-3005* intragenic recombination.



	Fold change in recombination			
ade6-3005	0	+ 1.4 (p<0.01)	+ 1.6 (p<0.01)	- 1.4 (p<0.01)
ade6-M26	0	+ 1.8 (p<0.01)	+ 2.5 (p<0.01)	- 2.3 (p<0.01)

Increases in mating temperature from 20°C to 30°C, result in significant increases in *ade6-3005* (**Dark line**) and *ade6-M26* (**Grey line**) intragenic recombination.
Increases in intragenic recombination are indicated by the gradient of each line of best fit (calculated in Microsoft Excel).
The gradients indicate that *ade6-M26* allele undergoes greater stimulation that the *ade6-3005* allele, although at each specific temperature, mean *ade6-M26* recombination figures are significantly lower than those attained in *ade6-3005* recombination (Tables 4.1 and 5.1, $p<0.01$ in all cases).
The increase in mating temperature from 30°C to 33°C, results in significant reductions in both *ade6-3005* and *ade6-M26* recombination.
The fold-changes in recombination are calculated from the mean recombination figures given in tables 4.1 and 5.1.
Error bars are derived from standard deviation figures given in tables 4.1 and 5.1.
Significance p values were determined via Student's t-test with a paired comparison of at least 6 (*ade6-3005*) or 14 (*ade6-M26*) individual repeats from each mating temperature.

5.7 The *ade6-3005* hotspot allele contains both *M26* and CRE hotspot sequences.

The *ade6-3005* hotspot allele is one of several *M26* heptamer containing alleles created within the *ade6* and *ura4* genes (Fox *et al.*, 1997). The *ade6-3005* hotspot allele contains four point mutations within the *ade6* gene that result in the creation of an *M26* heptameric sequence which is in an opposing orientation relative to the *M26* heptamer in the *ade6-M26* allele (Gutz, 1971, Ponticelli *et al.*, 1988; Fox *et al.*, 1997). However, we have determined that in addition to an *M26* heptamer, these mutations also created a CRE hotspot sequence that is in the same orientation as the *M26* heptamer in the *ade6-M26* allele (Table 5.3). Subsequent work by Smith and co-workers has identified an octameric CRE binding consensus sequence can act as a meiotic recombination hotspot and shares a high degree of sequence homology with the *M26* heptamer (Fox *et al.*, 2000). The study of one CRE hotspot allele has shown that the CRE sequence appears to utilise the same activation pathway as the *ade6-M26* hotspot as it undergoes a meiotic-specific *ade6-M26*-like chromatin remodelling that requires Atf1 and Pcr1 and their associated MAP Kinase pathway activators (Fox *et al.*, 2000). In this study we have shown that the *ade6-3005* hotspot also requires *atf1* for its activation (Figure 5.3). Therefore, although it still remains a possibility that *M26* and CRE sequences may have a level of independent activity, these results suggest that both *M26* and CRE sequences are variations of a similar meiotic recombination hotspot family.

Table 5.3

The *ade6-3005* hotspot allele contains both *M26* and CRE hotspot sequences.

Allele	5′-3′ sequences	Description
ade6	TGGAGGACGT	Wild type sequence
ade6-M26	TGGATGACGT	5′- 3′ *M26* heptamer
ade6-3005	TTGACGTCAT	3′- 5′ *M26* heptamer and 5′- 3′ CRE sequence
CRE	NTGACGTC/A	CRE consensus (Fox *et al.*, 2000)

Comparison of base sequences of the *ade6-3005* and *ade6-M26* hotspot alleles.
Mutated bases which create each allele are depicted in red.
M26 heptamer sequences are highlighted in boxes
In the *ade6-3005* allele (Fox *et al.*, 1997), the point mutations create an additional CRE sequence in an inverted orientation to the *M26* heptamer. CRE sequence is highlighted in yellow.

5.8 Discussion

The aim of the work described in this chapter was to determine whether inverting the orientation of an *M26* heptamer could suppress the significant reduction in *ade6-M26* hotspot activation recorded in *rec20-144* and *rec10-155* mutants. The *ade6-3005* allele contains an *M26* heptamer that maps to the same seven base pair region within *ade6*, but is in an inverted orientation relative to the *M26* heptamer of the *ade6-M26* allele (Figure 5.1, Fox *et al.*, 1997). We therefore assayed the level of activation of the *ade6-3005* hotspot in the *rec10*$^{+}$ mutants, *rec20-144* and *rec10-155*. During this analysis we determined that Rec10 is not required for full activation of the *ade6-3005* hotspot (Figure 5.2). However, we also discovered two important features pertaining to *ade6-3005* hotspot activation and the *ade6-3005* hotspot sequence. Firstly, at a mating temperature of 30°C, the *ade6-3005* hotspot is fully activated in *rec20-144* mutants only if strains are grown in adenine rich media prior to meiosis (Figure 5.2). Secondly, the *ade6-3005* allele (Fox *et al.*, 1997) contains not one, but two, *M26*/CRE hotspot sequences (Fox *et al.*, 2000) and that these sequences are in opposing orientations (Table 5.3). These data are possibly important and informative observations.

In recent studies it has been shown that the nutritional state of a cell can influence the levels of meiotic hotspot recombination through the modulation of transcription factor activity (Abdullah and Borts, 2001). Also in higher eukaryotes the ATF2 transcription factor (the closest homologue of *S. pombe* Atf1) plays an important role in response to amino acid starvation via its interaction with promoters that contain an amino acid response element (AARE) (Bruhat *et al.*, 2000) and other transcription factors (Averous *et al.*, 2004). It is therefore possible that for full hotspot activation the *ade6-3005* hotspot has a conditional requirement for a function(s) of Rec10 due to modulation of transcription factor interactions (see final discussion section 8.4). We have demonstrated that in *rec10*$^{+}$ strains, the *ade6-3005* hotspot has an increased level of activation relative to the *ade6-M26* hotspot (Figure 5.5). As the sequences of the *ade6-M26* and *ade6-3005* alleles differ by four nucleotide alterations (Table 5.3; Fox *et al.*, 1997) the nucleotide contexts within which their *M26* heptamers are embedded are different. Therefore the nucleotide context within which the *ade6-3005 M26*

heptamer is embedded may also be influencing whether Rec10 is required for full *ade6-3005* hotspot activation.

In further experiments we demonstrated that both the *ade6-M26* and *ade6-3005* hotspots undergo a statistically indistinguishable partial deactivation in the sister chromatid cohesion mutant, *rec11-156* (Li *et al.*, 1997, figure 5.5) and total inactivation in *atf1*Δ mutants (Figures 5.3). A recent study has also shown that a CRE sequence hotspot that maps to the same location with the *ade6* gene as the *ade6-M26* and *ade6-3005* sequences, and is in the same orientation as the *M26* heptamer of the *ade6-M26* allele, also requires Atf1 for its activation (Fox *et al.*, 2000). This study and our data demonstrate that, regardless of their orientation, *M26*/CRE hotspots are fully inactivated in *atf1*Δ mutants and that the differential requirement for Rec10 in the activation of the *ade6-M26* and *ade6-3005* hotspots is not due to a unique *atf1*-independent method of *ade6-3005* hotspot activation.

In our analysis of *ade6-3005* hotspot activation we determined that raising the temperature at which matings were preformed from 20°C to 30°C significantly increased the level of *ade6-3005* hotspot intragenic recombination but had no significant effect on the level of the *ade6-3006* non-hotspot intragenic recombination (Figure 5.5). In *S. pombe*, potential post-translational modifications to Atf1 and or Pcr1 can alter their *in vivo* and *in vitro* binding affinities for *M26* and CRE target sequences (Kon *et al.*, 1998, Fox *et al.*, 2000 Mizuno *et al.*, 2001, Yamada *et al.*, 2004). In a study into global transcriptional responses to environmental stresses, Jones and co-workers demonstrated that Atf1 is required for stimulation of heat shock genes (Chen *et al.*, 2003). Hence, it is possible, that temperature stimulates Atf1 binding to *M26*/CRE consensus sequences. Furthermore, *pcr1*Δ strains are cold sensitive (Watanabe and Yamamoto, 1996), suggesting temperature may also positively regulate Pcr1-dependent binding and/or activation at its target sequences. However, although we have demonstrated that *atf1* is required for both *ade6-M26* and *ade6-3005* hotspot activation and that temperature directly affects the level of *ade6-M26* and *ade6-3005* hotspot activity, we have not formally demonstrated that this is directly due to an enhanced binding or activation of Atf1 and or Pcr1.

At 33°C, *ade6-3005* intragenic recombination is increased approximately 2.2-fold compared to *ade6-M26* recombination (Tables 4.1 and 5.1). During meiosis at 34°C, a Pcr1-dependent DSB increases in intensity by 2.6-fold in *ade6-3005* strains compared to *ade6-M26* strains (Steiner *et al.*, 2002). This indicates a correlation between an increase in a Pcr1-dependent hotspot-specific DSB, and an almost equivalent increase in recombination frequency. As a result a possible explanation for why the *ade6-M26* and *ade6-3005* alleles show a strong correlation between increases in mating temperature and increases in intragenic recombination (Figure 5.5) could be that temperature increases enhance formation of Atf1•Pcr1-specific DSBs.

Our data has shown that at each tested mating temperature, the *ade6-3005* hotspot allele displays greater recombinogenic ability than the *ade6-M26* allele (Figure 5.5, tables 4.1, 5.1). The disparity in recombinogenic ability is such that the highest *ade6-M26* and lowest *ade6-3005* mean intragenic recombination values, recorded at 30°C and 20°C respectively, are virtually identical ($p=0.08$, $n=7$). One possible explanation for this is the *ade6-3005* allele contains a CRE sequence that may have a higher "basic" affinity for complexes that contain Atf1/Pcr1. Such an affinity level for Atf1•Pcr1 may dispense with the function(s) which appears to be required to stimulate *ade6-M26* intragenic recombination and is lost in *rec20-144* mutants (see chapter 4 discussion). Furthermore, this could also explain why increases in temperature cause greater reductions in *ade6-M26* than *ade6-3005* intragenic recombination (Tables 4.1, 4.2, 5.1 and 5.2) as a potential function of Rec10 could be to stabilise/activate Atf1•Pcr1 interactions with hotspot sequences with lower Atf1•Pcr1 affinities.

Together the results so far presented in this study and the studies of other laboratories suggest that factors that may determine whether a function(s) of Rec10 is required to achieve full hotspot activation could include the orientation of an *M26* heptamer, the modulation of Atf1 transcription factor activity and/or the nucleotide context within which an *M26* heptamer is located. Furthermore, these factors may in turn be further influenced by the temperature at which matings are performed and the nutritional state of a cell prior to meiosis.

5.9 Summary of main findings

- A function(s) of Rec10 that is required for full activation of the *ade6-M26* hotspot is not required for full *ade6-3005* hotspot activity indicating that the *ade6-M26* and *ade6-3005* alleles have differential requirements for at least one trans activating factor.
- The *ade6-3005* hotspot allele does not contain an exact inverted *M26* heptamer but contains two CRE hotspot sequences which are present in opposing orientations (Table 5.3).
- The *ade6-M26* and *ade6-3005* hotspots are total inactivated in *atf1*Δ mutants (Figure 5.4) and display statistically indistinguishable partial deactivation in the sister chromatid cohesion mutant, *rec11-156* (Figure 5.5, Li *et al.*, 1997).
- In *rec10*$^{+}$ strains, the *ade6-M26* and *ade6-3005* alleles undergo a temperature-dependent stimulation of intragenic recombination and the *ade6-3005* allele is intrinsically more recombinogenic than *ade6-M26* (Figure 5.6).

5.10 Conclusions

The data presented in this chapter conclusively shows that full *ade6-3005* and *ade6-M26* hotspot activation has a differential requirement for a function(s) of Rec10. Although we have proposed that this differential requirement may be linked to enhanced binding/activity of Atf1 and or Pcr1, we cannot rule out that it is the orientation of the *M26* heptamer that determines whether some function(s) of Rec10 is required for full hotspot activation. To establish if the orientation an *M26* heptamer or the context within which is located determines whether a function(s) of Rec10 is required to achieve full hotspot activation, a further series of experiment using hotspot alleles with differing *M26* orientations and nucleotide contexts is required.

Chapter 6

The *M26* nucleotide context and hotspot activity in *rec10*$^{+}$ mutants.

6.1 Introduction

The higher order context in which an *M26* heptamer is embedded influences its hotspot activity. For example, a 3.0 or 4.9 kb fragment of DNA containing the whole *ade6-M26* allele has no hotspot activity on a plasmid and may or may not be active when moved to other locations within the *S. pombe* genome (Ponticelli and Smith, 1992; Virgin *et al.*, 1995). Further studies in *S. pombe* have highlighted that a meiotic-specific modulation of chromatin occurs at both *M26* and CRE hotspots (Mizuno *et al.*, 1997, Fox *et al.*, 2000). Similar studies in *S. cerevisiae* have demonstrated that, in many cases, remodelled chromatin structures correlate with the location of meiotic DSB and recombination hotspots (Ohta *et al.*, 1994, Wu and Lichten, 1994, Mizuno *et al.*, 1997, Ohta *et al.*, 1998). As a result, it has been proposed that an early event in the activation of certain meiotic recombination hotspots is the remodelling of local chromatin structure (reviewed in Petes, 2001).

A study performed prior to this work determined that the *M26* heptameric sequence forms a meiotic recombination hotspot independent of its orientation (Fox *et al.*, 1997). In the previous chapter, (Chapter 5), we demonstrated that the point mutations that created the *ade6-3005* hotspot allele also created a CRE hotspot sequence that is in opposing orientation to the *M26* heptamer. A CRE consensus sequence can act as a meiotic hotspot (Fox *et al.*, 2000). It is therefore possible that the other hotspots created by Fox and co workers (1997) also contain potential hotspot sequences in opposing orientations and therefore the conclusion that the *M26* heptamer is capable of forming a meiotic recombination hotspot independent of its orientation requires confirmation.

In this chapter we aim to confirm the *M26* heptamer is capable of forming an orientation-independent hotspot and establish whether the orientation of an *M26* heptamer or the context within which it is embedded determines if the function(s) lost in the *rec20-144* and *rec10-155* mutants is required for full activation of an *M26* hotspot.

Figure 6.1
Locations and designations of hotspot sequences within the *ade6* gene.

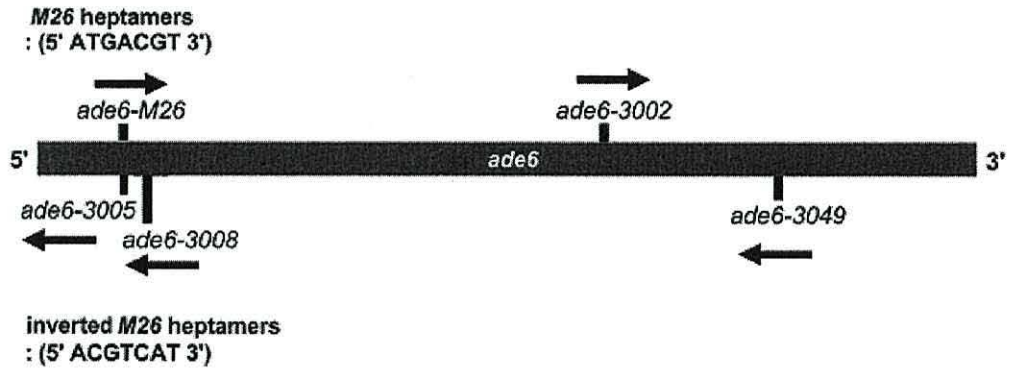


Figure 6.1 shows the locations and orientations of the *M26* heptamers within the *ade6* gene that constitute the *ade6* hotspot alleles created by Fox *et al.*, (1997).
Arrows indicate the direction of the *M26* heptamer relative to the direction of transcription of *ade6*

6.2 Results

6.3 An *M26* heptamer containing sequence can operate as an orientation-independent hotspot.

We analysed the sequences of all the *M26* heptamer containing hotspot alleles created by Fox and co workers (1997) to ascertain if they also contained additional CRE hotspot sequence(s). This analysis demonstrated that the *ade6-3049* hotspot allele contains a ten base pair region within which there are two overlapping hotspot sequences with opposing orientations. The *ade6-3008*, *ura4-167*, *ura4-169* and *ade6-3002* alleles however contain only a single *M26* or *M26*/CRE hotspot sequence (Table 6.1). As the *M26* heptamers of the *ade6-3008* and *ade6-M26* alleles are in opposing orientations (Fox *et al.*, 1997), this confirms that an *M26* heptamer is capable of creating an orientation-independent meiotic recombination hotspot.

6.4 The orientation of an *M26* heptamer does not determining whether the function(s) of Rec10 lost in the *rec20-144* mutant is required for full *M26* hotspot activation

The *ade6-3002* hotspot allele contains only a single *M26*/CRE hotspot sequence that is in the same orientation as the *M26* heptamer of the *ade6-M26* allele (Table 6.1). We assayed the level of *ade6-3002* hotspot activity in *rec20-144* and *rec10-155* mutants. These assays demonstrated that the *ade6-3002* hotspot is fully active in the *rec20-144* mutant (Figure 6.2), demonstrating that the orientation of an *M26* heptamer does not determine if the function(s) lost in the *rec20-144* mutant is required for full activation of an *M26* hotspot. However, a significant loss of *ade6-3002* hotspot activity was recorded in *rec10-155* mutants (see section 6.6).

Table 6.1
Sequence analysis of *S. pombe ade6* and *ura4* hotspot alleles

allele	5′- 3′ sequence	Description
	NTGACGT(C/A)	CRE consensus sequence, N= any base
ade$^+$	TGGAGGACGTG	Wild type sequence
ade6-M26	TGGATGACGTG	5′- 3′ heptamer no CRE consensus
ade$^+$	TGGAGGACGTG	Wild type sequence
ade6-3005[a]	TTGACGTCATG	3′- 5′ heptamer with CRE consensus plus additional 5′- 3′ CRE[b]
ade$^+$	CACGTTATT	Wild type sequence
ade6-3008	CACGTCATA	3′- 5′ heptamer no CRE consensus
ade$^+$	TATGTGGTC	Wild type sequence
ade6-3002	TATGACGTC	5′- 3′ heptamer with CRE consensus
ade$^+$	CTGCCGTCATT	Wild type sequence
ade6-3049	CTGACGTCATT	3′- 5′ heptamer with CRE consensus plus additional 5′- 3′ CRE[c]
ura4$^+$	TGGGACGTG	Wild type sequence
ura4-167	TATGACGTG	heptamer sequence no CRE consensus
ura4$^+$	GACGTGGTCTCTT	Wild type sequence
ura4-169	GACGTCATCTCTA	Inverted heptamer sequence no CRE consensus

Table 6.1 shows alignments of regions of wild type *ade6* coding DNA against the hotspot sequences of the *ade6-M26* and *ade6* alleles created by Fox *et al.* (1997).
Bases mutated from wild type sequence are depicted in red
Heptamers are outlined in boxes, CRE sequences are highlighted in yellow
The *ade6-M26* sequence is from Szankasi *et al.* (1988). All other hotspot sequences are from Fox *et al.*, (1997).
[a] The *ade6-3005* sequence was analysed in Chapter 5 (Table 5.6). It is presented here again to enable a complete comparison of all the hotspot alleles created by Fox *et al.* (1997) that have been analysed in this study.
[b]The palindromic 5′-TGACGTCA-3′ sequence contained with the *ade6-3005* and *ade6-3049* alleles is typical of the CRE consensus sequence of higher eukaryotes.

Figure 6.2
The activity of the *ade6-3008*, *ade6-3002*, *ade6-3049* and *ura4-167* hotspots in the *rec20-144* mutant

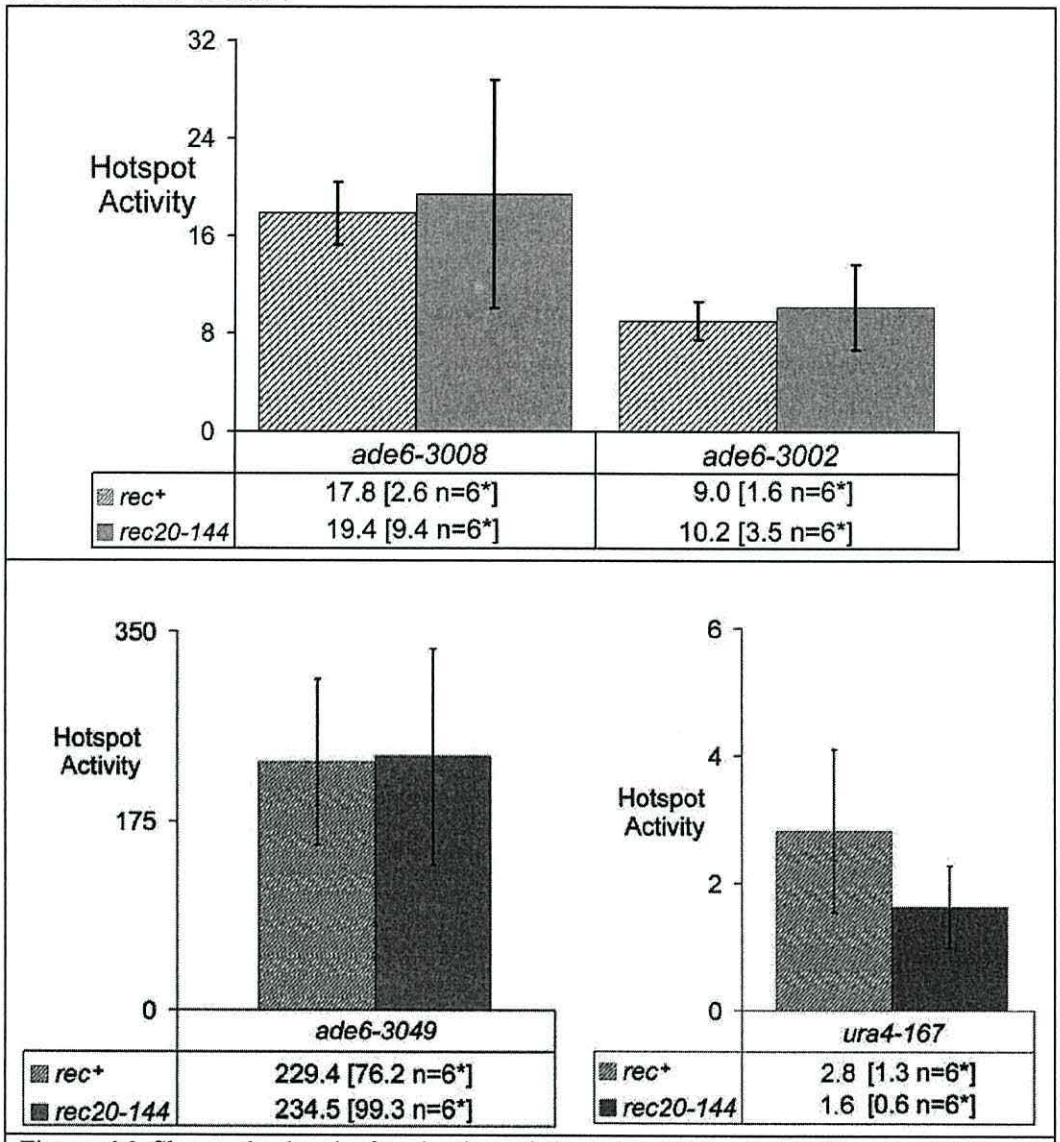


Figure 6.2 Shows the level of activation of the *ade6-3008* *ade6-3002*, *ade6-3049* and *ura4-167* hotspots determined at a mating temperature of 30°C.
In *rec20-144* mutants, the activity of *ura4-167* hotspot is significantly reduced ($p<0.01$) and the activity of the *ade6-3008*, *ade6-3002* and *ade6-3049* hotspots is not significantly different from that attained in rec^+ control strains ($p=0.46$, 0.05 and 0.86 respectively).
*n= number of independent repeats used to determine mean hotspot activities.
p values for each temperature were determined via Student's t-test comparison of all rec^+ and *rec20-144* hotspot values generated from a two experiments.
Figures in brackets and error bars are one standard deviation.
(See material and methods sections 2.2-2.3 for full explanations of hotspot value determinations and statistical analysis).

6.5 The sequence context of an *M26* heptamer may determine its level of activity in the *rec20-144* mutant

In addition to assaying the level of activation of the *ade6-3002* hotspot in *rec10*$^{+}$ mutants, we also analysed the *ade6-3008*, *ade6-3049*, *ura4-167* and *ura4-169* alleles (Fox *et al.*, 1997) for their level of activation in *rec10*$^{+}$ mutants. The level of *ade6-3008* and *ade6-3049* hotspot activations was assayed in *rec20-144* and *rec10-155* mutants, whilst the *ura4-167* and *ura4-169* hotspots were assayed in the *rec20-144* mutant alone. In these assays we determined that in *rec20-144* mutants, the *ade6-3008* and *ade6-3049* hotspots are fully activated (Figure 6.2). However, a significant loss of *ade6-3008* and *ade6-3049* hotspot activity was recorded in the *rec10-155* mutant (see section 6.6). In our assays of *ura4-169* and *ura4-167* hotspot activity, we could not detect any activation of the *ura4-169* hotspot in *rec10*$^{+}$ strains. This is in contrast to the data reported by Fox and co workers (1997). This may be due to the fact that the strains used in our assays also contained either an *ade6-M26* or *ade6-L52* allele and therefore were not isogenic to those used by Fox and co workers (1997). However, we did detect a low level of *ura4-167* hotspot activation and determined that in a *rec20-144* mutant background, the *ura4-167* hotspot is significantly reduced to only 57% of it's *rec10*$^{+}$ level of activation. Therefore, two *M26*-containing hotspots, namely the *ade6-M26* and *ura4-167* hotspots require a function(s) of Rec10 that is lost in the *rec20-144* mutant to achieve full activation.

6.6 Hotspot activation is lost in *rec10-155* mutants

As previously mentioned (see above) the *ade6-3002*, *ade6-3008* and *ade6-3049* hotspot are significantly reduced in activity in *rec10-155* mutants (Figure 6.3). The *ade6-3002* hotspot is reduced to 71.5% of the level of *rec10*$^{+}$ activity whilst the *ade6-3008* and *ade6-3049* hotspots are respectively reduced to 74% and 33% of the level of their *rec10*$^{+}$ activities (Figure 6.3). Therefore, to achieve full hotspot activation, it appears that these hotspot alleles require a function(s) of Rec10 that is lost in *rec10-155* mutants. Whether this is a true reflection of hotspot activity in *rec10-155* mutant is difficult to assess. The estimation of hotspot activation in *rec10-155* mutants may not be as reliable as in *rec20-144*

mutants. The *rec10-155* mutation reduces *ade6* intragenic recombination 10-100-fold more than does the *rec20-144* mutation. This means that the number of Ade^+ recombinant spores obtained from non-hotspot control crosses can often be very low, resulting in hotspot activation calculations that may be unreliable.

The determination of the levels of *ade6-3049* and *ade6-3009* intragenic recombination in *rec10-155* mutants did reveal an unexpected aspect of Rec10-dependent recombination. The decreases in *ade6-3049* and *ade6-3009* intragenic recombination in the *rec10-155* mutant are substantially less than those observed with the other *ade6* alleles investigated in this study (Tables 4.2, 5.2 and 6.4). As the mutations that create the *ade6-3049* and *ade6-3009* alleles are located downstream of all the other alleles employed in this study, this may suggest that Rec10 is less required for intragenic recombination towards the 3′ end of the *ade6* gene. Moreover, as this phenotype is not observed in the *rec20-144* mutant, this further indicates that *rec20-144* and *rec10-155* are hypomorphic.

Table 6.2
The effect of the *rec20-144* mutation on intragenic recombination at hotspot and hotspot control alleles of the *ade6* gene.

Temperature °C	*ade6* allele	Mean recombination frequency Ade$^+$ /10^6 viable spores[a]		Fold reduction in recombination[b]
		rec$^+$	*Rec20-144*	
30	*3008*	2459 (315) n=6	218 (41) n=6	11.3
	3010	140 (13) n=6	16 (11) n=6	8.8
	3002	3259 (815) n=6	211 (58) n=6	15.4
	3007	350 (64) n=6	21 (6) n=6	16.7
	3049	15550 (3486) n=6	1792 (494) n=6	8.7
	3009	74 (26) n=6	8 (3) n=6	9.3

Table 6.2 Shows the results of random spore analysis obtained from two-factor meiotic recombination assays carried out at a mating temperature of 30°C, employing the *ade6-3008*, *ade6-3002* and *ade6-3049* hotspot alleles and their respective *ade6-3010*, *ade6-3007* and *ade6-3009* control alleles in both a *rec*$^+$ and *rec20-144* mutant backgrounds. The *ade6-L52* marker allele was crossed against the *ade6-3008* and *ade6-3010* alleles. The *ade6-M375* marker allele was crossed against *ade6-3002*, *ade6-3007*, *ade6-3049* and *ade6-3009* alleles (Figures in parentheses are 1 standard deviation).
[a] See section 2.2 for protocol used to determine Ade$^+$ recombinants/viable spore.
[b] The *rec20-144*-dependent reduction in recombination is derived by dividing mean *rec*$^+$ Ade$^+$ values by mean *rec20-144* Ade$^+$ values. Reductions in recombination were highly significant with p values of < 0.01 in all cases derived via Student's t-test.

Table 6.3
The *ura4-167* heptamer and *ura4-168* control alleles

Temperature °C	*ura4* allele	Mean recombination frequency $Ura4^+ /10^6$ viable spores[a]		Fold reduction in recombination[b]
		rec$^+$	*Rec20-144*	
30	*167*	320 (171) n=6	9.2 (5.0) n=6	34.9
	168	123 (57) n=6	6.2 (3.4) n=6	19.7

Table 6.4 Shows the results of random spore analysis obtained from two-factor meiotic recombination assays carried out at a mating temperature of 30°C, employing the *ura4-294* marker allele in crosses against the *ura4-167* hotspot and *ura4-168* control alleles in both a *rec*$^+$ and *rec20-144* mutant backgrounds.
Figures in parentheses are 1 standard deviation.
[a] See section 2.2 for protocol used to determine $Ura4^+$ recombinants/viable spore.
[b] The *rec20-144*-dependent reduction in recombination is derived by dividing mean *rec*$^+$ $Ura4^+$ values by mean *rec20-144* $Ura4^+$ values. Reductions in recombination were highly significant with p values of < 0.01 in all cases derived via Student's t-test.

Table 6.4
The effect of *rec10-155* on intragenic recombination at hotspot and hotspot control alleles of the *ade6* gene.

Temperature °C	*ade6* allele	Mean recombination frequency Ade$^+$ /10^6 viable spores[a]		Fold reduction in recombination[b]
		rec$^+$	*Rec20-144*	
30	*3008*	1792 (128) n=3	4.1 (0.4) n=3	437
	3010	108 (92) n=3	0.4 (0.2) n=3	270
	3002	4223 (1508) n=6	12.6 (5.1) n=6	335
	3007	278 (52) n=6	1.4 (0.7) n=6	199
	3049	10987 (1388) n=3	229.7 (6.2) n=3	48
	3009	46 (10) n=3	3.7 (1.6) n=3	12

Table 6.3 Shows the results of random spore analysis obtained from two-factor meiotic recombination assays carried out at a mating temperature of 30°C, employing the *ade6-3008*, *ade6-3002* and *ade6-3049* hotspot alleles and their respective *ade6-3010*, *ade6-3007* and *ade6-3009* control alleles in both a *rec*$^+$ and *rec10-155* mutant backgrounds. The *ade6-L52* marker allele was crossed against the *ade6-3008* and *ade6-3010* alleles. The *ade6-M375* marker allele was crossed against *ade6-3002*, *ade6-3007*, *ade6-3049* and *ade6-3009* alleles.
(Figures in parentheses are 1 standard deviation).
[a] See section 2.2 for protocol used to determine Ade$^+$ recombinants/viable spore.
[b] The *rec10-155*-dependent reduction in recombination is derived by dividing mean *rec*$^+$ Ade$^+$ values by mean *rec20-144* Ade$^+$ values. Reductions in recombination were highly significant with p values of < 0.01 in all cases derived via Student's t-test.

Figure 6.3
The activity of the *ade6-3008*, *ade6-3002*, and *ade6-3049* hotspots in *rec10-155* mutant

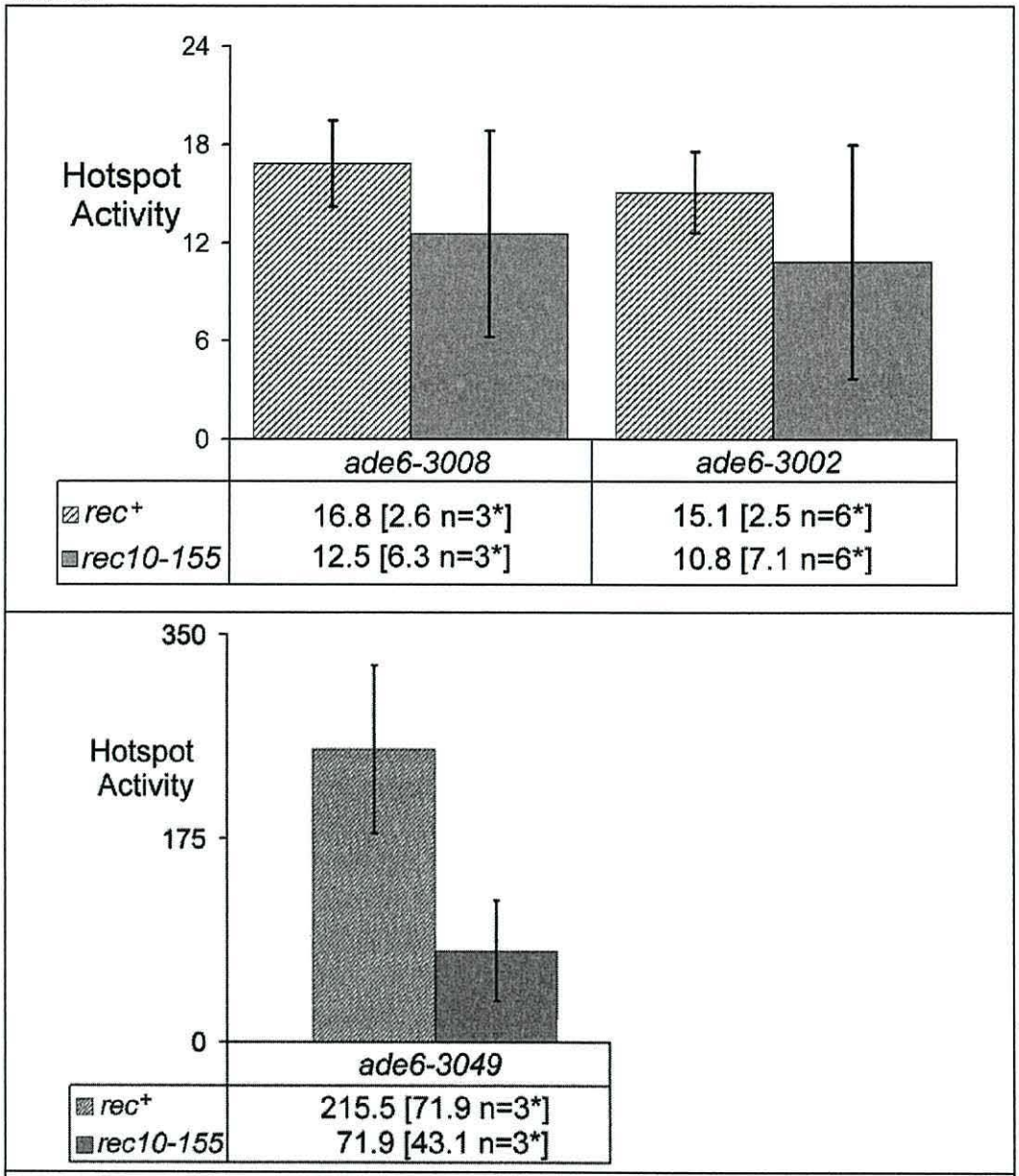


Figure 6.3 Shows the level of activation of the *ade6-3008 ade6-3002* and *ade6-3049* hotspots determined at a mating temperature of 30°C.
In *rec10-155* mutants, the mean activities of the *ade6-3008 ade6-3002* and *ade6-3049* hotspots are significantly reduced to 71%, 74%, and 33% of the level of that attained in *rec10*$^+$ control strains ($p<0.05$ in all cases).
*n= number of independent repeats used to determine mean hotspot activities.
p values for each temperature were determined via Student's t-test comparison of all *rec*$^+$ and *rec10-155* hotspot values generated from a two experiments.
Figures in brackets and error bars are one standard deviation.
(See material and methods sections 2.2-2.3 for full explanations of hotspot value determinations and statistical analysis).

6.7 Discussion

In the previous chapter (Chapter 5) we could not conclude whether the orientation of the *M26* heptamer determines if a function(s) of Rec10 is required for full hotspot activation. In this chapter we demonstrated that the orientation of an *M26* heptamer does not determine if a function(s) of Rec10 is required for full hotspot activation.

The hotspots analysed so far in this study vary in their dependency on whether the function(s) lost in either a *rec20-144* or *rec10-155* mutant is required for their full activation. The exact reason for this remains unclear. The hotspot sequences of the *ade6* and *ura4* alleles vary in the nucleotide context within which their *M26* heptamers are embedded, and in some instances with the *ade6* hotspots, their position within the *ade6* gene. As a result, either or both of these factors could affect if they require a function of Rec10 for full hotspot activation.

The *ade6-M26* and *ade6-3005* hotspot sequences are located within a 10 base pair region of the *ade6* gene (Figure 6.1, Fox *et al.*, (1997)) and yet have differential requirements for Rec10-mediated hotspot activation. In the *rec20-144* mutant, the *ade6-M26* hotspot does not achieve full activation (Chapter 4), whereas full activation of the *ade6-3005* hotspot can depend upon the nutritional state of cells prior to meiosis (Chapter 5). These data suggest that the requirement for Rec10 for full activation of an *M26* containing hotspot is not determined by the position of an *M26* containing hotspot sequence and is most likely influenced by the nucleotide context within which it is embedded. To further investigate this observation we utilised our sequence analysis (Table 6.1) to search for a feature that may determine why full activation of some but not all hotspot sequences require the function(s) of Rec10 lost in the *rec20-144* mutant.

The *ura4-167* and *ade6-M26* hotspot alleles contain a single *M26* sequence that does not conform to the CRE consensus (Table 6.1). Both these hotspots are significantly deactivated in the *rec20-144* mutant (Figure 6.2). Due to the presence of a C nucleotide at the 3′ flank of the *M26* heptamer, the *ade6-3002* hotspot allele conforms to the CRE consensus (Fox *et al.*, 2000, Table 6.1). The *ade6-3005* and *ade6-3049* hotspot alleles contain two merged hotspot sequences that individually conform to the CRE consensus described by Fox and co workers

(2000) and overall create an extended 5′- TGACGTCA- 3′ CRE consensus sequence typical of higher eukaryotes. The *ade6-3002*, *ade6-3005* and *ade6-3049* alleles are all fully active in the *rec20-144* mutant (Figure 6.2). These data indicate that the determining factor for whether or not an *M26*-containing hotspot can be fully activated in a *rec20-144* background may be the presence of a CRE consensus. The one hotspot allele that appears not to fit this model is the *ade6-3008* allele. The *ade6-3008* hotspot sequence does not correspond to the CRE consensus determined by Fox and co workers (2000) and so demonstrates that full activation of an *M26* containing hotspot sequence in the *rec20-144* mutant does not correlate with whether a hotspot sequence has a CRE consensus. However, the *ade6-3008* hotspot is located at a different position to the *ade6-M26* hotspot (Fox *et al.*, 1997; Szankasi *et al.*, 1988) and therefore must vary in the more expansive nucleotide context within which its *M26* heptamer is embedded. Therefore the *ade6-3008* hotspot result infers that any variation to the nucleotide context within which an *M26* heptamer is embedded may determine whether an *M26* containing hotspot requires the function(s) of Rec10 lost in the *rec20-144* mutant for full activation.

Although the position of a hotspot sequence within the *ade6* gene appears not to determine if full hotspot activation depends on a function(s) of Rec10, it may significantly affect its level of hotspot activity in *rec10*$^{+}$ strains. The *ade6-3005* and *ade6-3049* hotspots have strikingly different levels of activity (Figure 6.1). The extended hotspot sequences of the *ade6-3005* and *ade6-3049* alleles differ by a single nucleotide (Table 6.1). However, whilst the *ade6-3005* hotspot is near the 5′ end of *ade6*, the *ade6-3049* hotspot is closer to the 3′ end of *ade6* (Fox *et al.*, 1997).

The Rec11 cohesin is required for full hotspot activation of at least one *ade6* hotspot, namely *ade6-3005* (Chapter 5). The region covering the *ade6* gene has a high level of cohesin localisation (Lengronne *et al.*, 2004). Cohesins preferentially locate to regions between genes undergoing convergent transcription (Lengronne *et al.*, 2004). Between the 3′ ends of *ade6* and its neighbouring gene, C1322.14c, may be such a region (Wood *et al.*, 2002). It may be that the hotspot sequences towards the 3′ end of *ade6* are capable of greater

activity partially or wholly due to their proximity to sites of cohesion. Interestingly, in the *rec10-155* mutant, intragenic recombination appears to be less dependent upon Rec10 function towards the 3′ end of *ade6* (Table 6.4). This may also suggest that the requirement for Rec10 mediated intragenic recombination may be reduced at, or near too sites of high cohesin localisation.

6.8 Summary of main findings.

- The orientation of an *M26* heptamer does not determine if Rec10 is required for full activation of an *M26* containing hotspot.
- The *M26*/CRE sequences of the *S. pombe ade6* hotspot alleles can display significantly different levels of hotspot activity.
- The nucleotide context within which an *M26* heptamer is embedded and not its position within a gene most likely determines if the function(s) lost in the *rec20-144* mutant is required for full activation of an *M26*-containing hotspot.
- Intragenic recombination towards the 3′ end of the *ade6* gene is less dependent upon Rec10 function(s).
- The loss of full activation of *M26* containing hotspots in *rec10-155* mutants appears not be affected by position or possible proximity to sites of cohesin localisation.

6.9 Conclusions

In this chapter we proposed the nucleotide context within which an *M26* hotspot is embedded may affect whether the function(s) of Rec10 lost in the *rec20-144* mutant is required for full hotspot activation. We suggested nucleotides that have been shown to stimulate *ade6-M26* hotspot activity (Schuchert *et al.*, 1991) may suppress a requirement for Rec10 in full hotspot activation. If this is correct, a change to the nucleotide context of the *ade6-M26* hotspot allele that can stimulate hotspot activity should suppress a requirement for Rec10.

Chapter 7

The effect of a single nucleotide polymorphism on *ade6-M26* hotspot activity in *rec10*$^{+}$ mutants.

7.1 Introduction

In higher eukaryotes, members of the CREB transcription factor family recognise and bind to a palindromic 5′-TGACGTCA-3′ consensus sequence. In mammals the binding affinity and transcriptional stimulating activity of the CRE binding proteins, CREB1 and CREB2, are strongly effected by changes to nucleotides that immediately flank the known CRE consensus sequence (Benbrook and Jones, 1994).

The *S. pombe M26* and CRE sequences share a core 5′-TGACGT-3′ motif which requires the *S. pombe* CREB homologues, Atf1 and Pcr1 for activation as a meiotic recombination hotspot (Schuchert *et al.*, 1991, Kon *et al.*, 1997, Fox *et al.*, 2000). The hotspot activity of the *M26*/CRE sequences within *S. pombe* can vary in accordance with single nucleotide changes located immediately 5′ or 3′ of their core 5′-TGACGT-3′ sequence (Schuchert *et al.*, 1991, Fox *et al.*, 2000). For example, substitution of the 5′-**A** in the 5′-**A**TGACGT**G**-3′ sequence contained within the *ade6-M26* allele, either totally inactivates or substantially reduces hotspot activity. Furthermore, this effect can be reversed by alteration of the 3′ G to either an A or C (Fox *et al.*, 2000; Schuchert *et al.*, 1991). The 5′-N**T**GACGT(**C/A**)-3′ CRE hotspot sequence (Fox *et al.*, 2000) also displays a strong dependence on which nucleotide follows the core *M26*/CRE sequence. For example, the 5′-**G**TGACGT**T**-3′ sequence is not a hotspot, whereas the 5′-**G**TGACGT**G**-3′ sequence has approximately half the activity of the 5′-**G**TGACGT(**C/A**) -3′ sequences (Fox *et al.*, 2000; Schuchert *et al.*, 1991).

In previous chapters of this study we have shown that Rec10 is required for full activation of some but not all *M26*/CRE meiotic hotspots. Furthermore, the data obtained has indicated that the nature of the nucleotides that flank the *M26* 5′-ATGACGT-3′ sequence may be a factor which influences whether there is a requirement for some function(s) of Rec10 during hotspot activation. In the previous chapters this hypothesis could not be confirmed as the *M26*/CRE sequences contained in hotspot alleles studied so far vary in either chromosomal

locations or the extent of nucleotide substitutions that created them. The hotspot sequences of the *ade6-M26* and *ade6-M26-16C* alleles are located within the same 8 base pair region of *ade6* and differ only by a single G to C base substitution, which is located immediately 3′ to the *M26* heptamer (Schuchert *et al.*, 1991). A study into chromatin remodelling which takes place *in vivo* at *M26*/CRE heptamers has shown that the *ade6-M26* and *ade6-M26-16C* alleles have distinctly different meiotic-specific chromatin transitions (Mizuno *et al.*, 1997). In addition, utilising *in vitro* binding assays, Wahls and Smith (1994), demonstrated that the *ade6-M26-16C* hotspot sequence has an increased binding affinity for Atf1 and/or Pcr1 than does the *ade6-M26* sequence.

In this chapter we confirm that the function(s) of Rec10 which is lost in *rec20-144* mutants, and is required for activation of the *ade6-M26* hotspot, can be suppressed by a single nucleotide alteration to the context that 3′ flanks the *M26* heptamer of the *ade6-M26* hotspot allele.

7.2 Results

7.3 A single nucleotide polymorphism suppresses the function(s) of Rec10 required for full *ade6-M26* hotspot activation.

The hotspot sequences contained within the *ade6-M26-16C*, *ade6-M26* and *ade6-3005* alleles all map to the same location within the *ade6* gene (Schuchert *et al.*, 1991, Fox *et al.*, 1997). In previous experiments we had determined that the *ade6-3005* and *ade6-M26* hotspots have a differential requirement for the function(s) of Rec10 that enables full hotspot activation (Chapter 5). To allow a comparison of the level of *ade6-M26-16C* hotspot activation with these previous results, the same *ade6-L52* test allele was used in a series of zygotic crosses against the *ade6-M26-16C* and *ade6-M26* hotspot alleles and the *ade6-M375* non hotspot control allele. During this determination of *ade6-M26-16C* hotspot activity, only mating temperatures of 33°C and 30°C were employed in *rec20-144* mutants and 30°C in *rec10-155* mutants.

The mating temperature of 33°C was chosen for the *rec20-144* analysis because at this temperature *rec20-144* mutants exhibit the greatest reduction in *ade6* intragenic recombination (Tables 4.1 and 5.2) and loss of *ade6-M26* hotspot activation (Figure 4.1A), but still retain full *ade6-3005* hotspot activity (Figure 5.2). Matings were also performed at 30°C as this was the temperature at which we had obtained our maximum intragenic recombination level for the *ade6-M26* (Table 4.1).

In understanding the results presented in this chapter an important point is that we utilised the same *ade6-M375* non hotspot control recombination values for our determination of both *ade6-M26-16C* and *ade6-M26* hotspot ratios. Therefore, any difference between the activities of the *ade6-M26-16C* and *ade6-M26* hotspots is directly related to the level of *ade6-M26-16C* or *ade6-M26* intragenic recombination and independent of any fluctuations in non hotspot control recombination.

In *rec10*$^{+}$ strains, at a mating temperature of 30°C, there is no significant difference between the activity of the *ade6-M26* and *ade6-M26-16C* hotspots (Tables 7.1 and 7.2, Figure 7.1A). However, this parity in hotspot activity is not

repeated in *rec10*$^+$ mutants, as mean *ade6-M26-16C* hotspot activity is increased compared to mean *ade6-M26* hotspot activity by approximately 1.7-fold in *rec20-144* mutants and 1.5-fold in *rec10-155* mutants (Figure 7.1A, Table 7.2). The 1.5-fold increase in the activity of the *ade6-M26-16C* hotspot in *rec10-155* mutants is not significant, although this most likely reflects the low number of values used in the statistical analysis (Table 7.2). The 1.7-fold increase in *ade6-M26-16C* hotspot activity in *rec20-144* mutants is highly significant. Overall, *ade6-M26-16C* hotspot activity in *rec20-144* mutants is significantly reduced to approximately 60% of the *rec10*$^+$ value at 30°C and is not altered by growth of *rec20-144* mutants in adenine rich media prior to meiosis (Figure 7.1), as is the case for the *ade6-3005* hotspot (Chapter 5).

In *rec10*$^+$ matings performed at 33°C, the activity of the *ade6-M26-16C* hotspot is slightly reduced compared to that of the *ade6-M26* hotspot. However, in *rec20-144* mutants, the *ade6-M26-16C* hotspot retains 96% of its *rec10*$^+$ activity whilst the *ade6-M26* hotspot attains only 22% of its *rec10*$^+$ activity of (Figure 7.1B). Moreover, the level *ade6-M26-16C* intragenic recombination is 3.5-fold increased over the level of *ade6-M26* intragenic recombination (Table 7.1).

In summary, the results of mating performed at 30°C demonstrate that the *16C* single nucleotide polymorphism of the *ade6-M26-16C* allele has no significant effect on recombination in a *rec10*$^+$ background. However, in *rec20-144* mutants, it can significantly suppress the function(s) of Rec10 that is needed for full *ade6-M26* hotspot activation. The results of the mating performed at 33°C demonstrate that the *16C* SNP causes a slight but significant reduction in recombination in *rec10*$^+$ strains, but a total restoration of hotspot activation in *rec20-144* mutants.

Table 7.1.
The effect of *rec20-144* on *ade6-M26-16C* intragenic recombination.

Temperature °C	*ade6* allele	Mean recombination frequency Ade$^+$ /10^6 viable spores[a]				Fold reduction in recombination[b]
		rec$^+$		*rec20-144*		
30	*M26-16C*	5626 (710)	n=5	240 (38)	n=6	23.4
	M26	5732 (765)	n=6	142 (11)	n=6	40.4
	M375	317 (50)	n=6	23 (4)	n=6	13.8
33	*M26-16C*	3363 (770)	n=6	174 (50)	n=6	19.3
	M26	4111 (565)	n=6	49 (14)	n=6	83.9
	M375	215 (65)	n=6	12 (4)	n=6	17.9

Table 7.1 shows the results of random spore analysis obtained from two-factor crosses using the *ade6-M26-16C*, *ade6-M26* and *ade6-M375* alleles against an *ade6-L52* marker allele in both a *rec*$^+$ and *rec20-144* backgrounds.
In *rec*$^+$ strains at a mating temperature of 30°C there is no significantly difference between the mean level of *ade6-M26* and *ade6-M26-16C* recombination (p=0.41).
At a mating temperature of 33°C the mean level of *ade6-M26-16C* recombination is marginally significantly reduced compared to mean *ade6-M26* recombination (p=0.04).
In *rec20-144* mutants the mean level of *ade6-M26-16C* recombination is significantly higher than the mean level of and *ade6-M26* recombination at mating temperatures of both 30°C (1.7-fold, p<0.01) and 33°C (3.5-fold, p<0.01).
p values were derived via Student's t test from a pair wise comparison of recombination values from *ade6-M26* and *ade6-M26-16C* matings.
Figures in parentheses are 1 standard deviation.
[a] See section 2.2 for protocol used to determine Ade$^+$ recombinants/viable spore.
[b] The *rec20-144*-dependent reduction in recombination is derived by dividing mean *rec*$^+$ by mean *rec20-144* recombination values. Recombination reductions were highly significant at all temperatures p< 0.01 in all cases derived via Student's t-test.

Table 7.2.
The effect of *rec10-155* on *ade6-M26-16C* intragenic recombination.

Temperature °C	*ade6* allele	Mean recombination frequency Ade$^+$ /10^6 viable spores[a]				Fold reduction in recombination[b]
		rec$^+$		*rec10-155*		
30	*M26-16C*	5272 (901)	n=3	8.1 (2.3)	n=2	655
	M26	4730 (395)	n=3	5.6 (1.1)	n=3	850
	M375	216 (25)	n=3	0.5 (0.2)	n=3	411

Table 7.2 shows the results of random spore analysis obtained from two-factor crosses using the *ade6-M26-16C*, *ade6-M26* and *ade6-M375* alleles against an *ade6-L52* marker allele in both a *rec*$^+$ and *rec10-155* backgrounds.
Between *rec*$^+$ strains and *rec10-155* mutants there is no significant difference in the mean level of *ade6-M26* and *ade6-M26-16C* recombination (p=0.2, p=0.1 respectively)
Figures in parentheses are 1 standard deviation.
[a] See section 2.2 for protocol used to determine Ade$^+$ recombinants/viable spore.
[b] The *rec10-155*-dependent reduction in recombination is derived by dividing mean *rec*$^+$ by mean *rec20-144* recombination values. Recombination reductions were highly significant at all temperatures p < 0.01 in all cases derived via Student's t-test.

Figure 7.1
A single nucleotide polymorphism within the *ade6-M26-16C* hotspot allele suppresses a requirement for Rec10 in hotspot activation.

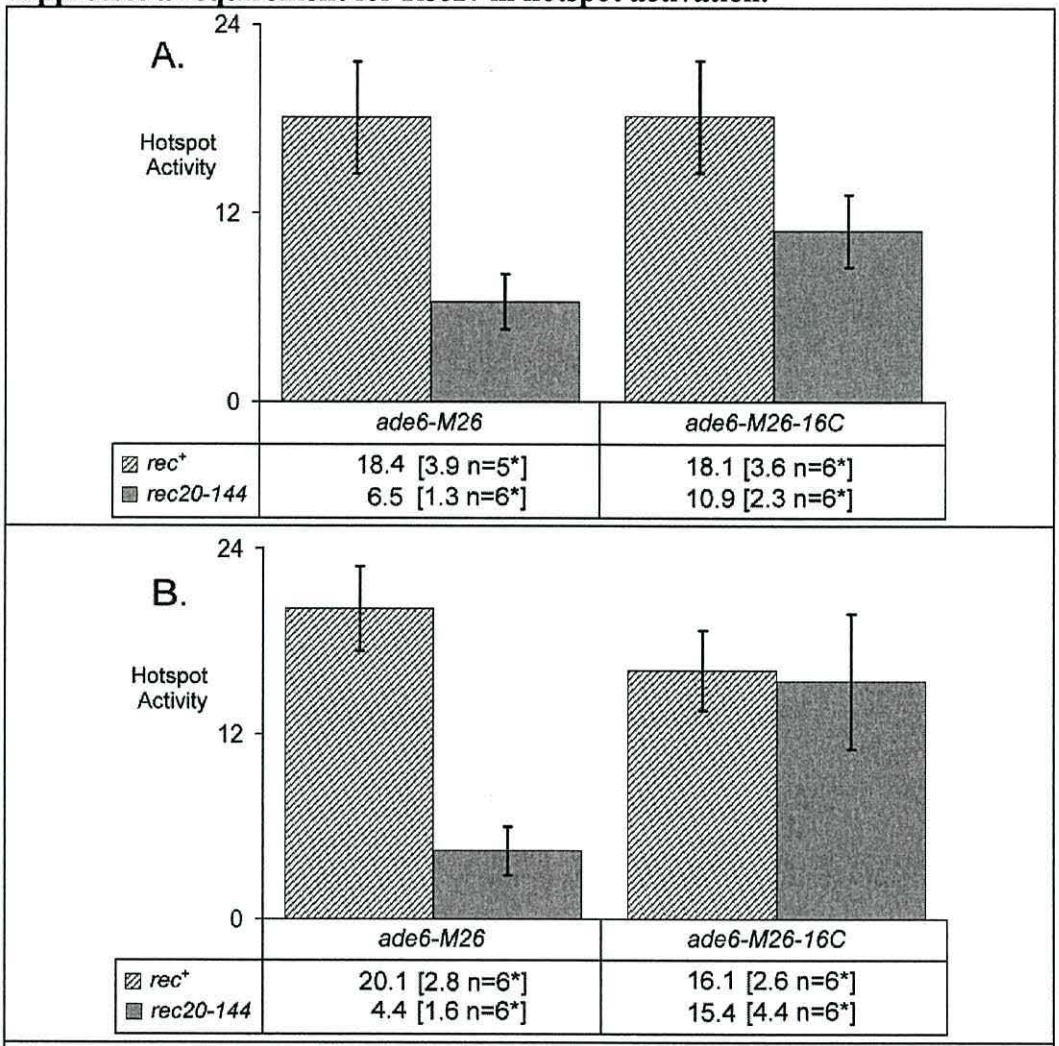


Figure 7.1 shows the level of activation of the *ade6-M26* and *ade6-M26-16C* hotspots in *rec*$^+$ strains and *rec20-144* mutants when matings are performed at temperatures of 30°C and 33°C.

A. At 30°C in *rec*$^+$ strains, the *ade6-M26* and *ade6-M26-16C* hotspot have no significant difference in hotspot activity ($p=0.41$).

In *rec20-144* mutants, a significant reduction ($p<0.01$) occurs in both *ade6-M26* and *ade6-M26-16C* hotspot activity. However, the level of *ade6-M26-16C* activity is 1.7-fold ($p>0.01$) higher than *ade6-M26* activity.

B. At 33°C in *rec*$^+$ strains, the *ade6-M26* and *ade6-M26-16C* hotspots have a slight, but significant difference in hotspot activity ($p=0.02$).

In *rec20-144* mutants a significant reduction takes place in *ade6-M26* ($p<0.01$) but not *ade6-M26-16C* hotspot activity ($p=0.23$).

*n= number of independent repeats used to determine mean hotspot activities.

p values for each temperature were determined via Student's t-test comparison of all *rec*$^+$ and *rec*$^-$ hotspot values generated from two independent experiments.

Figures in brackets and error bars are 1 standard deviation.

(See material and methods sections 2.2-2.3 for full explanations of hotspot value) determinations and statistical analysis)

7.4 Discussion

In previous chapters we demonstrated that the *ade6-M26* and *ura4-167* hotspots require a specific function(s) of Rec10 to achieve full hotspot activation, whereas four other *M26*-containing hotspots within *ade6* do not. In our analysis of these hotspots we noted a correlation between whether a function(s) of Rec10 is required for full hotspot activation and which nucleotides flank an *M26* heptamer. We noted that the hotspots that are fully active in the *rec20-144* mutant have an *M26* heptamer sequence with 5′ or 3′ flanking nucleotides that have been shown to increase *ade6-M26* hotspot activity and suggested that these *M26*/CRE sequences may have a level of interaction with Atf1 and/or Pcr1 that may compensate for the function(s) of Rec10 that is required for full activation of the *ade6-M26* and *ura4-167* hotspots. However, this model could not be confirmed because, of the hotspots we have so far studied, only the *ade6-M26* hotspot sequence has had the level of its Atf1•Pcr1 interaction assessed.

Using a reconstituted Atf1•Pcr1 heterodimer complex and a series of 215 bp regions of DNA, with homologies to various *ade6* alleles, it has been shown that the *ade6-M26-16C* hotspot sequence has an approximately 50% greater binding affinity for Atf1•Pcr1 *in vitro* than the *ade6-M26* hotspot sequence (Wahls and Smith, 1994). A recent study by Ohta and co workers has also demonstrated that during azygotic meiosis performed at 30°C, the pattern of nucleosome phasing within the *ade6-M26-16C* allele is more ordered in structure than it is within the *ade6-M26* allele (Mizuno *et al.*, 1997). As Ohta and co workers have also demonstrated that the Atf1 transcription factor is most likely directly involved in the remodelling of nucleosome phasing at *ade6-M26* (Yamada *et al.*, 2004), a possibility exists that the more ordered nucleosome phasing pattern of the *ade6-M26-16C* allele may be linked to the way in which its hotspot sequence interacts with Atf1•Pcr1.

In this chapter we demonstrated that in *rec10*$^{+}$ strains the *ade6-M26-16C* and *ade6-M26* hotspots have virtually identical levels of activity (Figure 7.1). Hence, in *rec10*$^{+}$ strains, a higher *in vitro* binding affinity for the Atf1•Pcr1 heterodimer and a more ordered chromatin configuration do not translate into

increased in hotspot activity *in vivo* when matings are performed at temperatures of 30°C or 33°C. However, in *rec20-144* mutants at a mating temperature of 30°C, the *ade6-M26-16C* hotspot is 60% more active than the *ade6-M26* hotspot (Figure 7.1). These data indicate that in *rec10*$^{+}$ mutants, a correlation between an increase in *in vitro* Atf1•Pcr1 binding capacity and *in vivo* hotspot activity does exist. We have not analysed the meiotic nucleosome configurations of the *ade6-M26-16C* or *ade6-M26* hotspots alleles in *rec20-144* mutants or assessed their levels of *in vivo* Atf1•Pcr1 interaction. However, the data presented in this chapter may suggest that in *rec20-144* and *rec10-155* mutants an enhanced Atf1•Pcr1 interaction and/or a more ordered chromatin configuration may suppress the function(s) of Rec10 that is required for full *ade6-M26* hotspot activation.

7.4 Summary of main findings.

- At mating temperatures of 30°C and 33°C, in *rec10*$^{+}$ strains, the *ade6-M26-16C* and *ade6-M26* hotspots are almost equivalent in their level of activation.
- At a mating temperature of 30°C, the *ade6-M26-16C* hotspot is significantly more active than the *ade6-M26* hotspot in the *rec20-144* mutant.
- At a mating temperature of 33°C, the *ade6-M26-16C* hotspot is fully active in the *rec20-144* mutant, whereas the *ade6-M26* hotspot is significantly reduced in its activation.

7.5 Conclusions

Compared to the *ade6-M26* allele, the *ade6-M26-16C* allele has a more ordered meiotic chromatin configuration and may have a potentially higher *in vivo* interaction with Atf1•Pcr1. In *rec20-144* mutants, experiments to analyse the meiotic nucleosome phasing configurations of the *ade6-M26-16C* or *ade6-M26* hotspots alleles and the level of Atf1•Pcr1 bound to the *ade6-M26* and *ade6-M26-16C* hotspots should be undertaken.

Chapter 8

Final discussion

8.1 Introduction

In this study we have made the following main findings. 1) The *S. pombe rec20-144* mutant is a temperature sensitive allele of the meiotic recombination gene *rec10*$^{+}$. 2) Rec10 has structural similarity to *S. cerevisiae* Red1, and weak homology to *C. elegans* XNP1 and Red1. 3) *M26*-containing hotspots can display significantly different levels of activation. 4) Factors that may contribute to the level of activation of an *M26*-containing hotspot include; the nucleotide context within which an *M26* heptamer is embedded, its position within a gene and the temperature at which matings are carried out. 5) The extent to which a function(s) of Rec10 is required for activation of an *M26*-containing hotspot also varies with the nucleotide context within which an *M26* heptamer is embedded and the temperature at which matings are carried out and can also be influenced by the nutritional state of cell prior to meiosis.

8.2 A speculative model for a function of Rec10 in meiotic recombination

In meiotic prophase chromosomes are structurally organised into loops of DNA attached to a proteinaceious chromosomal axis (reviewed in Zickler and Kleckner, 1997). In *S. cerevisiae*, meiotic DSB formation predominately takes place within these DNA loops and not at the chromosomal axis. However, many of the proteins involved in meiotic recombination are predominantly found at the chromosomal axis. A model to explain these observations proposes that during meiotic recombination DNA loops become tethered to the chromosomal axis by a protein complex called the "recombinasome" (Blat *et al.*, 2002). The recombinasome model implies that a degree of active structural deformation of DNA loops must occur, and presumably be stabilised long enough for DSB formation and processing to take place. How this might be achieved is unknown, although two of the proteins proposed to be required for this are Red1 and Hop1 (Blat *et al.*, 2002).

Red1 is required for the formation of axial elements (Bailis and Roeder, 1998; Rockmill and Roeder, 1990; Smith and Roeder, 1997), the precursors of the

lateral elements of the synaptonemal complex (SC) (reviewed in Zickler and Kleckner, 1998). The LEs of *S. pombe* are proposed to be evolutionary relics of *S. cerevisiae* lateral elements (Lorenz *et al.*, 2004). Rec10 is required for LE formation and is a structural component of LEs (Lorenz *et al.*, 2004; Molnar *et al.*, 2003). *S. cerevisiae* meiotic chromosome condensation also requires Red1 (Nag *et al.*, 1995). Rec10 has very weak domain homology to ATRX, a SNF2-like protein. SNF2 proteins can reposition histones (Whitehouse *et al.*, 1999). Rec10 could therefore be capable of stimulating chromosomal condensation and/or restructuring. In addition, both Red1 and Rec10 (Cervantes *et al.*, 2000) are required for full meiotic DSB formation. However, they are not required for recombination at all loci (Rockmill and Roeder, 1990; DeVeaux and Smith, 1994; Krawchuk *et al.*, 1999). Finally, cytological studies suggest *S. pombe* Hop1 locates to LEs in a manner reminiscent of that of *S. cerevisiae* Hop1 localisation to the SC (Lorenz *et al.*, 2004; Wan *et al.*, 2004). Therefore it appears *S. pombe* Rec10 and Hop1 and *S. cerevisiae* Red1 and Hop1 may be performing analogous functions (Lorenz *et al.*, 2004); whether these functions take place within a "recombinasome" complex in either *S. cerevisiae* or *S. pombe* remains to be proven. Finally, if activation of an *M26*-containing hotspot takes place within a recombinasome, the factors which influence the level of activation of an *M26*-containing hotspot in *rec10*$^{+}$ mutants (see section 8.1) must reflect the Rec10 function(s) that is required to fully activate this recombinasome.

8.3 Increased transcription factor activity may suppress the function(s) of Rec10 required for full activation of certain *M26*-containing hotspots

In *S. cerevisiae*, a chimeric protein consisting of the Spo11 endonuclease "fused" to a Gal4 transcription factor binding domain can confer meiotic-specific chromatin remodelling and DSB formation to loci that contain Gal4 binding motifs (Pecina *et al.*, 2002). This study demonstrates that a meiotic recombination initiation site can be created purely by targeting a "stable" endonuclease/transcription factor complex to a *cis*-sequence containing the transcription factors binding motif. Interestingly, in this analysis deletion of the

RED1 gene did not decrease the level of DSB formation at all loci (Figure 6 of Pecina *et al.*, (2002)).

At the *ade6-M26* hotspot, the Atf1 and Pcr1 transcription factors are required for remodelling the chromatin structure that facilitates *M26* hotspot activity and meiotic-specific DSB formation at *M26* (Mizuno *et al.*, 1997; Fox *et al.*, 2000; Mizuno *et al.*, 2001; Steiner *et al.*, 2003; Yamada *et al.*, 2004). The *ade6-M26-16C* hotspot also undergoes chromatin remodelling although at 30°C the resulting nucleosome phasing pattern significantly differs from that of the *ade6-M26* hotspot (Mizuno *et al.*, 1997). Interestingly, at 30°C there is no significant difference between the levels of *ade6-M26* or *ade6-M26-16C* hotspot activation (Chapter 7). Thus, although it appears there is no correlation between the pattern of nucleosome phasing and the absolute level of hotspot activity, it is possible that to reach full activation the *ade6-M26* and *ade6-M26-16C* hotspots must undergo substantially different chromatin modulations. If this is the case, the differential requirements at *ade6-M26* and *ade6-M26-16C* for a function(s) of Rec10 could intimately associated with the extent localised chromatin remodelling.

Wahls and co-workers (1997) noted that in heterozygous *atf1Δ/atf1*$^+$ and *pcr1Δ/pcr1*$^+$ crosses, hotspot activity was reduced respectively to 42% and 18% of that of homozygous *atf1*$^+$ and *pcr1*$^+$ crosses. This clearly indicates that a potential reduction in Atf1 or Pcr1 copy number can cause a reduction in hotspot activation. There is also evidence that the extent of Atf1•Pcr1 binding to *M26*-containing sequences may dictate their level of hotspot activity (Schuchert *et al.*, 1991; Wahls and Smith, 1994). In the *rec20-144* and *rec10-155* mutants, the *ade6-M26-16C* hotspot has a higher level of activation than the *ade6-M26* hotspot. This increase in activation correlates with a potential increase in Atf1 binding (Chapter 7, Wahls and Smith (1994)). Furthermore, increasing the mating temperature to 33°C totally suppresses the requirement for Rec10 in *ade6-M26-16C* hotspot activation (Chapter 7). Increased temperatures can also stimulate Atf1 activity (Chen *et al.*, 2003). We suggest it may be that increasing the hotspot-specific activity of Atf1 or Pcr1 can suppress a requirement for the function(s) of Rec10 in activation of certain *M26*-containing hotspots (Chapters 5 and 7). However, what

particular hotspot-specific function of Atf1 or Pcr1 would achieve this is as yet unknown. One possibility is as follows. In early meiotic prophase Rec10 locates to approximately 50 distinct foci which most likely mature into the more extensive LEs (Lorenz *et al.*, 2004). A possibility therefore exists that Rec10 and Atf1 could co-operate in the targeting and nucleation of LEs. If this were the case in the *rec20-144* mutant where LEs are disrupted but not fully absent, an *M26*-containing hotspot sequence with a high Atf1 interaction may be able to enhance the formation of LEs at its location. This would also mean that, the levels of LE function and hotspot activation may also be linked.

8.4 The modulation of specific DSB sites may determine the level of hotspot activation in the *rec20-144* mutant

In *atf1*Δ and *pcr1*Δ mutants, the frequency of intragenic recombination of an *M26*/CRE hotspot allele is reduced to that of its non-hotspot control allele, which remains unaffected by deletion of *atf1* or *pcr1* (Chapter 5, Kon *et al.*, 1997, Fox *et al.*, 2000). As the level of DSB formation of an *M26*/CRE hotspot allele correlates to its level of intragenic recombination (Steiner *et al.*, 2002), this indicates that *M26*/CRE hotspot activity relies on the formation and processing of one or more Atf1•Pcr-dependent DSBs and not on the stimulation of non Atf1•Pcr-dependent DSBs.

In an analysis of the *ade6-M26* and the *ade6* hotspot alleles created by Fox and co workers (1997), Steiner and co workers (2002) determined that the *ade6 M26*/CRE hotspots undergo meiotic-specific DSB formation at several prominent and in many cases common sites. However, one DSB site that occurs in the *ade6-M26* and *ade6-3005* alleles does not occur in any of the other *ade6* hotspot alleles (Steiner *et al.*, 2002). To achieve full hotspot activity the *ade6-M26* and *ade6-3005* hotspots are partially and conditionally dependent on the function(s) lost in the *rec20-144* mutant (Chapters 4 and 5). It is therefore possible that in a *rec20-144* mutant background the DSB unique to these hotspot alleles is specifically lost, conditionally reduced or perhaps incorrectly processed. This hypothesis can be tested by analysis of DSB formation in the *rec20-144* mutant (see section 8.5).

8.5 The implications of this study to hotspot recombination in higher eukaryotes

Atf1 and Pcr1 are homologues of the cAMP response element binding proteins (CREB) of higher eukaryotes (Takeda *et al.*, 1995; Watanabe and Yamamoto, 1996). The level of *M26*/CRE hotspot activity and the transcriptional control of CREB-dependent genes share many common regulatory processes. Many of the aspects of *M26*/CRE hotspot regulation (see section 8.1) are mirrored in the way CREB-dependent transcription is controlled in higher eukaryotes. For example, variations in the nucleotide context that surrounds a CRE sequence (Benbrook and Jones, 1994) and post-translational modification of CREB proteins (Johannessen *et al.*, 2004) can both alter the level of CREB-dependent transcription. Further similarities may also exist. Activation of CREB-dependent transcription requires that CREB proteins interact with specific co-activating proteins (Johannessen *et al.*, 2004). At least three *S. pombe* protein complexes are able to bind to *M26*/CRE sequence containing probes *in vitro* (Fox *et al.*, 2000; Jones and Jones, 1989; Wahls and Smith, 1994; Watanabe and Yamamoto, 1996). Formation of one of these Atf1•Pcr1 complexes may require an additional "activating" factor (Kon *et al.*, 1997; Wahls and Smith, 1994) whilst another complex may contain a protein other than Atf1 or Pcr1 (Watanabe and Yamamoto, 1996). CREB-dependent transcription can also be regulated by interaction with other transcription factors within close proximity and over relatively long distances. For example, CREB proteins can regulate transcription within the context of a cyclic AMP response unit (CRU), a region of DNA up to several hundred base pairs in length that contains several *cis*-acting transcription factor binding elements (Roesler, 2000) and within a short amino acid response element (AARE) (Averous *et al.*, 2004; Bruhat *et al.*, 2000). Kon and co workers (1998) employed an *in vivo* dimethyl sulfate footprint assay to determine that Atf1 binds to a region of approximately 35 base pairs that centres on the *M26* heptamer. In an analysis of DSB formation within the *ade6-M26* allele, Steiner and co workers (2004) proposed that Atf1 binding prevented DSB formation within an approximately 70 base pair region that contained the *M26* heptamer. Interestingly within the *ade6* gene, located 35 base pairs upstream of the end of

the *M26* Atf1 protected region is a nine base pair sequence that differs from the essential AARE sequence (Bruhat *et al.*, 2000) by a single nucleotide (Wood *et al.*, 2002). It may be that the approximately 70 base pair DSB free region observed by Steiner and co workers (2002) is due not only to Atf1 protection at the *M26* heptamer but also transcription factor binding at the potential AARE site. In the *ade6-M26* allele, the *M26* heptamer is located 179 bp from the end of the *ade6* promoter (Szankasi *et al.*, 1988). Deletion of the *ade6-M26* promoter eliminates hotspot activity (Zahn-Zabal *et al.*, 1995), whilst replacing it with the stronger *ADH1* promoter partially increases hotspot activity (Grimm *et al.*, 1991). In addition, even though the *M26*/CRE *ade6* hotspots, are located within an approximately 1400 bp region downstream of the *ade6* promoter they all stimulate DSB formation within the *ade6* promoter (Steiner *et al.*, 2002). There is therefore a possibility that potential transcription factor interacts both at and over 1400 bp from an *M26*/CRE binding site may influence hotspot activity. It may also be possible that in *S. pombe* Rec10 plays a role in establishing, stabilising or enhancing the hotspot-specific functions of Atf1•Pcr1, perhaps within the context of its role in the formation of LEs. If this is so, it is possible that Rec10-like proteins within the SC of higher eukaryotes play a similar role in the regulation of transcription-factor dependent hotspot activation.

8.5 Suggestions for future studies

Although we have direct evidence for how Rec10 modulates hotspot activation, we have proposed the following. 1) Rec10 may interact with other proteins that function within an *S. pombe* recombinasome-like complex. The isolation of Rec10 interacting proteins could be achieved via co-immuno precipitation (Co-IP) experiments using Rec10 specific antibodies or fractionation of the meiotic proteome on chromatographic columns that purify via complex size. Proteins that were isolated by these procedures could then be separated via SDS-PAGE electrophoresis and identified via mass-spectrometry. 2) The function(s) of Rec10 that is normally required for full activation of certain *M26*-containing hotspots may be suppressed by increased Atf1•Pcr1 activity and many of the aspects of *M26*-containing hotspot activation mirror the regulation of

CREB-dependent transcription. A study using chromatin immuno-precipitation (ChIP) could be used to compare the levels of Atf1•Pcr1 at *ade6-M26* and *ade6-M26-16C* at mating temperatures of 30°C and 33°C and in the *rec20-144* mutant. These experiments could determine if there was a correlation between increased binding of Atf1•Pcr1 and suppression of the function(s) of Rec10 required for full hotspot activation of the *ade6-M26* hotspot. In addition ChIP and micro-arrays could detect the levels of Atf1•Pcr1 at genome wide *M26*/CRE sequences under various mating conditions and in various mutant backgrounds. These experiments could determine if every *M26*/CRE sequence interacts with Atf1•Pcr1 during meiosis and hence is a potential recombination hotspot and if the distribution of these potential hotspots can be influenced by conditions that modulate CREB-dependent transcription of higher eukaryotes. 3) The fact that to achieve full hotspot activation, the *ade6-M26* and *ade6-M26-16C* hotspots have differential requirements for a function(s) of Rec10 may be linked to role Rec10 plays in the modulation of nucleosome phasing at the *M26* heptamers of these hotspots. This could be investigated by a comparative analysis of the nucleosome phasing patterns of the *ade6-M26* and *ade6-M26-16C* hotspots in the *rec20-144* mutant. 4) Rec10 may be required for formation or correct processing of a specific sub-set of hotspot-specific DSBs. To investigate these potential aspects of Rec10 function and *M26*-containing hotspot activation the following experiments would be informative. Pulse field gel electrophoresis and Southern blotting could be used to analyse DSB formation in the *rec20-144* mutant under varying mating conditions to determine if specific DSBs are differentially regulated by Rec10.

8.6 Closing remarks

In most organisms, genetic recombination is fundamental to the formation of gametes. The majority of meiotic recombination is initiated at a limited number of recombination "hotspots". We have demonstrated that the loss of a function(s) of Rec10 can result in a relative stimulation of certain *M26*-containing hotspot sequences. This could potentially lead to a switch in the distribution of hotspot recombination events. The activation of *M26*-containing *S. pombe* hotspots share many similarities to CREB-dependent transcriptional regulation in higher

eukaryotes and the activity of CREB homologues may be involved in the stimulation of certain *M26*-containing hotspot sequences in the *rec20-144* mutant. These findings can now be added to other aspects of *M26*/CRE hotspot regulation which include possible post-translational modification of Atf1 and/or Pcr1 (Kon *et al.*, 1997; Fox *et al.*, 2000; Mizuno *et al.*, 2001), meiotic-specific chromatin remodelling (Fox *et al.*, 2000; Mizuno *et al.*, 1997; Mizuno *et al.*, 2001; Yamada *et al.*, 2004) and the requirement for Rec11 (Chapter 5), a meiotic specific cohesin (Li *et al.*, 1997). The fact that Rec10 shares structural and sequence homology with other eukaryotic proteins indicates that Rec10 may belong to a family of functionally related proteins that operate in the regulation of the position, initiation, level of activation and processing of meiotic recombination hotspots in higher eukaryotes.

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Appendix

S. pombe strains

h^+	BP12	*ade6-M26*
h^+	BP84	*ade6-M26 rec20-144*
h^+	BP254	*ade6-M26 rec20-144*
h^+	BP695	*ade6-M26-16C*
h^+	BP697	*ade6-M26-16C rec20-144*
h^+	BP4	*ade6-M375*
h^+	BP130	*ade6-M375 rec20-144*
h^+	BP255	*ade6-M375 rec20-144*
h^-	BP364	*ade6-M375 rec20-144*
h^-	BP5	*ade6-L52*
h^-	BP83	*ade6-L52 rec20-144*
h^-	BP221	*ade6-L52 rec20-144*
h^+	BP363	*ade6-L52 rec20-144*
h^+	BP451	*ade6-3005*
h^+	BP453	*ade6-3005 rec20-144*
h^+	BP452	*ade6-3006*
h^+	BP454	*ade6-3006 rec20-144*
h^+	BP750	*ade6-3008*
h^+	BP739	*ade6-3008 rec20-144*
h^+	BP752	*ade6-3010*
h^+	BP751	*ade6-3010 rec20-144*
h^+	BP749	*ade6-3002*
h^+	BP753	*ade6-3002 rec20-144*
h^+	BP747	*ade6-3007*
h^+	BP748	*ade6-3007 rec20-144*
h^+	BP735	*ade6-3049*
h^+	BP754	*ade6-3049 rec20-144*
h^+	BP737	*ade6-3009*
h^+	BP829	*ade6-3009 rec20-144*
h^+	BP351	*ade6-M26 translin::kan^r*
h^-	BP354	*ade6-L52 translin::kan^r*
h^+	BP44	*ade6-M26 rec10-109*
h^-	BP746	*ade6-M216*
h^-	BP828	*ade6-M216 rec20-144*
h^+	BP465	*ade6-M26 ura4-169*
h^+	BP466	*ade6-M26 ura4-169 rec20-144*
h^+	BP482	*ade6-M26 ura4-170*
h^-	BP484	*ade6-M26 ura4-170 rec20-144*
h^+	BP378	*ade6-M26 ura4-167*
h^+	BP450	*ade6-M26 ura4-167 rec20-144*
h^+	BP379	*ade6-M26 ura4-168*
h^+	BP373	*ade6-M26 ura4-168 rec20-144*
h^{90}	BP151	*ade6-M26 ura4-294*
h^{90}	BP82	*ade6-M26 ura4-294 rec20-144*
h^-	BP375	*ade6-L52 ura4-294*
h^-	BP376	*ade6-L52 ura4-294 rec20-144*
h^+	BP787	*ade6-M26-16C leu1-32*

h^+	BP451	*ade6-3005*
h^+	BP452	*ade6-3006*
h^+	BP538	*ade6-3008 leu1-32*
h^+	BP542	*ade6-3010 leu1-32*
h^+	BP534	*ade6-3002 leu1-32*
h^+	BP536	*ade6-3007 leu1-32*
h^+	BP686	*ade6-3049 leu1-32*
h^+	BP540	*ade6-3009 leu1-32*
h-	BP584	*ade6-M216 leu1-32*
h^+	BP598	*ade6-M26 rec10-155::LEU2 leu1-32*
h^+	BP785	*ade6-M26-16C rec10-155::LEU2 leu1-32*
h^+	BP480	*ade6-M375 rec10-155::LEU2 leu1-32*
h^-	BP481	*ade6-L52 rec10-155::LEU2 leu1-32*
h^+	BP474	*ade6-3005 rec10-155::LEU2 leu1-32*
h^+	BP477	*ade6-3006 rec10-155::LEU2 leu1-32*
h^+	BP538	*ade6-3008 rec10-155::LEU2 leu1-32*
h^+	BP542	*ade6-3010 rec10-155::LEU2 leu1-32*
h^+	BP534	*ade6-3002 rec10-155::LEU2 leu1-32*
h^+	BP536	*ade6-3007 rec10-155::LEU2 leu1-32*
h^+	BP495	*ade6-3011 rec10-155::LEU2 leu1-32*
h^+	BP676	*ade6-3049 rec10-155::LEU2 leu1-32*
h^+	BP505	*ade6-3009 rec10-155::LEU2 leu1-32*
h-	BP585	*ade6-M216 rec10-155::LEU2 leu1-32*
h^+	BP639	*ade6-3005 rec11-156::LEU2 leu1-32*
h^+	BP642	*ade6-3006 rec11-156::LEU2 leu1-32*
h^+	BP637	*ade6-M26 rec11-156::LEU2 leu1-32*
h^+	BP588	*ade6-M375 rec10-156::LEU2 leu1-32*
h-	BP595	*ade6-L52 rec11-156::LEU2 leu1-32*

S. pombe meiotic linear elements contain proteins related to synaptonemal complex components

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Summary

The fission yeast *Schizosaccharomyces pombe* does not form synaptonemal complexes (SCs) in meiotic prophase nuclei. Instead, thin threads, the so-called linear elements (LEs), are observed at the corresponding stages by electron microscopy. Here, we demonstrate that *S. pombe* Rec10 is a protein related to the *Saccharomyces cerevisiae* SC protein Red1 and that it localizes to LEs. Moreover, a homologue to *S. cerevisiae* Hop1 does exist in *S. pombe* and we show by in situ immunostaining that it, and the kinase Mek1 (a homologue of which is also known to be associated with SCs), localizes to LEs. These observations indicate the evolutionary relationship of LEs with the lateral elements of SCs and suggest that these structures might exert similar functions in *S. cerevisiae* and *S. pombe*.

Key words: Chromosome pairing, Meiosis, Recombination, Yeast

Introduction

Cells of sexually reproducing eukaryotes normally contain two equal (homologous) sets of chromosomes, one contributed by the father and the other by the mother during the fusion of gametes and the formation of a zygote. Meiosis is the cell division that reduces the number of chromosomes by half. It produces gametes or their precursor cells, each of which contains a haploid set consisting of randomly assorted parental chromosomes. These chromosomes are mosaics, because the original parental homologues have exchanged corresponding pieces by crossing-over. Thus, the function of meiosis is twofold – it compensates for the doubling of the chromosome number at fertilization and it provides the progeny with newly assorted sets of alleles, which is the basis of their genetic diversity.

Crossing-over is initiated at multiple sites in recombining chromosomes by the enzymatic induction of double-strand breaks (DSBs). The resection of single strands at DSBs leads to the formation of gaps in the DNA. The missing bases are replenished by using the complementary sequence from the homologous chromosome as the template. This process is recombinogenic; that is, it can lead to the reciprocal exchange of DNA between the chromosomes involved (for review, see Keeney, 2001).

In order to allow crossing-over, homologous parental chromosomes must pair during meiotic prophase via a ladder-like proteinaceous structure, the synaptonemal complex (SC) (for reviews, see Loidl, 1990; Zickler and Kleckner, 1999). The SC consists of two parallel axes (the lateral elements), to each of which the two chromatin threads of a single replicated chromosome are attached. The lateral elements are connected and kept at a distance of ~100 nm by the so-called transversal filaments. The ultrastructure of the SC is evolutionarily well conserved from protists to humans, although its molecular composition is far more heterogeneous.

The fission yeast *Schizosaccharomyces pombe* features a meiosis that is unique in several respects. Most remarkably, it lacks an SC. Instead, so-called linear elements (LEs) appear during meiotic prophase (Olson et al., 1978; Bähler et al., 1993). LEs appear in the electron microscope (EM) as single lines of variable length, networks of interconnected lines or bundles of lines. These different morphological classes were found to prevail at different stages of meiotic prophase (Bähler et al., 1993), which suggests that their change in appearance is functionally related to chromosome pairing and/or recombination.

Because a *rec10* mutant lacks LEs, a structural or regulatory role of the Rec10 protein in LE formation has been proposed (Molnar et al., 2003). Apart from this indirect evidence, information on the molecular composition of LEs is scarce. Neither topoisomerase II nor Rec8 [which, in other organisms, constitute the cores along which lateral elements form (Klein et al., 1992; Klein et al., 1999)] delineate entire LEs (Hartsuiker et al., 1998; Parisi et al., 1999; Watanabe and Nurse, 1999), and *S. pombe* homologues of proteins present in SCs have not been described (Parisi et al., 1999; Davis and Smith, 2001). Therefore, the evolutionary relationship of LEs to SCs has remained unclear. Here, we have identified several molecular components of LEs and localized them in situ by immunocytochemistry. We demonstrate the similar molecular composition of the lateral elements of SCs and LEs, and we discuss possible functions of LEs.

Materials and Methods

Antibody production and testing

Amino acids 32-45 and 670-684 were selected from the translated

Table 1. Strain list

Name	Genotype	Source/reference
1-20	*h⁻ ade6-M210*	J. Kohli*
1-25	*h⁺ ade6-M216*	J. Kohli*
ALP3	*h⁺/h⁻ ade6-M216/ade6-M210*	1-20 × 1-25 / this paper
S1241	*h⁺ ade6-M216 leu1-32 mek1Δ::kanMX6*	Pérez-Hidalgo et al., 2003
S1242	*h⁻ ade6-M210 leu1-32 mek1Δ::kanMX6*	Pérez-Hidalgo et al., 2003
ALP22	*h⁺/h⁻ de6-M216/ade6-M210 mek1Δ::kanMX6/mek1Δ::kanMX6*	This paper
S1294	*h⁻/h⁻ pat1-114/pat1-114 ade6-M216/ade6-M210 leu1-32/leu1-32 mek1⁺::3HA-kanMX6/mek1⁺::3HA-kanMX6*	Pérez-Hidalgo et al., 2003
ED10	*h⁺/h⁻ ade6-M216/ade6-M210 ura4-D18/ura4-D18 rec8Δ::ura4⁺/rec8Δ::ura4⁺*	Eveline Doll*
BP841	*h⁺ ade6-M216 rec10-109*	This paper
BP843	*h⁻ ade6-M210 rec10-109*	This paper
ALP41	*h⁺/h⁻ ade6-M210/ade6-M216 rec10-109/rec10-109*	BP841 × BP843 / this paper
ED3	*h⁺/h⁻ rec10-155::LEU2/rec10-155::LEU2 leu1-32/leu1-32 ade6-M216/ade6-M210*	Molnar et al., 2003
ED4	*h⁺/h⁻ rec11-156::LEU2/rec11-156::LEU2 ade6-M210/ade6-M216 leu1-32/leu1-32*	Molnar et al., 2003
ED5	*h⁺/h⁻ rec12-152::LEU2/rec12-152::LEU2 leu1-32/leu1-32 ade6-M216/ade6-M210*	Molnar et al., 2003

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sequence of *rec10* (SPAC25G10.04c) as epitopes for the elicitation of antibodies in rabbits. Peptide synthesis and antibody production were performed by Eurogentec (Herstal, Belgium). Individual rabbits were immunized with a single peptide each. Antibodies were affinity purified on EAH-Sepharose columns to >85% purity and diluted in PBS containing 0.01% NaN_3 and 1% bovine serum albumin (BSA). The antibody against the C-terminus (amino acids 670-684) was used for immunostaining.

Amino acids 482-496 and 514-528 were selected from the translated sequence of SPBC1718.02 (Hop1) as epitopes for the elicitation of antibodies in guinea pigs. Peptide synthesis and antibody production were performed by Eurogentec. Two individuals were each injected with both peptides. The serum was purified by adsorption to acetone powder of sporulating *hop1Δ* cells (Harlow and Lane, 1988) (we will be publishing the details of construction of a *hop1Δ* strain in a future paper).

Strains, growth and sporulation

The strains used are listed in Table 1. Cells were maintained as prototrophic diploids on yeast extract agar (YEA) plates through the use of interallelic complementing auxotrophic markers *ade6-M210* and *ade6-M216* (Moreno et al., 1991). Sporulating cells for microscopic examination were prepared as follows (Molnar et al., 2003). Single colonies were transferred to yeast extract liquid (YEL) and cultivated overnight. (Liquid cultures were always kept shaking at 30°C.) This culture was used to inoculate *S. pombe* minimal medium (PM), and cells were grown to a density of 1×10^7-2×10^7 cells per ml. They were then pelleted by centrifugation and transferred to PM without NH_4Cl (PM-N) at a density of 1×10^7 cells per ml for sporulation. The strain carrying haemagglutinin (HA)-tagged Mek1 was *pat1-114*. Sporulation in this strain was induced according to a published procedure (Cervantes et al., 2000).

11 ml samples were taken at hourly intervals, of which 10 ml were used for the production of immunostained microscopic preparations and 1 ml to check meiotic progression by DAPI (4′,6-diamidino-2-phenylindole) staining (Molnar et al., 2003).

Microscopic preparation

Aliquots from sporulating cultures were centrifuged and the cells were resuspended in an enzyme solution for spheroplasting (Bähler et al., 1993; Molnar et al., 2003). 20 μl spheroplasted cells were put on a microscope slide and mixed with 40 μl fixative (4% paraformaldehyde, 3.4% sucrose) and 80 μl detergent ('Lipsol'; LIP, Shipley, UK), which causes the cells and nuclei to swell and to expose the nuclear contents. The spreading procedure was stopped after ~30 seconds by the addition of 80 μl fixative. The slides were then dried in air and were kept in the refrigerator until use.

For electron microscope (EM) inspection, slides were stained with $AgNO_3$ and the material was transferred to EM grids as described previously (Bähler et al., 1993).

Immunostaining and detection

Slides were washed three times for 15 minutes each in 1× PBS containing 0.05% Triton X-100. After shaking off excess liquid, primary antibody was applied under a coverslip and the slides were put in the refrigerator overnight. Concentrations were 1:2000 for rabbit anti-Rec10 antibody, 1:50 for Guinea pig anti-*S. pombe*-Hop1 antibody and 1:200 for mouse anti-HA antibody. Primary antibodies were applied alone or in appropriate combinations. After incubation, the coverslip was removed and washing steps were performed as above. Incubation in appropriate secondary fluorescence-tagged (FITC, Cy3, Alexa) antibodies was for 4 hours at room temperature. After another round of washing as above, slides were mounted in antifade solution (Vectashield, Vector Labs, Burlingame, CA) supplemented with 1 μg ml^{-1} DAPI for the staining of DNA.

Immunofluoresence was detected with a Zeiss Axioskop epifluorescence microscope equipped with single-band-pass filters for the excitation of blue, green and red fluorescence. Pictures were taken with a cooled CCD camera (Photometrics, Tucson, AZ). Black-and-white images were assigned false colours and merged with the help of IPLab Spectrum software (Scanalytics, Fairfax, VA).

Results

Rec10 shows architectural and partial sequence similarity to *Saccharomyces cerevisiae* Red1

S. pombe Rec10 was identified as a putative homologue of *Saccharomyces cerevisiae* Red1 from a sequence architecture-based database search. *S. cerevisiae* Red1 is an 827 amino acid protein with one clear sequence homologue found in the non-redundant database (nr) from NCBI, namely the 854 amino acid *Kluyveromyces lactis* Red1 (Smith and Roeder, 2000). Both proteins show similar sequence architecture: a lysine-rich region predicted to contain nuclear targeting signals is located around position 500-700 (residues 568-686 and 518-693) [determined by CAST (Promponas et al., 2000) and PROSITE (Sigrist et al., 2002)]. A highly helical region is found at the very C-terminus (the last ~90 residues) and contains a predicted coiled coil (in *S. cerevisiae*, amino acids 765-795)

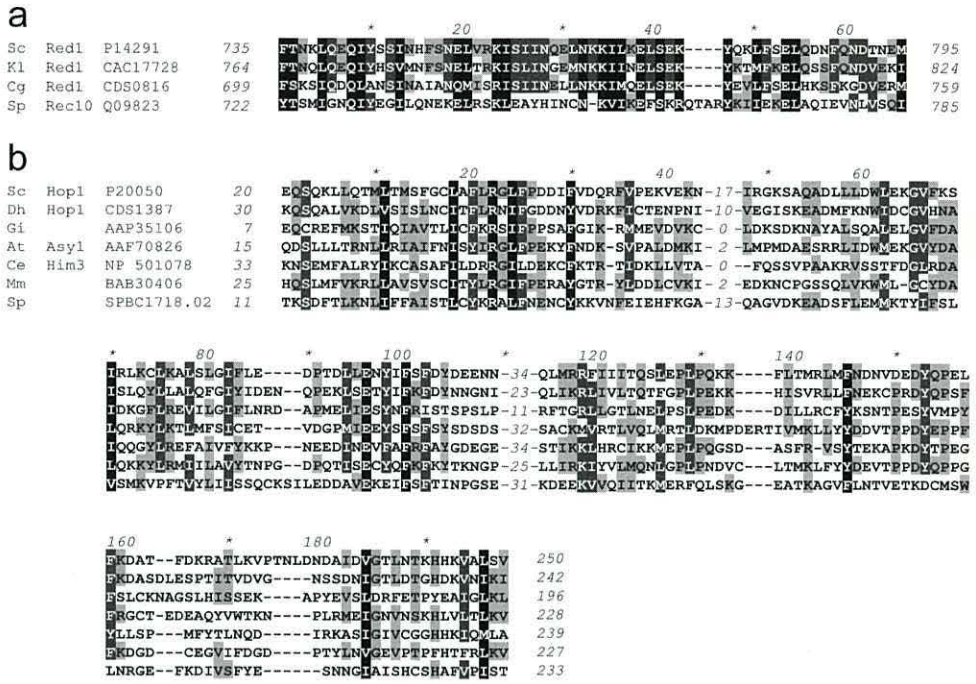

Fig. 1. (a) Multiple sequence alignment of the homologous C-termini of Red1-related proteins in *S. cerevisiae* (Sc), *Kluyveromyces lactis* (Kl), *Candida glabrata* (Cg) and *S. pombe* (Sp). Sequences are indicated with the species, followed by a database accession number [databases used are Genpept (Sc, Kl, Sp) and Génolevures (Cg) (Feldmann, 2000)]. Sequences are labelled with a four-level conservation shading using similarity groups. (b) Multiple sequence alignment of the HORMA domain (Aravind and Koonin, 1998) from Hop1-related proteins of Sc, *Debaryomyces hansenii* (Dh), *Giardia intestinalis* (Gi), *Arabidopsis thaliana* (At), *Caenorhabditis elegans* (Ce), *Mus musculus* (Mm) and Sp. Sequences are indicated with the species, followed by a database accession number [databases used are Génolevures (Dh) (Feldmann, 2000), Pompep (Sp) and Genpept (all remaining sequences)]. Sequences are labelled with a four-level conservation shading using similarity groups.

(Lupas et al., 1991). Functionally, the C-terminal 291 amino acids including the helical region are thought to mediate Red1 homo-oligomerization (Hollingsworth and Ponte, 1997; Woltering et al., 2000).

S. pombe is an ascomycete only distantly related to *S. cerevisiae*, so that the sequentially poorly conserved Red1 (*K. lactis* and *S. cerevisiae* Red1 proteins show just 26% identity over their entire length) is not found in *S. pombe* using full-length sequence similarity searches. The C-terminal 90 amino acids of the known Red1 proteins are better conserved (62%) and thus a potentially superior bait in sequence-based searches. The region from amino acid 764 to amino acid 834 in *K. lactis* (matching *S. cerevisiae* Red1 amino acids 735-805 with 66% identity) when used in WU-Blast against the *S. pombe* proteome (http://www.genedb.org/) obtains Rec10 as second best hit (E=0.017) and by far the top hit of a sequence of the expected length (791 amino acids) where the match is found at the C-terminal amino acids 722-785. [The first-listed hit in this search (SPAC7D4.14c, E=0.0052, has a similar range) is rejected as potential candidate because it is much shorter (551 residues) and the region of similarity is N-terminal (residues 41-90) in contrast to the required C-terminal location.] Shared sequence architecture was a criterion for selecting Rec10 as the potential *S. pombe* Red1 homologue (Fig. 1a). Rec10, much like Red1 proteins, contains a coiled-coil region (amino acids 760-782) and a K-rich region located in the second half of the sequence (amino acids 433-523 found with CAST) (Promponas et al., 2000), which hits a bipartite nuclear targeting sequence pattern in PROSITE (Sigrist et al., 2002).

Although a similar function has been suggested for the two animal protein families represented by vertebrate SCP3/COR1 (Heyting et al., 1987) and *Drosophila* c(2)M (DS02750.10) (Manheim and McKim, 2003), and for *S. cerevisiae* Red1 (Rockmill and Roeder, 1988), these are not conserved at the primary sequence level.

SPBC1718.02 is the likely *HOP1* orthologue

S. pombe SPBC1718.02 shows a meiotic S-phase expression peak (Mata et al., 2002). A sequence-architecture-based search similar to that applied for Rec10/Red1 identified it as the likely orthologue of budding yeast *HOP1*. Hop1, like Red1, is associated with the axial and lateral elements of the SC (Muniyappa et al., 2000). Over a length of 605 amino acids, Hop1 contains an N-terminal HORMA domain [amino acids 21-245, with a significant (E=4×10^{-27}) match in the Conserved Domain Database (CDD) (Aravind and Koonin, 1998)], and a central zinc finger motif, which is essential for its function and shows a nonsignificant hit against the PHD finger in Aravind's signalling database [amino acids 332-375, E=0.094, IMPALA – (Hollingsworth et al., 1990; Schaffer et al., 1999)]. Simple full-length WU-Blast of Hop1 against the *S. pombe* proteome (http://www.genedb.org/) obtains the 528 amino acids SPBC1718.02 as a nonsignificant hit (E=0.62) with three collinear regions of significant sequence similarity: one in the HORMA domain (amino acids 21-130), one in the PHD finger and one thereafter. Again, analogous sequence architecture is used to substantiate distant sequence similarity found between Hop1 and SPBC1718.02. SPBC1718.02, as indicated in the genedb annotation, is likely to contain an N-terminal HORMA domain (FFAS03: scop/d1goA score=–9.3) and a central PHD domain (Aravind's signalling database, E=5×10^{-5}). An N-terminal HORMA domain is typical of the Hop1 homologous group of proteins identified so far in *S. cerevisiae* (Hollingsworth et al., 1990), *Arabidopsis* (Asy1) (Caryl et al., 2000) and *Caenorhabditis elegans* (HIM-3) (Zetka et al., 1999) (Fig. 1b).

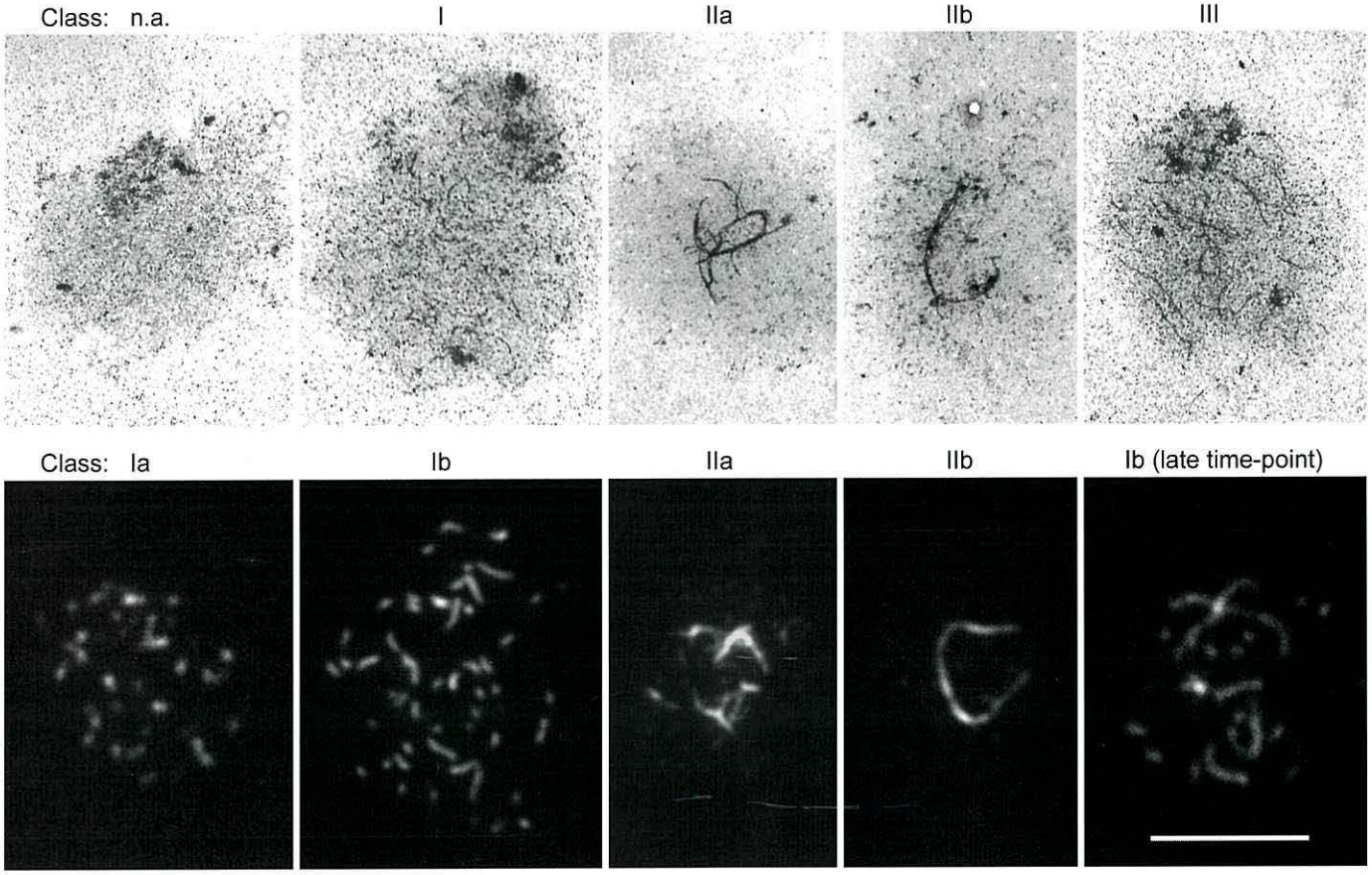

Fig. 2. LEs as observed after silver staining in the electron microscope (EM) (top) and by immunostaining of Rec10 (bottom). The classification of LEs in the EM is according to Bähler et al. (Bähler et al., 1993). Nuclei containing many (up to >50) Rec10-positive spots (designated here as class Ia) appear inconspicuous in the EM. The empty nucleus in the EM (top left, class assignment not applicable) might correspond by its shape and size to immunostained class Ia. Classes I (Ib) and IIa are identified by both immunostaining and EM as containing individual threads and meshes of threads, respectively. Class IIb appears in the EM as bundles of LEs that probably correspond to the long, thick Rec10-positive structures. Silver-stained individual LEs of heterogeneous lengths, which prevail during later stages of meiosis (7-10 hours), were designated as class III (Bähler et al., 1993). The corresponding Rec10-LEs are classified as Ib. A class-Ib nucleus from a late time point in meiosis (8 hours in sporulation medium) is shown for comparison. Scale bar, 5 μm.

Rec10 is a component of LEs and similar classes of LE morphology are observed by EM and Rec10 immunostaining

Immunostaining with Rec10 antiserum highlighted dot-shaped and linear structures in spread wild-type meiotic nuclei (Fig. 2), whereas there was no immunostaining with preimmune serum (not shown). The Rec10-positive structures correspond well with the classes of LEs that have been defined by their appearance in the EM (Bähler et al., 1993) (Fig. 2). Over a meiotic time course, there is a change in the proportion of the different Rec10 LE classes (Fig. 3). The earliest Rec10-positive structures, observed at 3 hours in sporulation medium, were dots or very short lines. Up to 58 Rec10-positive spots were present in nuclei that we denote as class Ia (63 nuclei from three different experiments were evaluated). They have no parallels in the EM. Possibly at this stage of development, the structures are not yet sufficiently elaborated to be discriminated from the high background of Ag-positive grains. Class Ia was present at ~3-10% of nuclei in all time points. Class Ib features individual LEs of various lengths. There are fewer lines than in class Ia (a maximum of 29 counted in 55 nuclei from three different experiments), which suggests that they originate by the fusion of class Ia LEs. Their frequency showed a peak at 5 hours in sporulation medium, followed by a decline and an accumulation towards 10 hours. Class IIa nuclei contain a network of connected LEs. Class IIa was the most prevalent at 5-7 hours. Class IIb appears in the EM as a dense bundle of LEs and it can be assumed that it corresponds to the thick bar seen after Rec10 immunostaining. They constitute a minor portion of nuclei at all time points. Bähler et al. (Bähler et al., 1993) had introduced a class III consisting of long single LEs appearing at late times during meiosis. Here, we did not make this distinction and assigned all single LEs to class Ib because we cannot decide whether long individual LEs observed at late times originate by the steady growth of Ia and Ib LEs or whether they are a degradation product of class II LEs. Likewise, for a class Ia nucleus at a late time, it cannot be determined whether it is the end point of a structural transformation or if it has not developed beyond the initial state. However, the fact that class Ib nuclei come in two peaks and that class IIa is most abundant in the valley between the peaks, suggests that class IIa derives from early class I and

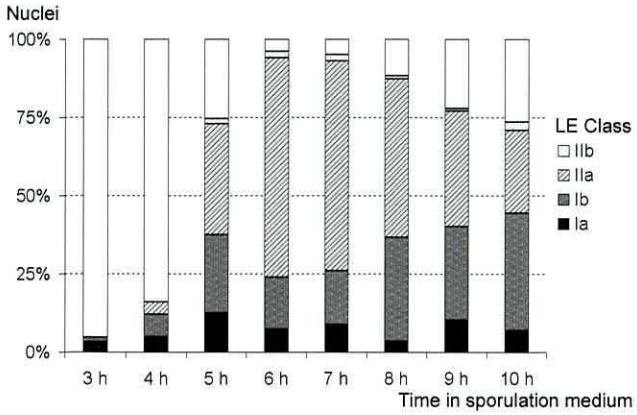

Fig. 3. Proportions of different LE classes in nuclei of the wild type at different times in sporulation medium. A typical time course is shown. For the classification of LEs, see Fig. 2. 200 nuclei were evaluated for each time point.

transforms to late class I. Neither can it be determined whether the rare class IIb represents a step in the development of all nuclei going through meiosis or if only a subset of nuclei adopt this appearance. In the former case, it must be of very short duration.

Morphology of Rec10 structures in meiotic mutants

To confirm the identity of immunostained structures and LEs as seen by EM and to determine the conditions for LE formation, we studied Rec10 localization in various mutants. In the *rec10-155* mutant, in which no LEs had been detected by EM (Molnar et al., 2003), the corresponding structures were also completely missing after immunostaining (Fig. 4b), whereas, in another mutant (*rec10-109*), up to ten Rec10-positive dots or short lines were observed (Fig. 4c) and similar rudimentary structures were also seen by EM (not shown). This is a considerably reduced LE formation compared with the wild type.

Because there is now evidence from a range of organisms that meiotic cohesins underlie the lateral elements (Klein et al., 1999; Pasierbek et al., 2001; Prieto et al., 2001), it would be interesting to know whether LE formation also depends on a cohesin scaffold. Molnar et al. (Molnar et al., 2003) reported that strains that lack meiotic cohesin components develop aberrant LEs (Molnar et al., 1995). Here, we found that the rudimentary LEs formed in a *rec8*Δ strain contain Rec10 (Fig. 4d). In spite of their superficial similarity to class IIb LEs (which are likely to develop from class I LEs), the origin of these structures must be different, because the normal precursor stages (class I) were not detected. It remains to be tested whether these aberrant LEs are at all associated with chromatin or represent aggregates of LE components that are not properly localized in the absence of chromosomal cores to which they would normally attach. Because Rec8 partners with Rec11 in arm-associated meiotic cohesin (Kitajima et al., 2003), a *rec11* mutant showed structures very similar to those in *rec8*Δ both in the EM (Molnar et al., 2003) and after Rec10 immunostaining (not shown), as expected.

In the recombination-deficient *rec12-152* mutant [*rec12* encodes the homologue of the DSB-inducing protein Spo11 (Lin and Smith, 1994)], Molnar et al. (Molnar et al., 2003) detected LEs but the frequencies of the different classes deviated from those found in the wild type, with long single threads being the most frequent phenotype. Here, we confirm that these LEs are normal in the sense of being endowed with Rec10 (Fig. 4e) and thus that LE formation is independent of the initiation of DSB formation.

We next tested the effect of the loss of Mek1 on LE formation. Mek1 was discovered in *S. cerevisiae* as a meiosis-specific protein kinase required for chromosome synapsis (Rockmill and Roeder, 1991). When the *S. pombe mek1* homologue is deleted, spore viability and recombination are reduced (Pérez-Hidalgo et al., 2003). Rec10 LEs appeared morphologically wild-type in the *mek1*Δ strain (Fig. 4f) but class IIa LEs were under-represented compared with the wild type. Whereas, in the wild type, up to ~45% of meiotic nuclei (n=619 nuclei after 7 hours in sporulation medium) were of this class, at most 3.2% of *mek1*Δ nuclei (n=400 nuclei after 6 hours in sporulation medium) contained class IIa LEs.

S. pombe Hop1 and Mek1 localize to LEs

To determine the cellular localization of Hop1, we produced antibodies against the protein for immunostaining in spread meiotic nuclei. Hop1 staining produced dotted and linear

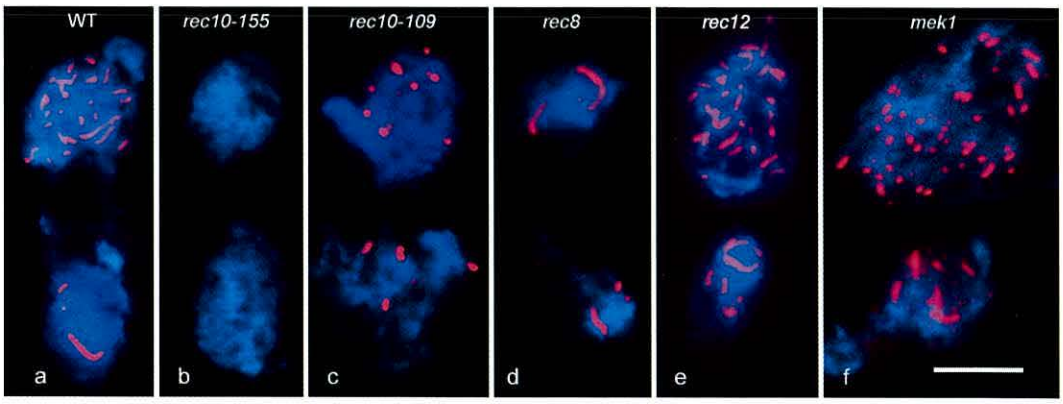

Fig. 4. Examples of Rec10-positive LEs (red) in (a) the wild type (WT) and various mutants. Two typical nuclei are shown for each strain. (b) In the *rec10-155* mutant, LEs are completely missing, whereas, in the *rec10-109* mutant (c), LEs develop into a few dots or short lines. (d) In a *rec8*Δ strain, Rec10 is organized into thick threads resembling class IIb LEs. (e) In the *rec12-152* mutant strain, all classes of LEs occur (a class IIa and a class IIb nucleus are shown). (f) In a *mek1*Δ strain, Rec10 assembles into wild-type-like LEs, whereby classes Ia and Ib are more abundant than the others. Chromatin is stained blue with DAPI. Scale bar, 5 μm.

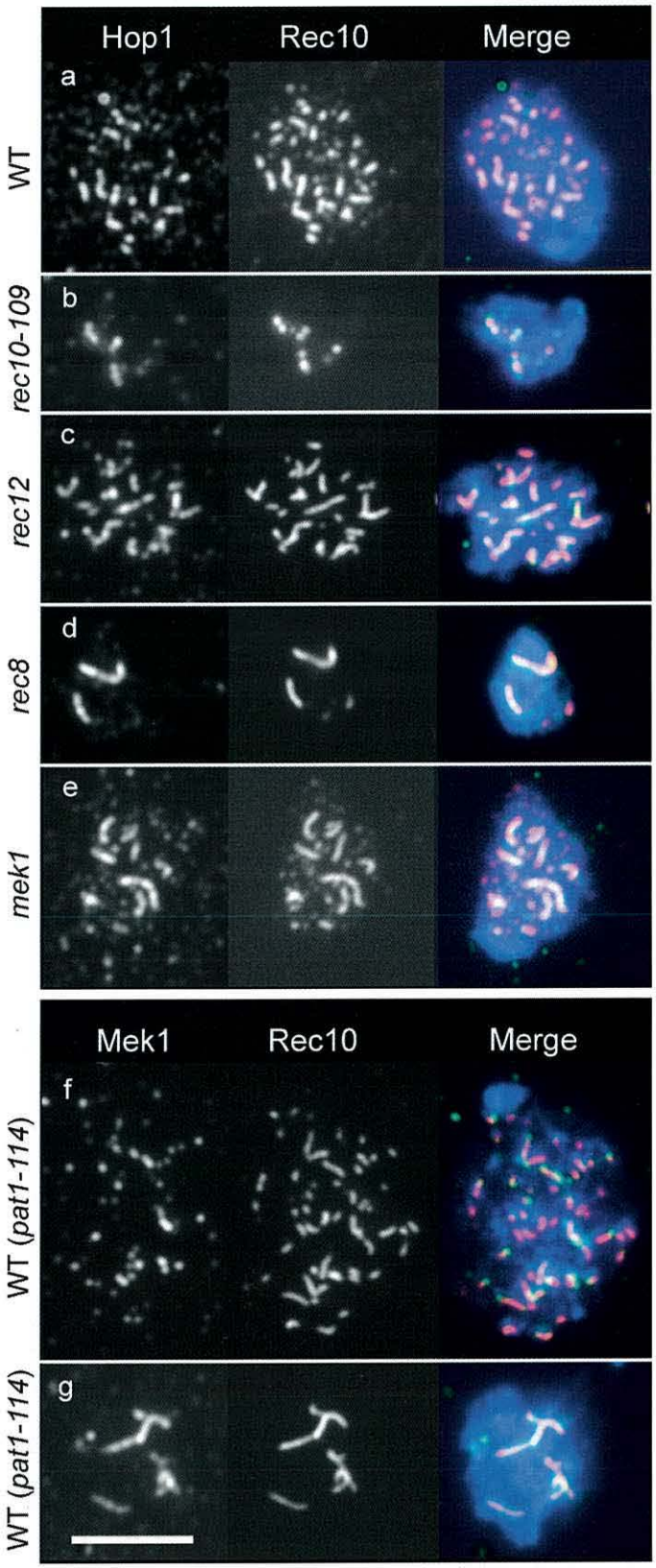

Fig. 5. Hop1 and Mek1 localization to Rec10 LEs shown by immunostaining of Rec10, Hop1 and Mek1-HA. Regions where Hop1 or Mek1 (green) colocalize with Rec10 (red) appear orange in the merge image. Chromatin is stained blue with DAPI. The images show Hop1 localization to: (a) class Ib LEs of the wild type (WT); (b) residual LEs in the *rec10-109* mutant; (c) LEs in a *rec12-152* mutant strain; (d) aberrant LEs in the *rec8*Δ mutant; and (e) LEs in a *mek1*Δ strain. Some short LEs are lacking Hop1 and Hop1 localization to longer LEs is discontinuous. Mek1 forms dots on short LEs in class I nuclei (f) but covers most parts of LE bundles in (rare) class IIb nuclei (g). Scale bar, 5 μm.

patterns similar to Rec10 LEs (Fig. 5a). Hop1 immunostaining of a *hop1* mutant (A. Lorenz et al., unpublished) was negative (not shown), which confirms that the antibody is specific.

Double immunostaining revealed that, in class I nuclei, often only a subset of Rec10-positive dots is highlighted with Hop1 antibody. Of the longer class II LEs, virtually all were Hop1 positive, although Hop1 localization was somewhat discontinuous (Fig. 5a-e). The presence of class I LEs without Hop1 suggests that Hop1 is loaded onto LEs somewhat later than Rec10. In nuclei of the *rec10-155* mutant strain, which lacks LEs, no Hop1 threads were detected. However, Hop1 was present in the fragmentary LEs of the *rec10-109* mutant (Fig. 5b), supporting the notion that Hop1 associates with a pre-existing Rec10-containing scaffold.

In the *rec12-152* mutant, Hop1 delineated LEs (Fig. 5c), suggesting that wild-type LEs are independent of the formation of meiotic DSBs and, in the *rec8*Δ strain, Hop1 localized to the aberrant LEs (Fig. 5d), confirming that their molecular composition resembles that of the wild type.

Because the *S. cerevisiae* protein kinase Mek1 has been found to localize to SCs (Bailis and Roeder, 1998) and it is known that its *S. pombe* homologue is present in meiotic nuclei (Pérez-Hidalgo et al., 2003), we wanted to determine the spatial relationship of *S. pombe* Mek1 with LEs in spread nuclei. To do this, we used a strain that expresses HA-tagged Mek1 (Pérez-Hidalgo et al., 2003). In nuclei with short LEs, Mek1-HA staining highlighted many spots that localized to Rec10 LEs, whereas linear signals were rarely observed. In class II nuclei, Mek1 delineated stretches along LE bundles (Fig. 5f,g).

It had been suggested that, in *S. cerevisiae*, the interaction between Red1 and Hop1 is facilitated by Mek1-dependent phosphorylation of Red1 (De los Santos and Hollingsworth, 1999), and so we tested whether *S. pombe* Mek1 has an influence on the localization of Hop1 to LEs. In a *mek1* deletion strain, we did not observe notably reduced Hop1 immunostaining of LEs (Fig. 5e). This is in accordance with recent evidence from budding yeast that Red1 is not a Mek1 substrate (Wan et al., 2004).

Discussion

S. pombe linear elements and the axial/lateral elements of synaptonemal complexes contain similar proteins

In the budding yeast, in which the molecular composition of SCs is known best, Red1 was shown to be a basic component of SCs. However, Red1 might not be the primary building block of axial/lateral elements, because Red1 staining is discontinuous along pachytene bivalents (Smith and Roeder, 1997) and, in its absence, fragmentary axial and lateral elements are still formed (Rockmill and Roeder, 1990). It is

likely that it attaches to a pre-existing core of topoisomerase II and cohesin (Klein et al., 1992; Klein et al., 1999). The localization of Hop1, in turn, depends on Red1 (Smith and Roeder, 1997). In the absence of Hop1, extensive stretches of axial elements can be formed (Loidl et al., 1994) but both Red1 and Hop1 are required for the full development of axial elements. These are then linked by the transversal filaments consisting of Zip1 and become the lateral elements of the SC, but Hop1 dissociates from chromosomes at or before pachytene, as chromosomes synapse. The serine/threonine protein kinase Mek1 and the protein phosphatase type 1 Glc7 are also loaded onto axial elements (Bailis and Roeder, 2000; Wan et al., 2004).

Here, we show that, in *S. pombe*, immunostaining of meiotic nuclei with Rec10 antiserum highlighted structures that closely resemble those that have been visualized after silver staining in the EM (Bähler et al., 1993). Moreover, mutants with reduced or abnormal LE formation as seen by EM (Molnar et al., 2003) showed identical deficiencies of Rec10-stained structures. This is evidence that Rec10-positive structures correspond to LEs.

Our observation that *S. pombe* LEs contain Rec10 (which has some similarity to *S. cerevisiae* Red1) and homologues of *S. cerevisiae* Hop1 and Mek1 suggests that LEs are equivalents (and probably evolutionary relics) of the lateral elements of SCs. Unlike lateral elements, however, LEs do not seem to extend along the entire length of chromosomes. Moreover, it is possible that, at some time in their development, LEs become detached from the chromosomes. This is suggested by the observation that DAPI-stained chromatin occupies a much larger area than the dense LE bundle in class IIb nuclei.

The existence or nature of any underlying axial core structure to which Rec10 and Hop1 are attached is not entirely clear. Although Rec8 is part of the core structure in *S. cerevisiae* (Klein et al., 1999) to which the structural components of lateral elements are attached, Rec8 was not observed to be organized as linear structures in *S. pombe* meiotic nuclei by either green-fluorescent-protein tagging or immunostaining (Parisi et al., 1999; Watanabe and Nurse, 1999), as would be expected of a chromosomal axial scaffold. However, in the absence of Rec8, Rec10 and Hop1 form only aberrant structures resembling class IIb-like LE bundles. It is therefore possible that, in the *rec8*Δ mutant, LEs are not associated with chromosomes and resemble SC polycomplexes (Goldstein, 1987) in that they are self-organizing aggregates of unused LE components. Alternatively, the rudimentary LEs in the mutant could contain Rad21/Scc1, Rec8's mitotic counterpart, which also seems to be expressed at a low degree in meiosis (Yokobayashi et al., 2003). It therefore remains to be determined whether, in *S. pombe*, Rec8 underlies the LE structure.

Rec10 promotes homologous pairing and crossing-over

Homologous pairing was found to be reduced in the absence of Rec10 but interstitial regions of chromosomes are more severely affected than loci near the centromeres and telomeres (Molnar et al., 2003). It was proposed by Molnar et al. (Molnar et al., 2003) that the meeting of homologous centromeres and telomeres might benefit from the rigorous centromere-telomere polarization (bouquet formation) of meiotic chromosomes (Chikashige et al., 1994) and rely to a lesser extent on LEs (Scherthan et al., 1994).

In addition to pairing, Rec10 has also been implicated in recombination because genetic assays demonstrated a variable reduction in recombination for several marker pairs in the *rec10-109* mutant (De Veaux and Smith, 1994; Krawchuk et al., 1999). Here, we have found that this mutant possesses a few short LE fragments (Fig. 4b), which might account for the residual recombination detected. However, in the *rec10-155* mutant with no LEs, recombination in the single interval tested was dramatically reduced (~500 times less than the wild type) (Lin and Smith, 1995; Krawchuk et al., 1999). More recently, it was found that *rec10*Δ mutants have strongly reduced meiotic recombination in multiple intervals and lack detectable meiotic DSBs (C. Ellermeier and G. R. Smith, pers. commun.). By contrast, in the *rec8* mutant, in which only aberrant LEs (which are possibly not associated with chromosomes) are formed, recombination is less affected near chromosome ends. Therefore, it is possible that the requirement for LEs in recombination is site specific (Parisi et al., 1999).

Although LE formation is independent of DSBs (Molnar et al., 2003) and both Rec10 and Hop1 delineate wild-type LEs in a DSB-deficient *rec12* mutant (this paper), there is a similar number of incipient LEs (>50 Rec10-positive spots in class Ia nuclei) and of estimated crossovers [~45 (Munz, 1994)] per meiosis. Also, the estimated 50-150 DSB sites (Cervantes et al., 2000) per meiosis would roughly correspond to early short LEs. This invites the speculation that DSBs and the initiation of crossing-over might preferentially take place in regions where LEs start to load onto chromatin.

Possible functions for LEs

The SC has been attributed a range of functions, including strengthening the connection between homologues and mediating crossover interference (for reviews, see Loidl, 1994; Roeder, 1997; Zickler and Kleckner, 1999). Axial elements mediate the attachment of chromatin strands to transversal filaments by which the former become intimately paired. (The axial elements are referred to as lateral elements in the mature SC.) Apart from this obvious role in building the SC, axial elements have a more intricate function in conferring a loop structure on the chromatin strands and thereby providing the structural basis for the mutual exposure of homologous DNA tracts for recombination (Zickler and Kleckner, 1999; Blat et al., 2002). Moreover, in *S. cerevisiae*, the axial element components Red1, Hop1 and Mek1 direct crossing-over towards homologous non-sister chromatids (perhaps in part by actively preventing sister-chromatid recombination) (Kleckner, 1996; Roeder, 1997; Thompson and Stahl, 1999; Wan et al., 2004).

S. pombe LEs, unlike the lateral elements of canonical SCs, do not seem to be connected by transversal filaments. Therefore, the role of LEs in the stabilization of chromosome pairing is questionable. In accordance with the presynaptic structural role of axial elements, the function of LEs in *S. pombe* could be the organization of chromatin loops presenting homologous DNA regions for homology recognition and recombination and/or the prevention of recombination between sisters. For this purpose, they need not be organized into canonical tripartite SCs or extend all along the chromosomes.

If LEs have this function then one would expect them to be present at the sites of crossing-over. If, by contrast, the function of LEs was in the sensing of DSBs and the activation of a putative pachytene checkpoint (see below) then it would probably be sufficient if they were formed around only a random subset of recombination sites, because these would be representative of the status of the recombination process in the nucleus.

Recently, it was reported that DSB accumulation and other defects can cause delays in meiotic progression in fission yeast. Shimada et al. (Shimada et al., 2002) and Pérez-Hidalgo et al. (Pérez-Hidalgo et al., 2003) proposed the involvement of the fission yeast Mek1 homologue in a meiotic recombination checkpoint in *S. pombe*. Although its existence has been disputed by others (Catlett and Forsburg, 2003), the present identification of homologues of Red1 and Hop1 [which were implicated in triggering the pachytene checkpoint in budding yeast (Bailis and Roeder, 2000)], at least suggests that a putative checkpoint might use similar chromosomal components in the two yeasts.

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