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Interactions between Mei4, Rec114, and other proteins required for meiotic DNA double-strand break formation in *Saccharomyces cerevisiae*

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Abstract

In most sexually reproducing organisms, meiotic recombination is initiated by DNA double-strand breaks (DSBs) formed by the Spo11 protein. In budding yeast, nine other proteins are also required for DSB formation, but roles of these proteins and interactions among them are poorly understood. We report here further studies of the behaviors of these proteins. Consistent with other studies, we find that Mei4 and Rec114 bind to chromosomes from leptotema through early pachynema. Both proteins showed only limited colocalization with the meiotic cohesin subunit Rec8, suggesting that Mei4 and Rec114 associated preferentially with chromatin loops. Rec114 localization was independent of other DSB factors, but Mei4 localization was strongly dependent on Rec114 and Mer2. Systematic deletion analysis identified protein regions important for a previously described two-hybrid interaction between Mei4 and Rec114. We also report functional characterization of a previously misannotated 5' coding exon of *REC102*. Sequences encoded in this exon are essential for DSB formation and for Rec102 interaction with Rec104, Spo11, Rec114, and Mei4. Finally, we also examined genetic requirements for a set of previously described two-hybrid interactions that can be detected only when the reporter strain is induced to enter meiosis. This analysis reveals new functional dependencies for interactions among the DSB proteins. Taken together, these studies support the view that Mei4, Rec114, and Mer2 make up a functional subgroup that is distinct from other subgroups of the DSB proteins: Spo11-Ski8, Rec102-Rec104, and Mre11-Rad50-Xrs2. These studies also suggest that an essential function of Rec102 and Rec104 is to connect Mei4 and Rec114 to Spo11.

Introduction

Homologous recombination is essential for accurate chromosome segregation during meiosis in most sexual organisms, including the budding yeast *Saccharomyces cerevisiae* (Page and Hawley 2003; Petronczki et al. 2003). Meiotic recombination is initiated by DNA double-strand breaks (DSBs) formed by the evolutionarily conserved Spo11 protein, which cuts DNA through a topoisomerase-like reaction (reviewed in Keeney 2001; Keeney 2007). However, Spo11 alone is not sufficient for DSB formation in vivo: in budding yeast at least nine other

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proteins are also required to generate a DSB (Keeney 2001; Keeney 2007). Interactions among these factors and the roles they play in promoting Spo11 activity remain poorly understood.

Five of the proteins required for DSB formation (Spo11, Mei4, Rec102, Rec104, and Rec114) are meiosis-specific and their expression is controlled primarily by transcription (reviewed in Kassir et al. 2003). Mer2 (also known as Rec107) is also meiotically induced, but its expression is controlled differently. The *MER2* message is constitutively expressed but contains an intron that is spliced efficiently only in the presence of a meiosis-specific splicing factor, Mer1 (Engbrecht et al. 1991; Nandabalan and Roeder 1995; Spingola and Ares 2000). As a result of this regulation, Mer2 protein is present at low levels during vegetative growth and at substantially higher levels during meiosis (Henderson et al. 2006; Li et al. 2006).

The remaining four DSB proteins in *S. cerevisiae* also have roles in vegetative cells. This category includes the evolutionarily conserved Mre11-Rad50-Xrs2 complex, which has multiple functions in meiotic and nonmeiotic cells, including DSB repair, telomere maintenance, and DNA damage checkpoint activation (reviewed in Assenmacher and Hopfner 2004; Krogh and Symington 2004; Stracker et al. 2004). The last protein in this category is Ski8, which has at least two separable functions. In vegetative cells it is part of a multiprotein complex in the cytoplasm that is involved in degradation and translational repression of non-polyadenylated RNA (Masison et al. 1995; Jacobs Anderson and Parker 1998; van Hoof et al. 2000; Araki et al. 2001). During meiosis, however, Ski8 relocates to the nucleus and associates with meiotic chromosomes where it acts as a direct partner of Spo11 (Tessé et al. 2003; Arora et al. 2004).

Aside from the DNA cleaving activity of Spo11, the biochemical functions of the DSB proteins are not well understood. Ski8 is a WD-repeat protein suggested to promote Spo11 interactions with other DSB proteins (Tessé et al. 2003; Arora et al. 2004). The nuclease activity of Mre11 is required to remove Spo11 that has become covalently bound to DNA ends during DSB formation (Neale et al. 2005), but this activity is dispensable for DSB formation per se (Alani et al. 1990; Nairz and Klein 1997; Prinz et al. 1997; Moreau et al. 1999). Roles of the other members of the Mre11-Rad50-Xrs2 complex are similarly unclear. The other meiosis-specific DSB proteins do not have sequence motifs to suggest their biochemical roles.

How do these proteins work together to promote DSB formation? One possibility is that they are stoichiometric subunits of a defined DSB-forming holoenzyme. However, recent studies seem inconsistent with this simple model. For example, genetic, cytological, and physical analyses suggest that Rec102 and Rec104 interact to form a single functional unit (Salem et al. 1999; Jiao et al. 2003; Kee et al. 2004). Although this Rec102-Rec104 complex interacts with other DSB proteins, including Spo11, there are numerous differences between Rec102-Rec104 and the other proteins with respect to their genetic dependencies for protein-protein and protein-chromosome interactions (Kee et al. 2004; Wells et al. 2006) (discussed in more detail below). Moreover, whereas Mre11 and Spo11 appear to localize specifically to preferred sites of DSB formation as assessed by chromatin immunoprecipitation, Rec102 appears to be more broadly associated with both hotspot and non-hotspot regions (Borde et al. 2004; Kee et al. 2004; Prieler et al. 2005).

The picture emerging from these and other studies suggests that, rather than making up a single holoenzyme of defined stoichiometry, the DSB proteins instead form distinct functional subcomplexes that collaborate to promote Spo11-dependent DSB formation. If so, many questions remain about the composition of the subcomplexes and the relationships among them. Here, we describe studies that uncover new details about physical and functional interactions among these factors and between these proteins and meiotic chromosomes.

Materials and methods

Yeast strains, plasmids, and culture methods

Yeast strains used in this study are isogenic diploid derivatives of SK1 and are listed in Supplemental Table S1. Gene deletions or disruptions were introduced by mating or transformation and were confirmed by Southern blot analysis of genomic DNA. *Rec114* and *Mei4* were epitope-tagged by integrating a previously described *myc8-URA3-myc8* cassette (Kee et al. 2004) at the 3' ends of the open reading frames at their normal genomic loci. Correct targeting of tagging constructs was verified by PCR and Southern blot analysis of genomic DNA.

Two-hybrid fusions of DSB proteins to the transcription activating domain of Gal4 (Gal4AD) or the DNA binding domain of bacterial LexA were constructed as described (Arora et al. 2004). Serial truncations and internal deletions of *REC114* and *MEI4* were generated by PCR amplification of desired portions of the open reading frames and ligation into pACT2 or pCA1, respectively (vector descriptions were previously provided (Arora et al. 2004)). For *REC102* expression constructs for return-to-growth analysis, either full-length or the open reading frame in exon 2 were amplified by PCR using either genomic DNA or cDNA from an SK1 strain, as appropriate. The amplification products were cloned into the previously described 2 μ *TRP1* vector pCA11 (Arora et al. 2004) under the control of the constitutive *ACT1* promoter. Plasmid inserts were confirmed by DNA sequence analysis.

Meiotic cultures were prepared by growing yeast cells in liquid YPA (1% yeast extract, 2% Bacto Peptone, and 1% potassium acetate) for 13.5 hr at 30°C. The cells were then washed and resuspended in the indicated sporulation medium (either 2% potassium acetate or SPM, which is 0.3% potassium acetate and 0.02% raffinose) and incubated at 30°C with vigorous aeration. Return-to-growth assays for recombination between *arg4-N* and *arg4-B* heteroalleles were conducted as described (Diaz et al. 2002).

Two-hybrid, RT-PCR, and protein analyses

Two-hybrid assays were conducted as described previously (Arora et al. 2004). Briefly, the two-hybrid reporter strains in the SK1 background contain *E. coli lacZ* preceded by two LexA binding sites integrated at the *URA3* locus. The strains also carry the *ndt80* mutation, which causes arrest of cells in prophase of meiosis I (Xu et al. 1995). Two-hybrid fusion constructs were introduced individually into haploid reporter strains by lithium acetate transformation (Gietz and Woods 1998), and then strains were mated in appropriate pairwise combinations. The resulting diploids were assayed for LacZ expression after vegetative growth in selective medium lacking tryptophan and leucine, or after culturing in SPM for ≥ 12 hr at 30°C.

For RT-PCR, total RNA was prepared from meiotic cultures 6 hr after transfer to sporulation medium (2% potassium acetate) using the RNeasy kit according to manufacturer instructions (Qiagen). First-strand cDNA synthesis was carried out with AMV reverse transcriptase according to manufacturer instructions (Invitrogen SuperScript III). PCR primers for amplification of *REC102* cDNA were 5'-GTTGTGGCGCTGTAAATAATG and 5'-AATCATGGAGAAGACCATCGG; *MER2* primers were 5'-ATTCTCCCACAGTGGGAAAGT and 5'-TAGGCGTGATCTGCCTTTTCT.

Protein extracts were prepared by lysing cells with glass beads in SDS sample buffer or in 20% cold trichloroacetic acid, as described (Kee et al. 2004). Samples were analyzed by SDS-PAGE and western blotting with chemiluminescent detection. Primary antibodies were mouse monoclonal anti-myc (1:1000, Covance), mouse monoclonal anti-HA clone F7 (1:1000, Santa Cruz), rat monoclonal anti-tubulin (1:500; Harlan Sera-Lab, Ltd.), or affinity-purified polyclonal goat anti-LexA (1:500, Santa Cruz). Peroxidase-conjugated secondary antibodies

were goat anti-mouse IgG (1:10,000; Jackson Laboratory), and donkey anti-goat IgG and donkey anti-rat IgG (both 1:1000; Accurate Chemicals and Scientific Corp., NJ).

Cytology

Meiotic chromosome spreads were prepared using a modified version (Kee et al. 2004) of a method originally described by (Loidl et al. 1998). For indirect immunofluorescence staining the following antibodies were used: primary antibodies were mouse monoclonal anti-myc (1:1000 dilution, Covance); rat monoclonal anti-HA (1:100, Boehringer); rabbit polyclonal anti-Red1 (a generous gift of G.S. Roeder, Yale Univ.; 1:200); or guinea pig polyclonal anti-GSTZip1 (1:500; K. Henderson, this laboratory). Secondary antibodies were from Molecular Probes and were used at 1:1000 dilution: goat anti-mouse Alexa-488; goat anti-rat Alexa-594; donkey anti-rabbit Alexa-594; goat anti-guinea pig Alexa-546. Slides were mounted with cover slips in Prolong antifade (Molecular Probes) containing 50 ng/ml DAPI. Images were captured on a Zeiss Axiophot microscope with a 100x objective using a Cooke Sensicam cooled CCD camera. Data capture and image processing were performed using the Slidebook software package (Intelligent Imaging Innovations). Colocalization of Rec114myc and Mei4myc with Rec8-HA was quantified as described (Kee et al. 2004). Briefly, from visual inspection of the fluorescence images captured by the CCD camera, we set thresholds to define individual pixels as positive or negative for anti-HA (Rec8). The total anti-myc fluorescence signal over background was measured (Mei4myc or Rec114myc), and then the fraction of this signal that lay within Rec8-positive pixels was calculated (“% overlap”). To estimate the contribution of fortuitous overlap, the process was repeated after the anti-myc fluorescence image was rotated 180°. As we previously described (Kee et al. 2004), control experiments demonstrated ~90% overlap between Rec8 and Zip1 in this type of analysis (data not shown). Alignment of the fluorescence light paths for our microscope was assessed by imaging 0.2 µm diameter fluorescent beads (Tetraspek, Molecular Probes), as described (Kee et al. 2004).

Results

Association of Mei4 and Rec114 with meiotic chromosomes

Tagged versions of Mei4 and Rec114 were generated with 8 copies of the myc epitope fused to their C termini and were expressed from their normal promoters at the endogenous loci. Rec114myc8 appeared fully functional in vivo: intragenic recombination frequencies at *arg4* in return-to-growth assays were 0.076 ± 0.023 in *REC114myc8* vs. 0.079 ± 0.020 for wild type (prototrophs per viable cell, mean $\pm$ s.d., N=3 cultures) and spore viability was 97% vs. 98% for wild type (≥ 70 tetrads). Mei4myc8 supported high levels of meiotic recombination but was not fully normal: intragenic recombination frequencies at *arg4* were 0.052 ± 0.012 (66% of wild type) and spore viability was 79% (107 tetrads). Expression of both proteins was meiosis-specific, as expected (Fig. 1a). Both proteins accumulated to similar steady-state levels (compared to wild type) in mutant strains lacking each of the other DSB proteins (Fig. 1b).

To define association of these proteins with meiotic chromosomes, spread nuclei were double-stained with antibodies to the myc epitope and to Zip1, the protein that makes up the transverse filaments of the synaptonemal complex (SC) (Sym et al. 1993). The stage in meiotic prophase can be determined for any given nucleus based on the extent of SC formation (Fig. 2). Both Mei4 and Rec114 localized to chromosomes at or prior to leptotema (i.e., before formation of detectable SC; Fig. 2a, e). This result places these proteins on chromatin at or prior to DSB formation. Both proteins showed a punctate or patchy staining pattern that increased in intensity as cell progressed through zygonema (Fig. 2b, f) and persisted into pachynema (Fig. 2c, g). For both proteins, two types of pachytene nuclei were observed: those with strong staining for Rec114 or Mei4 (Fig. 2c, g) and those with weak or no staining (Fig. 2d, h). Strong- and weak-staining nuclei often lay side by side in the same microscope field, so the difference is unlikely

to be an artifact of the spreading or immunostaining. This pattern is similar to that observed earlier for Rec102 and Rec104 (Kee et al. 2004), and we infer that the Rec114-negative or Mei4-negative pachytene spreads represent a later sub-stage in pachynema. Consistent with this interpretation, meiotically dividing nuclei were also negative for Rec114 and Mei4 (data not shown). Interestingly, however, the decrease in chromatin-bound Rec114 and Mei4 in late prophase appears not to reflect degradation of these proteins because steady-state levels remained high well past the time of the meiotic divisions (Fig. 1a).

While this work was in progress, an independent study demonstrated that Mei4 and Rec114 localize to chromosomes in discrete foci or patches, and that the extent of staining for both proteins declines with progression through prophase (Li et al. 2006). This earlier study was conducted with different tagged versions of these proteins (Rec114myc3 and Mei4-GFP) and in a different strain background (BR). The good agreement between the two sets of findings confirms and reinforces the significance of the observed patterns for these proteins.

Spatial organization of Mei4 and Rec114 on chromosomes

Meiotic chromosomes are organized into linear arrays of chromatin loops emanating from a proteinaceous axis along each chromatid (reviewed in Moens et al. 1998; Zickler and Kleckner 1999; van Heemst and Heyting 2000). Sister chromatid axes are closely joined and, at pachynema, are synapsed along their lengths with the axes of the homologous sister pair to form the SC. Cohesins are associated with chromosome axes, whereas DSB formation is thought to occur preferentially in DNA sequences in the chromatin loops (Klein et al. 1999; Blat et al. 2002). Indeed, an inverse correlation has been observed between sites of DSB formation and sites enriched for cohesin binding (Gerton et al. 2000; Blat et al. 2002; Mieczkowski et al. 2006).

Immunofluorescence signals for both Mei4 and Rec114 showed only partial overlap with Zip1 in zygotene and early pachytene cells, suggesting that a significant fraction of both proteins was located on chromatin loops rather than on chromosome axes (Fig. 2b, c, f, g). To address this issue in more detail, we compared localization of Mei4 and Rec114 with that of Rec8, a meiosis-specific cohesin subunit that is axis-associated prior to SC formation (Klein et al. 1999; Blat et al. 2002; Eijpe et al. 2003). By this criterion as well, much of the Mei4 and Rec114 appeared not to be localized to chromosome axes (Fig. 2i, j).

Because staining patterns for Mei4 and Rec114 were often irregular rather than always forming discrete foci, we evaluated overlap of these proteins with Rec8 on a pixel-by-pixel basis in the images recorded by the CCD camera (for more detail, see Materials and Methods and Kee et al. 2004). By this method, $54.3 \pm 6.4\%$ of the total Mei4 immunofluorescence signal overlapped with the chromosome axes in late zygonema or early pachynema (mean $\pm$ s.d., $N=10$ nuclei; Fig. 2k). For Rec114, the overlap was $48.1 \pm 7.6\%$ ($N=10$; Fig. 2k). Difference between the two proteins was not statistically significant ($p=0.065$, unpaired t test). Put another way, approximately half of the chromosome-bound population of both proteins was clearly distinct from the cohesin-defined axes. To test whether the remaining fraction that did overlap with Rec8 might reflect fortuitous colocalization of two unrelated protein distributions within the confined space of a chromosome spread, we also measured overlap in the same nuclei after rotating the anti-myc image 180° . The rotated Rec114 spreads showed similar levels of overlap as the true images ($47.2 \pm 5.4\%$; $p=0.744$) (Fig. 2k). Overlap in the rotated Mei4 spreads was significantly reduced compared to the true images, but was still substantial ($44.0 \pm 7.6\%$, $p=0.004$) (Fig. 2k). These findings suggest that much of the colocalization of Mei4 and Rec114 with the axes may be due to fortuitous overlap. Because of limits on the resolution of light microscopy, this study cannot rule out the possibility that there are truly axis-associated subpopulations of these proteins, but it appears that most if not all of Mei4 and Rec114 is bound to chromatin loops.

Genetic requirements for chromosome association

Neither the chromosomal association nor nuclear localization of Rec114 were affected by mutations in any of the other DSB genes, suggesting that Rec114 binds independently to chromatin (data not shown). These results agree with earlier findings by Li et al. (2006). In contrast, normal subcellular localization of Mei4 was dependent on the presence of Rec114 and Mer2. In the absence of either of these proteins, Mei4 association with chromosomes was greatly reduced (Fig. 3). These effects were specific because Mei4 localization to chromosomes was indistinguishable from wild type in each of the other DSB-defective mutants (Fig. 3 and data not shown). These findings differ from those of Li et al. (2006), who found that chromosomal association of a different tagged version of Mei4 (Mei4-GFP) was unaffected by *rec114* mutation and only slightly reduced in a *mer2* mutant. Possible reasons for these differences are addressed in the Discussion.

Amino acid sequence alignments of highly diverged yeast Mei4 and Rec114 homologs

No motifs to suggest biochemical function have been identified in Mei4 or Rec114. To identify conserved regions of the proteins, we aligned homologs of Mei4 and Rec114 from yeasts closely related to *S. cerevisiae* (specifically, subphylum Saccharomycotina, which includes the genera *Saccharomyces*, *Kluyveromyces*, *Ashbya/Emothecium*, and *Candida*). Rec114 and Mei4 are highly divergent. For example, in the very closely related species *S. cerevisiae* and *S. paradoxus*, Mei4 and Rec114 homologs share only 84.1% and 73.4% amino acid identity, respectively (Malone et al. 1997; Keeney 2007). In contrast, the nucleotide sequence across all coding regions in these species is >88% identical and ~65% of proteins have ≥90% amino acid identity (Cliften et al. 2001). Perhaps because of this divergence, we have been unable to identify clear homologs of these proteins in most organisms other than those related to *S. cerevisiae*. Similar divergence is seen for the other meiosis-regulated DSB proteins (Nau et al. 1997; Jiao et al. 2002; Henderson et al. 2006; Keeney 2007), but this feature is not unique to the DSB proteins, as it has been observed that meiotic recombination proteins in general tend to be among the most rapidly diverging groups of proteins in the cell (see Ramesh et al. 2005; Richard et al. 2005).

Mei4 homologs showed relatively weak similarity spread throughout the length of the proteins, with no extended regions of identical sequences. Fig. S1a shows an example of a ClustalW output for illustrative purposes. Other alignment methods yielded similar overall results, but with only partial agreement about precisely which residues were conserved (data not shown). The most distant pairwise comparisons showed only ~15-23% identity (data not shown).

Rec114 alignments showed three discrete regions of relatively well conserved sequence separated by large regions with little or no conservation (Fig. S1b, c). *S. cerevisiae* *REC114* has an intron near the 3' end (Malone et al. 1997; Juneau et al. 2007), a placement that is very rare in yeast (Lopez and Seraphin 1999; Lopez and Seraphin 2000). This intron splits the C-terminal conserved domain and is found in many of the *REC114* homologs (of the homologs shown, only those from *C. glabrata* and *S. castellii* lack the intron) (arrow in Fig. S1c). Modest sequence similarity has been described between Rec114 and Rec7 (Malone et al. 1997; Molnar et al. 2001), a protein required for initiation of meiotic recombination in *Schizosaccharomyces pombe* (Davis and Smith 2001; Young et al. 2004; Lorenz et al. 2006). The main region of similarity centers on the sequence RFQ (residues 109-111 in Rec114 and 93-95 in Rec7). This triplet is well conserved in Rec114 homologs (asterisk in Fig. S1b). However, the larger conserved motifs in Rec114 are not clearly identifiable in the Rec7 sequence (data not shown).

Deletion mapping of protein domains required for interaction between Mei4 and Rec114

In systematic analysis of two-hybrid interactions among the DSB proteins, Mei4 and Rec114 showed one of the highest LacZ signals (up to 600-900 fold over background) (Arora et al.

2004). This interaction was observed in a vegetatively growing reporter strain, thus was independent of other meiosis-specific proteins or meiosis-specific posttranslational modifications. In support of the physiological significance of this interaction, Mei4 and Rec114 have also been shown to interact in meiotic cells by co-immunoprecipitation from whole-cell extracts and colocalization on meiotic chromosomes (Li et al. 2006) (MJN, unpublished observations).

We further characterized this interaction by two-hybrid analysis of truncated Mei4 and Rec114 proteins. We constructed serial deletions of ~50 amino acid segments from the N and C termini and from central regions of each protein, then assayed for interaction of each truncated version with its full-length partner. When the N-terminal 48 amino acids of Mei4 were removed, interaction signal with Rec114 was reduced ~8-fold (Fig. 4a, Mei4(49-408)). Deletion of up to 165 amino acids reduced the interaction further (>50-fold decreased relative to full length), although LacZ expression remained significantly above background (Fig. 4a, Mei4(166-408)). Importantly, the interaction-defective truncations were expressed at levels comparable to the full-length LexA fusion protein (Fig. 4b). In contrast to the N-terminal truncations, neither a C-terminal deletion of up to 162 amino acids nor internal deletion of residues 166-246 had significant effect on the two-hybrid signal (Fig. 4a). Taken together, these results suggest that the N-terminal region of Mei4 (~1-165) is necessary and sufficient for robust two-hybrid interaction with Rec114. The C terminal half of Mei4 is dispensable for this interaction, but may contain a weaker interaction ability.

For Rec114, deletion of up to 152 amino acids from the N terminus or 202 amino acids from the C terminus had little effect on interaction with Mei4 (Fig. 4c, Rec114(153-428) and Rec114(1-227)). These results indicate that both the N-terminal and C-terminal regions of Rec114 are sufficient by themselves to support interaction with Mei4, suggesting the possibility that more than one region of Rec114 makes contact with Mei4. In contrast, the middle ~one-third of Rec114 was neither necessary nor sufficient for interaction with Mei4 (Fig. 4c). Thus, the poorly conserved central portion of Rec114 appears not to play a significant role in the interaction between these two proteins. (Note that the interaction-defective fusion proteins were expressed at even higher steady-state levels than the full-length protein; Fig. 4d).

Essential sequence encoded by a previously unrecognized 5' exon in REC102

Whereas several studies document physical and functional interactions connecting Ski8, Rec102, and Rec104 to Spo11, thus far relatively few direct connections are known to link Mer2, Mei4, and Rec114 to these other factors (Salem et al. 1999; Kee and Keeney 2002; Jiao et al. 2003; Arora et al. 2004; Kee et al. 2004; Henderson et al. 2006; Li et al. 2006). Recent findings concerning the structure of the *REC102* gene help to address this lack.

While this work was in progress, a curator of the *Saccharomyces* Genome Database alerted us to the likely presence of a previously unsuspected intron and 5' exon in *REC102* (D. Fisk, pers. commun.), identified from comparative genomic analyses of *Saccharomyces* species (Cliften et al. 2001; Cliften et al. 2003; Kellis et al. 2003). Inspection of DNA sequence alignments revealed that the codon corresponding to the originally annotated initiator ATG (Jiao et al. 2002) was GTT in the *S. mikatae REC102* homolog, with no in-frame ATG nearby (data not shown). It was noted, however, that all of the sequenced *REC102* homologs from *Saccharomyces* species contained matches to 5' and 3' splice signals and a branch point sequence (respectively 115, 21, and 69 bp upstream of the originally annotated ATG in *S. cerevisiae*), as well as a conserved ATG at the beginning of a potential coding exon (289 bp upstream). The updated genomic structure for *S. cerevisiae REC102* predicts 174 bp of coding sequence in the 5' exon, a 97 bp intron, and 618 bp of coding sequence in the 3' exon (Fig. 5a). The predicted mature mRNA encodes an additional 63 amino acids at the N-terminus of the protein. The amino acid sequence encoded by the 5' exon is about as well conserved among

Saccharomyces species as is the full-length Rec102 protein (Fig. 5b) and includes several invariant residues (Fig. S1d and data not shown).

To confirm this predicted intron/exon structure, we amplified *REC102* message by RT-PCR using primers flanking the putative intron. An RT-PCR product of the size expected for the spliced mRNA was observed (Fig. 5c), and the expected sequence at the splice junction was confirmed by sequencing of the RT-PCR product (data not shown). Splicing of the *REC102* message was also demonstrated by large-scale microarray and RT-PCR analyses in recent independent studies (Miura et al. 2006; Juneau et al. 2007). Interestingly, splicing was relatively inefficient in that the intron had been removed in only ~70% of the amplified message from RNA samples isolated 6 hr after transfer to sporulation medium (Fig. 5c). Unlike for the *MER2* transcript, *REC102* mRNA splicing efficiency was not affected by a *mer1* mutation (Fig. 5c), as expected because the *REC102* intron lacks a match to the consensus binding sequence for Mer1 (Spingola and Ares 2000). More surprising, however, splicing was frequently inaccurate, as ~40% of the spliced message had a splice junction indicative of use of an alternative 3' splice signal (UAG in the transcript) located 29 nucleotides upstream of the correct splice signal (Fig. 5a, c). This alternative splicing disrupts the *REC102* reading frame and places an in-frame stop codon just after the end of exon 1. The inefficiency and inaccuracy of *REC102* mRNA splicing are addressed further in Discussion.

We also tested whether exon 1 is required for *REC102* function in vivo. Plasmids were constructed to express the originally annotated *REC102* ORF (i.e., starting with the ATG in exon 2), full-length *REC102* containing the intron, or a cDNA copy of full-length *REC102*, all under the control of the constitutive *ACT1* promoter. The plasmids were tested for complementation of the *rec102Δ* recombination defect in return-to-growth experiments. The genomic and intronless versions of full-length *REC102* fully complemented *rec102Δ*, whereas the version lacking exon 1 was indistinguishable from a vector control (Fig. 5d). Thus, sequences encoded by exon 1 are essential for *REC102* function in meiotic recombination. The previously annotated version lacking exon 1 will be referred to hereafter as *rec102-ΔN* to reflect that fact.

Requirement for the *REC102* 5' exon for physical interactions with other DSB proteins

In several previous studies, epitope-tagged versions of Rec102 were generated by integration of the tagging constructs at the 3' end of the *REC102* genomic locus (Kee and Keeney 2002; Jiao et al. 2003; Kee et al. 2004; Li et al. 2006). These constructs were functional because they still had the intron and 5' exon. However, previous attempts to assess interactions of Rec102 with other proteins used two-hybrid fusions containing only the *rec102-ΔN* portion of the coding sequence. No strong two-hybrid signals involving Rec102-ΔN in either vegetative or meiotic cells were observed, with at best only modest signals over background for interaction of Rec102 with Rec104 (Arora et al. 2004; Kee et al. 2004). Because *rec102-ΔN* was not sufficient for meiotic recombination in vivo, we repeated our previous two-hybrid analysis using fusion constructs containing full-length *REC102*.

Notably, Rec102 demonstrated a robust interaction signal with Rec104 (Fig. 5e). This interaction was readily observed in the vegetatively growing reporter strain, so it does not require the presence of any of the other meiosis-specific DSB proteins. We previously observed that many of the interactions between DSB proteins were orientation-specific in that they were observed in only one or a few of the eight possible pairwise combinations of N-terminal and C-terminal LexA and Gal4AD fusions (Arora et al. 2004). In contrast, the Rec102-Rec104 interaction was relatively insensitive to orientation, yielding LacZ signals ranging from 20-fold to 600-fold over background in six of the eight combinations of orientations (only combinations involving Rec102-LexA failed to support interaction; Fig. 5e and data not shown). These findings agree well with numerous lines of evidence indicating that these

proteins work together as a functional unit (Salem et al. 1999;Kee and Keeney 2002;Jiao et al. 2003;Kee et al. 2004).

More importantly, the use of full-length Rec102 also revealed a new interaction with Rec114 that was detectable in a vegetatively growing reporter strain (Fig. 5f) as well as previously undetectable meiosis-specific interactions with Spo11 and with Mei4 (described in more detail in the next section). Fig. 5g summarizes these new two-hybrid interactions along with the most robust interactions from our previous study. The strong dependence of these interactions on sequences in exon 1 of *REC102* may be sufficient to explain the recombination defect of *rec102-ΔN* strains.

Genetic requirements for meiosis-specific two-hybrid interactions among DSB proteins

Many of the two-hybrid interactions previously described between DSB proteins were meiosis-specific, i.e., they were not observed when the reporter strain was growing vegetatively but could be observed when the strain was induced to enter meiosis (Fig. 5g) (Arora et al. 2004). This pattern could reflect a requirement for meiosis-specific posttranslational modification or, alternatively, stabilization or indirect mediation of interactions through binding of an endogenous protein(s) that is only expressed in meiosis. To address the latter possibility, we characterized the genetic requirements for the meiosis-specific two-hybrid interactions diagrammed in Fig. 5g, including those involving full-length Rec102. For each interaction, we measured expression of the LacZ reporter in wild type and in strains in which each of the other meiosis-induced DSB genes had been deleted in turn (*SPO11*, *REC102*, *REC104*, *MEI4*, *MER2*, or *REC114*). For a given interaction, we did not delete the endogenous genes for the proteins being tested.

Interaction of Spo11-Ski8 with Rec102-Rec104—When *REC104* was deleted, Spo11 was no longer able to interact with Rec102 (Fig. 6a). We previously demonstrated the reciprocal result, i.e., that meiosis-specific interaction of Spo11 with Rec104 required endogenous Rec102 (Fig. 6b) (Arora et al. 2004). We also demonstrated previously that Ski8 interaction with Rec104 required endogenous Spo11 and Rec102 (Fig. 6c) (Arora et al. 2004). These results are consistent with other studies implicating Rec102 and Rec104 as a functional unit (see above). In contrast, *MEI4*, *REC114*, and *MER2* were dispensable for interaction of Spo11 with either Rec102 or Rec104 (Fig. 6a, b). Likewise, Ski8 was still able to interact with Rec104 in the absence of *MEI4*, *REC114* or *MER2*, although LacZ signal was reduced ~2.5-fold in the *rec114Δ* strain (Fig. 6c). These findings further emphasize functional distinctions between the DSB proteins.

Interaction of Mei4 with Rec102-Rec104—LexA-Mei4 interacted with both Rec102 and Rec104 (Fig. 6d, e). Both interactions were affected similarly by deletions of other DSB genes, again emphasizing the similar behaviors of Rec102 and Rec104. However, particular gene deletions differed in unusual ways. In a *rec114Δ* strain, interactions with both Rec102 and Rec104 were abolished, suggesting that these interactions are stabilized or mediated by endogenous Rec114 (Fig. 6d, e). In contrast, both interactions gave a LacZ signal that was elevated ~2-fold in a *mer2Δ* strain (Fig. 6d, e). Strikingly, deleting *MER2* had the same relative effect on both the Mei4-Rec102 and Mei4-Rec104 interactions despite ~5-fold differences in the absolute strength of the LacZ signals (compare the β-galactosidase units for Fig. 6d and e). The reason for this stimulation of the two-hybrid signal is not clear. One possibility is that Mer2 antagonizes or competes with Rec102-Rec104 for binding to Mei4. Alternatively, presence of endogenous Mer2 may inhibit the ability of the two-hybrid fusion constructs to interact with basal transcription machinery.

Strains carrying *spo11Δ*, *rec102Δ*, or *rec104Δ* mutations showed a very different pattern. In these strains, the Mei4-Rec102 and Mei4-Rec104 combinations no longer activated LacZ expression significantly above controls containing the LexA-Mei4 fusion alone (Fig. 6d, e). Because none of these mutants supports a specific two-hybrid interaction signal, we infer that a higher order complex containing Spo11, Rec102, and Rec104 is necessary for interaction with Mei4. Note, however, that LacZ expression induced by LexA-Mei4 alone was elevated ~5-fold in these mutants, with each mutant yielding approximately the same absolute LacZ expression level (Fig. 6d, e). Interestingly, this elevated meiotic lacZ signal was quantitatively similar to the signal that LexA-Mei4 alone gives when the wild-type reporter strain is growing vegetatively (Arora et al. 2004, and data not shown). We interpret these unusual findings as follows. It appears that the LexA-Mei4 fusion is capable of low-level activation of the *lacZ* reporter in vegetative cells, but this inherent ability to activate transcription is inhibited during meiosis. In the absence of *SPO11*, *REC102*, or *REC104*, however, meiosis-specific inhibition of the LexA-Mei4 transcriptional activity does not occur. Implications of this pattern are discussed below.

Interaction of Mer2 with Rec114—Mer2 and Rec114 showed a two-hybrid interaction only in meiotic cells (Arora et al. 2004). Because both proteins interacted individually with Mei4 in a vegetative reporter strain, it was possible that the meiosis-specific Mer2-Rec114 interaction was bridged or stabilized by endogenous Mei4 protein. However, we found that Mer2 still interacted with Rec114 in a *mei4Δ* strain, although with a LacZ signal reduced ~2-fold (Fig. 6f). Similar two-hybrid signals were obtained in each of the other mutants tested (Fig. 6f). These results reveal that Mer2 and Rec114 can interact in meiotic cells independent of the other meiosis-specific DSB proteins, although the reason for the attenuation of the two-hybrid signal in the mutants is not clear. Mer2 is phosphorylated by cyclin-dependent kinase during meiotic prophase, and mutant Mer2 protein lacking the CDK target sites was no longer able to interact with Rec114 (Henderson et al. 2006). Together with the current observations, these findings suggest that meiosis-specificity of the Mer2-Rec114 interaction can be attributed largely to meiosis-specific posttranslational modification rather than a requirement for the presence of one or more of the meiosis-specific DSB proteins.

Discussion

Spo11 requires nine other proteins in order to make DSBs to initiate meiotic recombination, but the functions of these proteins and the interactions among them remain poorly understood. We report here further characterization of interactions of Mei4 and Rec114 with chromosomes, with each other, and with other DSB proteins including a newly annotated full-length Rec102. These studies confirm and extend conclusions from other recent studies about the physical and functional interactions that interconnect the DSB proteins.

Functional subgroups among the DSB proteins, and the network of interactions that connects them

The findings in this study reinforce the view that has emerged from several lines of inquiry that there are at least four functionally distinct subgroups among the DSB proteins: Spo11-Ski8; Rec102-Rec104; Mer2-Mei4-Rec114; and Mre11-Rad50-Xrs2 (see Introduction) (Ohta et al. 1998; Jiao et al. 2003; Arora et al. 2004; Kee et al. 2004; Prieler et al. 2005; Henderson et al. 2006; Li et al. 2006; Sasanuma et al. 2007). To date, there is little support for the possibility that all of the DSB proteins together form a discrete “DSB holoenzyme,” although available data cannot rule out this possibility.

Among the meiotically induced DSB proteins, Rec102 and Rec104 share perhaps the most intimate relationship with one another: they interact physically and genetically, they are

mutually interdependent for proper chromosome localization, their behaviors are affected (or not) in parallel ways by mutations in other genes, and absence of either one exerts the same kind of effect (or not) on the behavior of other proteins (Salem et al. 1999; Jiao et al. 2003; Arora et al. 2004; Kee et al. 2004; Prieler et al. 2005; Henderson et al. 2006; Li et al. 2006; Sasanuma et al. 2007). The yeast two-hybrid results presented here further support this conclusion. Specifically, *rec102* and *rec104* mutations had identical effects on other two-hybrid interactions, and in turn interactions involving either Rec102 or Rec104 responded in parallel fashion to mutations in other DSB genes.

Several lines of evidence link Mer2, Mei4, and Rec114 to one another as a group that is functionally distinct from the other DSB proteins (Fig. 5g). First, physical interactions among them are revealed by co-immunoprecipitation in pairwise combinations, significant colocalization on spread meiotic chromosomes, and extensive two-hybrid interactions with one another independent of other DSB proteins (this study and Arora et al. 2004; Henderson et al. 2006; Li et al. 2006). Second, functional interdependence is revealed by effects of *mer2* and *rec114* mutations on normal chromosomal localization of Mei4 and by effects of *rec114* mutation on the meiosis-specific two-hybrid interaction of Mei4 with Rec102 and Rec104 (this study and Li et al. 2006). Third, unlike Spo11 and Ski8, none of the Mer2-Mei4-Rec114 group is required for normal chromosomal association of Rec102 and Rec104, and none is required for physical association of Rec102-Rec104 with Spo11 or Ski8 (this study and Arora et al. 2004; Kee et al. 2004).

Nevertheless, there are also significant differences between Mer2, Mei4, and Rec114 that indicate that the three proteins do not behave as a single functional unit in the same way Rec102 and Rec104 appear. For example, each pair is able to physically interact in the absence of the third, as assessed by co-immunoprecipitation, two-hybrid analysis, and colocalization on spread chromosomes (this study and Li et al. 2006). Rec114 and Mer2 interact with several other protein partners independently (revealed, e.g., by two-hybrid interactions in vegetative cells and/or in mutant strains in meiosis) (this study and Arora et al. 2004; Henderson et al. 2006). Moreover, Mer2 and Rec114 each localize to chromosomes independently of the other members of the group, and indeed Mer2 associates with chromosomes even in vegetative cells (this study and Henderson et al. 2006; Li et al. 2006). Finally, Rec114 but not Mei4 or Mer2 is required for association of Spo11 with DSB hotspot sequences as assessed by chromatin immunoprecipitation (Prieler et al. 2005) and for the ability of Gal4BD-Spo11 to recruit other Spo11 molecules to a Gal4 binding site on chromatin (Sasanuma et al. 2007). The available data thus reveal a complex set of partially dependent and partially independent relationships between these proteins.

Physical and functional connections of Rec102-Rec104 with Spo11 are well established (Salem et al. 1999; Kee and Keeney 2002; Jiao et al. 2003; Arora et al. 2004; Kee et al. 2004; Prieler et al. 2005; Sasanuma et al. 2007). In contrast, it has been less clear how Mei4 and Rec114 are connected to Spo11, and thus how they are able to influence DSB formation. Several features of the analysis presented here suggest that the Rec102-Rec104 complex is a bridge that connects Mei4 and Rec114 to Spo11-Ski8 (Fig. 5f, g). Both Rec104 and Rec102 interacted with Rec114 in vegetatively growing cells (i.e., in the absence of other meiosis-induced DSB proteins), raising the possibility that Rec102 and Rec104 may both directly contact Rec114. Thus, the Rec102-Rec104 complex appears to interact both with Rec114 and with Spo11. Rec104 and Rec102 also interacted with Mei4 in a meiosis-specific fashion, dependent on both Rec114 and Spo11 (Fig. 6d, e). These findings are consistent with the hypothesis that interaction of Rec102-Rec104 with Mei4-Rec114 occurs in conjunction with Spo11. This interpretation is also consistent with the unusual patterns we observed for the background LacZ expression induced by LexA-Mei4 (see Results; Fig. 6d, e). Moreover, this hypothesis is consistent with the

observation that both Rec114 and the Rec102-Rec104 complex are required for binding of Spo11 to hotspots (Prieler et al. 2005; Sasanuma et al. 2007).

These observations provide a more detailed picture of interactions among the different DSB proteins, but many questions remain. For example, whereas data discussed above connect Rec114 and Mei4 to Spo11 and Rec102-Rec104, other data fail to support this connection. For example, Mer2 colocalized extensively and co-immunoprecipitated with Mei4 and Rec114, but not with Rec102 (Li et al. 2006). One possibility is that a Mer2-Mei4-Rec114 complex interacts with Rec102 only transiently or only in cytologically invisible subpopulations. Another possibility is that the proteins interact at different times, e.g., Mei4-Rec114 might interact with Mer2 or with Rec102-Rec104, but not both simultaneously. Such a scenario might account for the apparently antagonistic effect of Mer2 on interaction of Mei4 with Rec102-Rec104 (Fig. 6d, e).

Association of Mei4 and Rec114 with chromosomes

Mei4 and Rec114 localize to meiotic chromosomes in leptotema (this study and Li et al. 2006), placing both proteins on chromatin at or prior to DSB formation. Much if not all of the Mei4 and Rec114 localizes to chromatin domains that are spatially distinct from chromosome axes, suggesting that these proteins bind preferentially to the chromatin loops. DSBs are thought to form preferentially in DNA sequences in the loops (e.g., Blat et al. 2002), so these findings are consistent with a correlation between the sites of DSB formation and localization of Mei4 and Rec114. The potential Rec114 ortholog in *S. pombe*, Rec7, also localizes to chromosomes, but Rec7 foci appear closely associated with chromosome axes and these foci do not form in mutants defective for the axis-associated protein Rec10 (Lorenz et al. 2006). These differences may reflect the closer functional dependency between DSB formation and the cohesin-containing axes in *S. pombe* (Ellermeier and Smith 2005; Loidl 2006; Lorenz et al. 2006; Wells et al. 2006).

Mei4 and Rec114 remained bound to chromatin past the time of DSB formation (this study and Li et al. 2006), a pattern that has also been observed for *S. cerevisiae* Spo11, Rec102, Rec104, Mer2, and Ski8 (Arora et al. 2004; Kee et al. 2004; Prieler et al. 2005; Henderson et al. 2006; Li et al. 2006), and has been described for Ski8 and/or Spo11 in other organisms as well (Romanienko and Camerini-Otero 2000; Storlazzi et al. 2003; Tessé et al. 2003). It is not yet clear whether this persistence reflects additional functions after DSB formation. It is also not yet known what controls the dissociation of these proteins from chromosomes, which in most cases is nearly complete by the end of pachynema.

We found that Rec114 localized to chromosomes independently of the other DSB proteins, in agreement with prior analysis in a different strain background (Li et al. 2006). However, we found that association of Mei4myc with chromosomes was substantially reduced in *rec114* and *mer2* mutants, whereas Li et al. found that localization of a Mei4-GFP construct was only partially reduced in a *mer2* mutant and was unaffected by *rec114* mutation (Li et al. 2006). The reasons for the differences are not yet certain. One possibility is the difference in tags, because localization of Mei4myc13 to chromosomes was reported to be completely dependent on *MER2* (Li et al. 2006), in contrast to Mei4-GFP but comparable to our findings with Mei4myc8. Because the *MEI4myc8* genomic construct used in our study appears to be only partially functional, it is possible that Mei4 association with chromatin is only partially dependent on Rec114 and Mer2, and that the myc tag further destabilizes Mei4 when these other proteins are missing. Differences in strain background and/or spreading methods between the studies could also contribute to variation in the findings. Despite the differences, however, it is important to stress that the studies agree that chromosomal association of Mei4 is at least partially defective in the absence of Mer2. Our results suggest that the same is true, albeit to a lesser extent, in the absence of Rec114.

An intron in *REC102*

REC102 has a previously uncharacterized 5' coding exon such that full-length Rec102 has 63 additional amino acids at its N terminus. The new annotation places the correct ATG 99 bp downstream of a previously identified *URSI* sequence which is required to repress expression of *REC102* in vegetative cells (Mitchell 1994; Jiao et al. 2002). The N-terminal sequence is essential for Rec102 function, at least partially because this sequence is essential for physical interactions with other proteins. These findings bring the total of intron-containing DSB genes to 4 out of the 6 meiosis-induced genes (only *SPO11* and *REC104* lack introns). Reasons for the relatively high prevalence of introns in these and other meiotic genes is not known. It has been suggested that this difference may simply reflect less opportunity (on an evolutionary time scale) or less selection pressure for meiotic transcripts to be subject to intron loss through reverse transcription and recombination (Malone et al. 1997). An alternative possibility was suggested by the observation that the majority of meiotic introns (including the one in *REC102*) show meiosis-specific regulation of their splicing efficiency (Juneau et al. 2007). In this model, there is selective pressure to maintain introns in meiotic genes because their inefficient splicing in vegetative cells serves as an additional layer of repression to prevent expression of proteins that might be deleterious in vegetative cells (Juneau et al. 2007). Notably in this regard, intron structure is conserved among the DSB proteins despite very poor overall amino acid conservation (Malone et al. 1997; Henderson et al. 2006). A final interesting feature is that *REC102* splicing is inaccurate, with a substantial fraction of the splicing events in vivo utilizing a 3' splice site that yields a transcript encoding a truncated protein. It is not yet clear why *REC102* is subject to these alternative splicing outcomes. In fact, it is not even clear why the proper 3' splice site is chosen as frequently as it is: the "incorrect" 3' splice signal is UAG, which in other introns would usually be highly preferred over the "correct" *REC102* site (which is AAG) (Umen and Guthrie 1995). It will be interesting to determine whether the meiotically regulated splicing efficiency and the unusual 3' splice site selection process for *REC102* are mechanistically related.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Epitope-tagged Mei4 and Rec114 proteins. **(a)** Western blot analysis of time courses of Mei4myc and Rec114myc expression. Migration positions of protein size standards are shown. **(b)** Steady-state levels of Mei4myc and Rec114myc in DSB-defective mutants. Whole-cell extracts at 4 hr in meiosis were analyzed by anti-myc western blot. Blots probed with anti-tubulin antibody served as loading controls.

Fig. 2. Temporal and spatial patterns of Mei4 and Rec114 association with chromosomes. **(a–d)** Localization of Mei4. **(e–h)** Localization of Rec114. Nuclear spreads from meiotic cultures of *MEI4myc* or *REC114myc* strains were stained with anti-Zip1 (red) and anti-myc (green) antibodies. Equivalent exposures of representative nuclei are shown, staged according to the extent of SC formation. **(i–k)** Localization of Mei4 or Rec114 with respect to chromosome axes. Nuclear spreads of *MEI4myc REC8HA* (**i**) or *REC114myc REC8HA* (**j**) strains were stained with anti-HA (red) and anti-myc (green) antibodies. Equivalent exposures of representative late zygotene nuclei are shown. **(k)** Quantification of the extent of overlap of Mei4myc or Rec114myc immunofluorescence with Rec8HA. Overlap was also assessed for

the same nuclei with the green fluorescence channel rotated 180° in order to estimate random colocalization. Error bars are mean $\pm$ s.d. Scale bars, 2 μ m.

Fig. 3.

Genetic requirements for stable chromosomal association of Mei4myc. Nuclear spreads were prepared from meiotic cultures of a *MEI4myc* strain (wild type) and *MEI4myc* strains carrying the indicated DSB gene mutations. Zip1 staining could not be used to stage the nuclei because DSB-defective mutants are defective for SC formation. Therefore, spreads were double-stained for Mei4myc (green) and a different chromosome structure protein, Red1 (red). Exposures equivalent to the ones in Fig. 2 are shown. Scale bar, 2 μ m.

Fig. 4.

Deletion analysis of the interaction between Rec114 and Mei4. **(a)** Serial deletions were constructed containing the indicated amino acid residues of Mei4 fused to the C terminus of LexA. For comparison, the schematic of *S. cerevisiae* Mei4 showing conservation with homologs from other yeasts is reproduced from Fig. S1. Each construct was assayed in vegetatively growing reporter cells for two-hybrid interaction with full-length Rec114. Units of β -galactosidase expressed when the LexA-Mei4 construct was paired with Gal4AD-Rec114 and when paired with empty Gal4AD vector ("Control") are shown (single measurement for each). **(b)** Western blot analysis of steady-state expression levels for full-length LexA-Mei4 and interaction-defective N-terminal deletions. Bands corresponding to LexA-Mei4 fusion proteins are marked with open circles; asterisks indicate cross-reacting bands. Extract from equivalent numbers of cells was loaded in each lane. **(c)** As for panel (a), except serial deletions of Rec114 fused to the C terminus of Gal4AD were assayed for interaction with full-length Mei4. **(d)** Western blots probed with anti-HA antibody to detect full-length and interaction-defective Gal4AD-Rec114 fusion proteins, with or without co-expression of LexA-Mei4 as indicated (LexA-Mei4 was not detected in this blot). Different amounts of extract were loaded because the smaller fusion proteins accumulated to higher steady-state levels than the full-length fusion. Gal4AD fusions (or Gal4AD alone) are marked with open circles; the asterisk marks a set of nonspecific cross-reacting bands apparent when larger amounts of extract were loaded. The reason the fusion proteins migrate as doublets or smears is unknown, but it may reflect proteolysis, translational read-through of the stop codon, and/or post-translational modification.

Fig. 5. *REC102* genomic structure and requirement for the 5' exon. **(a)** Schematic of *REC102* genomic locus. Exons are denoted by gray boxes and the intron by a horizontal line. Positions of the correct ATG and the previously annotated start codon (asterisk) are indicated. The circled area highlights the genomic sequence around the 3' end of the intron (lower case, intron sequence; upper case, coding sequence). The two alternative 3' splice signals are indicated. **(b)** Sequence conservation of Rec102 homologs. The amino acid sequence of *S. cerevisiae* Rec102 was compared to that of homologs from the indicated *Saccharomyces* species, both for the full-length protein and for just the sequence encoded in exon 1. **(c)** RT-PCR analysis of *REC102*. Total RNA was isolated from wild-type and *mer1Δ* strains six hr after transfer to sporulation

medium. *REC102* (left panel) and *MER2* (right panel) were amplified by RT-PCR with (+) or without (–) reverse transcriptase, as indicated. Negative images of an ethidium-stained gel are shown. PCR products from unspliced precursor RNA (P) and spliced mRNA (S) are shown. The bands marked by asterisks are template-switching artifacts generated by the reverse transcriptase (data not shown). **(d)** Exon 1 is required for *REC102* function. Return-to-growth assays were conducted in a *rec102Δ* strain carrying the indicated *REC102* sequences under the control of a constitutive promoter on a 2μ plasmid. Values are the mean ± sd for three independent cultures. Premeiotic recombinant frequencies are often lower for DSB-deficient strains because some Arg⁺ recombinants arise from cells that prematurely entered meiosis during the premeiotic growth regimen. **(e)** Two-hybrid interactions of full-length Rec102 with Rec104 were assayed in vegetative cultures in various orientations: 1, LexA-Rec102 × Gal4AD-Rec104; 2, LexA-Rec102 × Rec104-Gal4AD; 3, Rec104-LexA × Gal4AD-Rec102 (single measurement of each). Background controls contained the LexA fusion protein plus Gal4AD alone. For comparison, interaction of LexA-Rec102ΔN and Gal4AD-Rec104 yielded less than 0.8 units of β-galactosidase (Kee et al. 2004). **(f)** Two-hybrid interaction of full-length LexA-Rec102 with Gal4AD-Rec114 was assayed in vegetative cultures. Error bars are mean ± range for two cultures. **(g)** Two-hybrid interaction network, incorporating new Rec102 interactions (adapted from Arora et al. 2004). Gray arrows are interactions observed in a vegetative reporter strain. Black arrows are interactions observed only in a meiotic reporter strain. For clarity, the figure omits interactions among Rad50, Mre11 and Xrs2 and also omits several interactions that gave relatively weak LacZ signals (less than 10-fold over background) that have not been evaluated by independent methods (see Arora et al. 2004 for further details).

Fig. 6.

Genetic requirements for meiosis-specific two-hybrid interactions. Each reporter strain carried a deletion of one of the meiosis-specific DSB genes, as indicated. ("WT", wild type for the DSB genes. Note that all strains also carry the *ndt80* mutation to block meiosis in prophase I.) Dark gray bars are two-hybrid signals for LexA and Gal4AD fusions to the indicated DSB proteins; light gray bars are controls carrying the LexA fusion with an empty Gal4AD vector. Values are the mean $\pm$ range for 2–3 independent measurements. Variation was low enough that some error bars are not visible. Fusion orientations were as follows: (a) Spo11-LexA $\times$ Gal4AD-Rec102; (b) Spo11-LexA $\times$ Rec104-Gal4AD; (c) LexA-Ski8 $\times$ Rec104-Gal4AD; (d) LexA-Mei4 $\times$ Gal4AD-Rec102; (e) LexA-Mei4 $\times$ Rec104-Gal4AD; (f) LexA-Mer2 $\times$ Gal4AD-Rec114.