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Role of *RAD52* Epistasis Group Genes in Homologous Recombination and Double-Strand Break Repair

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INTRODUCTION

Recombination refers to the exchange or transfer of information between DNA molecules and is found in all organisms that have been studied in detail. Homologous recombination involves the exchange of DNA between sequences of perfect or near perfect homology over several hundreds of base pairs. In contrast, nonhomologous recombination occurs between sequences with little or no sequence homology. The process of homologous recombination plays essential roles in the mitotic and meiotic cell cycles of most eukaryotic organisms. In meiosis, the primary function of recombination is to establish a physical connection between homologous chromosomes to ensure their correct disjunction at the first meiotic division. In addition, meiotic recombination contributes to diversity by creating new linkage arrangements between genes, or parts of genes. It is now widely recognized that the primary function of homologous recombination in mitotic cells is to repair double-strand breaks (DSBs) that form as a result of replication fork collapse, from processing of spontaneous damage, and from exposure to DNA-damaging agents. Recombination is also required to repair the DSBs that initiate programmed rearrangements, such as mating-type switching in *Saccharomyces cerevisiae.*

Most of the genes in the *RAD52* epistasis group (*RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RDH54* [*TID1*], *MRE11* [*RAD58*], and *XRS2*) were identified by their requirement in the repair of ionizing-radiation (IR)-induced DNA damage. Mutations in these genes lead to defects in meiotic and/or mitotic recombination, providing evidence for a link between DSB repair (DSBR) and homologous recombination. Homologues of the *RAD52* group of genes have been identified in many other eukaryotes, and in some cases in prokaryotes and archaea, indicating high conservation of the recombinational repair pathway. The recent discovery that several human cancer-prone syndromes, for example, Nijmegen breakage syndrome (NBS) and ataxia-telangiectasia-like disorder (A-TLD), are caused by defects in DSBR has highlighted the importance of this repair pathway in maintaining genome integrity and cancer avoidance (47, 358, 411).

The goal of this review is to provide an update of the role of the *RAD52* group genes in spontaneous and DSB-induced homologous recombination. Other aspects of DSBR, cell cycle checkpoints, and meiosis are beyond the scope of this review, and the reader is referred to several excellent reviews on these topics (212, 271, 432). Most of the studies described in this review were performed with *S. cerevisiae* and with proteins corresponding to the *S. cerevisiae* gene products. When studies with other organisms are described, the gene or protein is prefixed by the abbreviated species name. The first part of the review describes models for DSBR and physical and genetic assays used to monitor repair and recombination. In the second part, the biochemical functions of the Rad52 group proteins and phenotypes of the *rad52* group mutants in various recombination assays are described.

CURRENT VIEW OF THE MECHANISMS OF HOMOLOGOUS RECOMBINATION

Much of our understanding of the mechanisms of recombination is based on organisms, such as *S. cerevisiae,* in which all of the products of an individual meiosis can be recovered for analysis in the form of asci containing four haploid spores. Two types of recombination events have been identified based on the segregation of heterozygous markers during meiosis: crossing over and gene conversion. A crossover between linked heterozygous markers results in new linkage arrangements for two spore products, but the markers are still recovered in Mendelian ratios. Gene conversion represents the nonreciprocal transfer of information between two homologous sequences to duplicate one of the alleles, with the corresponding loss of the other, resulting in a non-Mendelian segregation. Studies with yeast and other fungi have shown that gene conversion events are frequently associated with the exchange of flanking markers (145). This association is thought to be due to alternate resolution of a Holliday junction containing intermediate to generate crossover or noncrossover products (135). Several models have been proposed to explain the molecular mechanisms of recombination, and of these the DSBR and synthesis-dependent strand-annealing (SDSA) models are most consistent with the available genetic data (135, 236, 257, 381).

Double-Strand Break Repair Model

The observation that IR stimulates recombination suggested that recombination is initiated by DSBs. Studies by Orr-Weaver et al. demonstrated that a nonreplicating plasmid containing a DSB made within a region of the plasmid with homology to genomic sequences recombined with high efficiency following transformation of yeast (269). The plasmid DNA integrated at the homologous locus and was flanked by a duplication of the region of yeast DNA carried on the plasmid. When plasmid DNA was digested with restriction endonucleases to delete an internal fragment from the yeast DNA, it was still found to transform yeast at high frequency. The resulting transformants were indistinguishable in structure from those obtained using uncut DNA, indicating that repair of the gapped region had occurred during integration. This repair of a double-strand gap is equivalent to gene conversion without mismatch repair. Using linearized replicating plasmids, Orr-Weaver and Szostak reported the recovery of approximately equal numbers of integrated and non-integrated plasmids and concluded that gene conversion by DSBR can occur with or without crossing over (268). These observations formed the basis for the DSBR model of recombination (381) (Fig. 1A). In this model, the ends of the break are resected to form 3′ single-stranded tails that are active in strand invasion with a homologous duplex. Following strand invasion, the 3′ end is extended by DNA synthesis. The D-loop formed by strand invasion is able to pair with the other side of the DSB, and the 3′ end of the noninvading strand is also extended by DNA synthesis, forming a double-Holliday-junction (dHJ) intermediate. Random resolution of the two Holliday junctions is expected to yield equal numbers of crossover and noncrossover products.

Further support of the DSBR model as a general model for recombination came from the observation of transient DSBs at meiotic recombination hot spots (46, 369). The ends of meiotic DSBs are processed to yield 3′ single-stranded tails of about 600 nucleotides (370). DSBs made by the HO endonuclease in

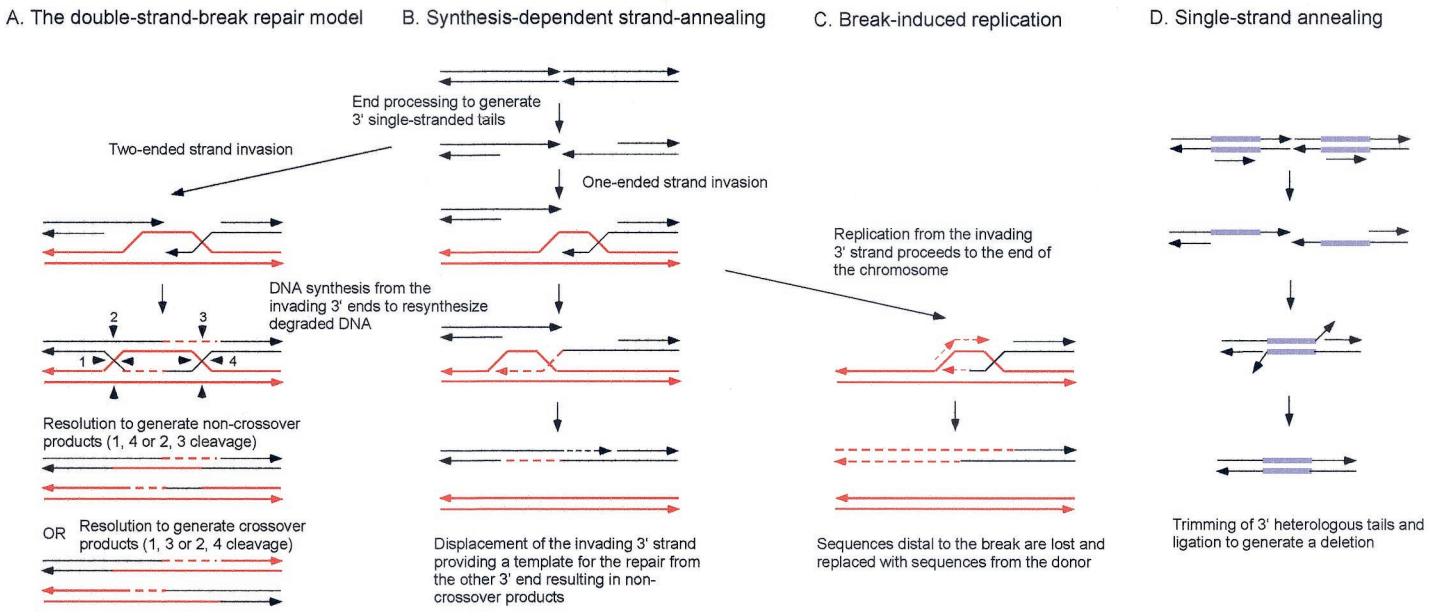

FIG. 1. Models for the repair of DSBs. (A) In the DSBR model, the ends are processed to yield 3′ single-stranded tails. The 3′ ends invade the homologous duplex, priming DNA synthesis. After ligation, a dHJ intermediate is formed, which can subsequently be resolved by endonucleolytic cleavage of the two Holliday junctions to generate crossover or noncrossover products. (B) In the SDSA model, the ends are processed to yield 3′ single-stranded tails, one of which invades the homologous duplex, priming DNA synthesis. The displacement loop (D-loop) formed by strand invasion could be extended by DNA synthesis or could migrate with the newly synthesized DNA. After displacement from the donor duplex, the nascent strand pairs with the other 3′ single-stranded tail and DNA synthesis completes repair. (C) The initial steps in the BIR model are the same as in the SDSA model, but DNA synthesis from the invading strand continues to the end of the DNA molecule. (D) In the SSA model, a DSB made between direct repeats is subject to resection to generate 3′ single-stranded tails. When complementary sequences are revealed due to extensive resection, the single-stranded DNA anneals, resulting in deletion of one of the repeats and the intervening DNA. The 3′ tails are endonucleolytically removed, and the nicks are ligated. The 3′ ends are indicated by arrowheads.

mitotic cells are also resected to generate long 3′ single-stranded tails (419). The length of the single-stranded tails at the *ARG4* locus correlates well with the length of gene conversion tracts within the *ARG4* gene, suggesting that most conversion events arise from repair of heteroduplex DNA and that four-strand branch migration is limited (370). Branched DNA molecules with the topology expected from dHJ intermediates have been detected by two-dimensional agarose gel electrophoresis of meiotic DNA, providing further support for the model (61, 327, 328).

Synthesis-Dependent Strand-Annealing Model

Subsequent studies of plasmid gap repair in yeast and *Ustilago maydis* and chromosomal DSBR following P element excision in *Drosophila melanogaster* have shown a lower association of crossing over with gene conversion (20, 96, 257, 291). Similarly, mating-type switching in *S. cerevisiae,* which is a gene conversion event initiated by a site-specific DSB at the *MAT* locus made by the HO endonuclease (183, 360), is rarely associated with crossing over. To account for the low levels of associated crossing over observed for some DSB-induced gene conversion events, the SDSA/migrating D-loop model was proposed (96, 257, 360) (Fig. 1B). In this model, strand invasion occurs as envisioned in the DSBR model, but after extensive DNA synthesis primed from the invading strand, the elongated invading strand is displaced and pairs with the other side of the break. DNA synthesis can then be primed from the noninvading 3′ end to repair the DSB or gap. An alternative scenario for gap repair involves coupling of lagging-strand DNA synthesis to leading-strand synthesis from the invading strand (137). Further evidence in support of the SDSA model comes from the observation that the donor sequences are generally unchanged during DSB-induced gene conversion (273).

Recent studies of meiotic recombination intermediates have identified a second class of branched molecules, termed single-end invasion intermediates (SEI) (144). These could be precursors to dHJ intermediates or could be processed directly into noncrossover products as predicted by the SDSA model. The relatively long-lived dHJ intermediates appear to be precursors for crossover products. The observation that formation of crossover, but not noncrossover, products is blocked by an *ndt80* mutation is consistent with the suggestion that these two classes of recombinants are derived from different intermediates (11). Allers and Lichten propose that noncrossovers are generated by SDSA and crossovers are generated from the resolution of dHJ intermediates that are formed as envisioned by the DSBR model (11).

Other Mechanisms for Homology-Dependent Double-Strand Break Repair

When a DSB is induced at the *MAT* locus on one chromosome *III* homologue of diploid cells, the break is generally repaired by gene conversion with about 20% associated crossing over (217). In certain genetic backgrounds, gene conversion is eliminated and repair occurs by invasion of the donor duplex by the broken chromosome followed by replication to the end of the donor chromosome (217) (Fig. 1C). This process is known as break-induced replication (BIR). This nonreciprocal process is likely to be important for telomere maintenance in the absence of telomerase (190, 388). Homology-dependent duplication of an entire chromosome arm to the end of a transformed linearized plasmid has also been detected in yeast and is presumed to occur by BIR (248). Another pathway of homology-dependent repair, single-strand annealing (SSA), is restricted to DSBs that occur between direct repeats (Fig. 1D). These events have been detected in yeast and animal cells by using artificial direct repeats and could conceivably be important for repair in genomes of higher eukaryotes that contain many repeated sequences (101, 202, 224). After formation of the DSB, the ends are resected to produce 3′ single-stranded tails, which can anneal when resection is sufficient to reveal complementary single-stranded regions. Single-stranded tails are removed by nucleases, and the resulting gaps and nicks are filled in by DNA repair synthesis and ligation. This process is accompanied by deletion of one of the repeats and the DNA between the direct repeats and is therefore considered to be mutagenic.

GENETIC AND PHYSICAL ASSAYS FOR RECOMBINATION

Although sensitivity to IR is a universal feature of *rad52* group mutants, the mutants show considerable heterogeneity in different assays for recombinational repair of DSBs and spontaneous mitotic recombination. A brief description of the commonly used recombination assays and the products that are recovered from wild-type cells follows. Later sections discuss in detail the phenotype of the *rad52* group mutants in these assays.

Spontaneous and Induced Heteroallelic Recombination

The term "spontaneous recombination" refers to events that occur during normal growth rather than following treatment of cells with DNA-damaging agents such as UV light or IR. These events most probably result from recombination initiated by spontaneous lesions that arise from replication fork arrest or collapse, intermediates in base excision repair, aberrant repair, or transcription. Strand breaks that occur in the context of the replication fork are most likely to be repaired using the sister chromatid, an event that is normally genetically silent. Spontaneous recombination can be detected between chromosome homologues in diploid cells or between artificial duplications in haploids or diploids. In most cases, the two recombining sequences contain mutant alleles (heteroalleles) of a selectable gene to allow selection for recombination events during growth of a culture. Heteroallelic recombination in diploids generally occurs by gene conversion with low (10 to 20%) associated exchange of flanking markers. Understanding the mechanisms of spontaneous recombination is difficult because these events occur at low frequency (around 10^{-6}/cell/generation) and are usually selected. Although both products of a G_1 event should be retained, the products of an event initiated during the S or G_2 phase of the cell cycle will segregate to different daughter cells 50% of the time and only one of these will be recovered by selection (Fig. 2). The frequency of heteroallelic recombination can be increased by up to 1,000-fold when cells are treated with UV light or IR; these events are referred to as

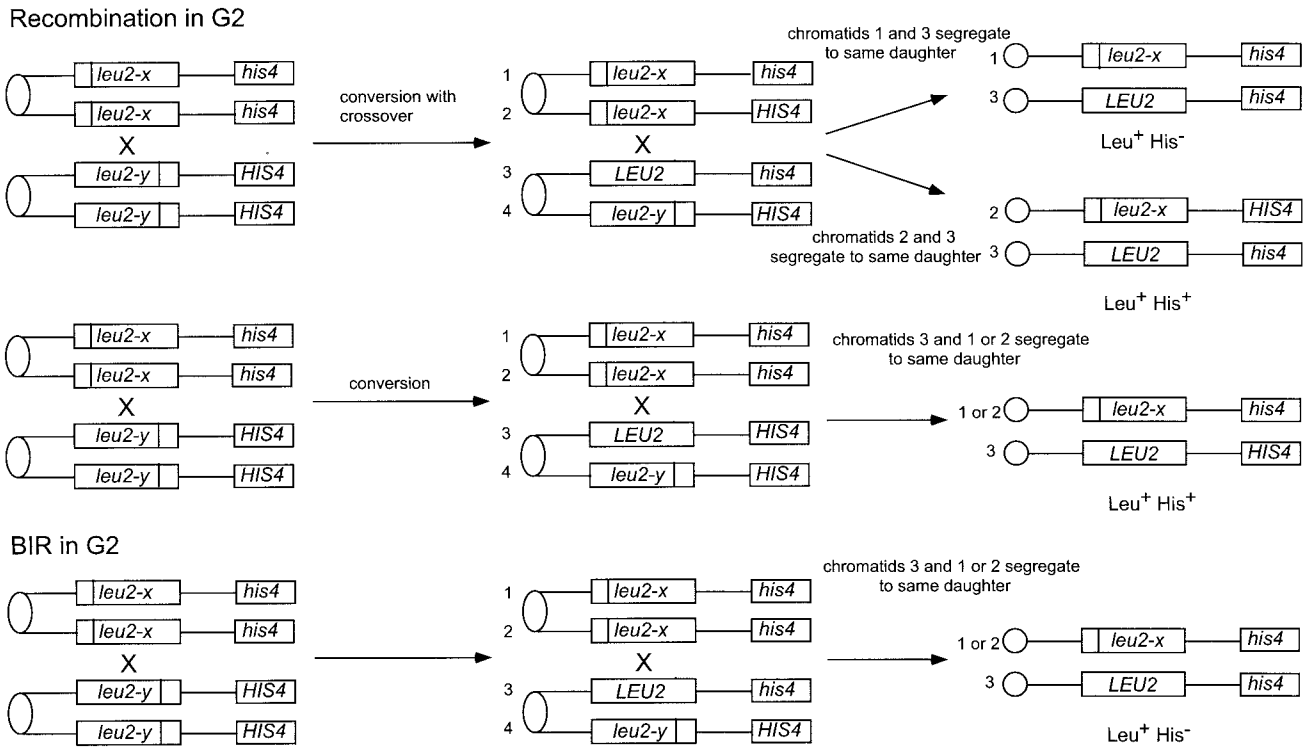

FIG. 2. Mitotic recombination in diploids. A diploid containing *leu2* heteroalleles and heterozygous for *HIS4* is shown. Gene conversion associated with crossing over gives rise to Leu^+ His^+ or Leu^+ His^- recombinants, whereas gene conversion events maintain heterozygosity at *HIS4*. A BIR event cannot be distinguished from a G_2 crossover unless the reciprocal product is recovered.

induced recombination. Insertion of the recognition sequence for a rare-cutting endonuclease, such as HO, within one of the repeats results in very high levels of recombination when HO endonuclease is induced and allows the analysis of unselected events. Analysis of HO-induced gene conversion has shown that the level of associated crossing over is similar to that for spontaneous events (217, 261).

Haploid strains containing duplicated genes have been used extensively to study mechanisms and genetic control of mitotic recombination (177). Chromosomal direct repeats can be generated by targeting the integration of a plasmid containing a yeast gene to the homologous chromosomal location to form a nontandem duplication (Fig. 3). Recombination between the repeats can occur by conversion, maintaining the structure of the duplication, or by a deletion event that removes one repeat and the intervening DNA (176, 319, 391). Deletion events between direct repeats can be directly selected if the intervening DNA contains a marker with counterselection, for example *URA3,* or if the repeats are truncated genes that, by deletion, restore a functional copy of the gene. Conversion events between repeats can occur by an intrachromatid or sister chromatid interaction. Gene conversion between misaligned sister chromatids can generate a triplication on one sister while retaining the direct repeat on the other chromatid. Triplications can also occur by unequal crossing over between sister chromatids, but in this case the reciprocal product is equivalent to a deletion event. Because spontaneous recombination events occur at low frequency, events are usually selected and therefore the reciprocal product is not recovered. Intrachromatid gene conversion associated with crossing over should generate a deletion on the chromosome and an extrachromosomal circular product. It is clear that deletions are produced at high frequency between direct repeats, but the reciprocal product expected from a conservative crossover event is produced in only about 7% of deletions (316). This suggests that intrachromatid gene conversion is rarely associated with crossing over or that most gene conversion events occur between misaligned sisters rather than by intramolecular interactions.

HO-induced recombination between repeats can occur at high enough frequency to be studied nonselectively in order to recover both products of a recombination event and to monitor the kinetics of DSBR in synchronized populations of cells (260, 312). When the HO-cut site is within one of the repeats, the same spectrum of recombination events found for spontaneous events is recovered, including conversions, deletions, and triplications. The high frequency of triplication or deletion events recovered in one study suggests that sister chromatid interactions are at least as frequent as intrachromatid events (416). Spontaneous deletion events between direct repeats are assumed to occur by SSA, mispairing during replication, or replication slippage rather than by a reciprocal exchange between the repeats. A study of HO-induced recombination involving direct repeats of *lacZ* contained on a *CEN* plasmid supports the idea that SSA is a major pathway for DSB-induced deletion formation between direct repeats (101). However, in another study, evidence for DSB-induced reciprocal recombination between direct repeats was presented. An *ARS1* element and *TRP1* marker were introduced between direct repeats so that

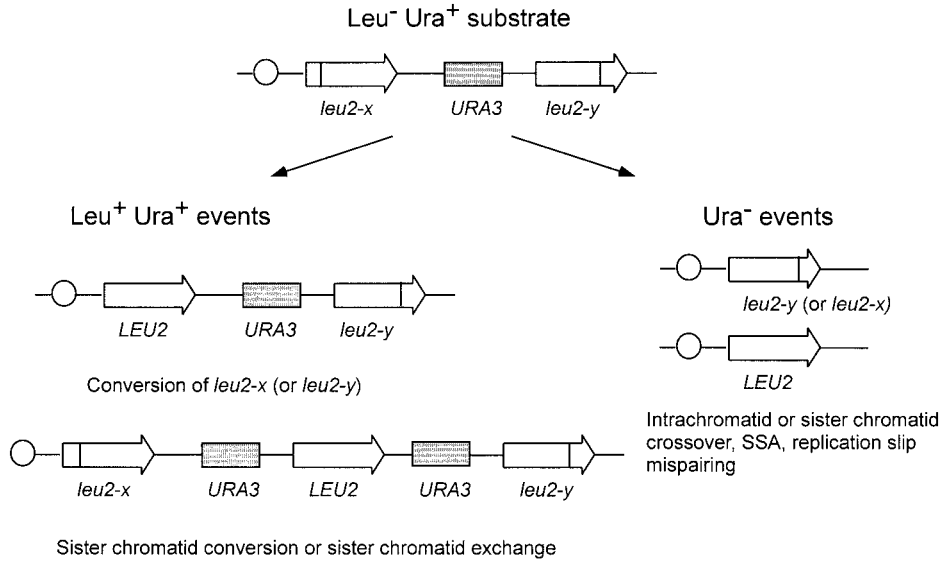

FIG. 3. Use of a direct-repeat substrate to measure mitotic recombination. The substrate contains *leu2* heteroalleles separated by a copy of the *URA3* gene. Leu^+ Ura^+ recombinants can be generated by intrachromatid or sister chromatid conversion or by unequal sister chromatid exchange. Ura^- recombination events can occur by an intrachromatid or unequal sister chromatid crossover, by SSA, or by replication slip mispairing.

an intrachromatid reciprocal exchange could be detected as unstable Trp^+ colonies (300). No stable Trp^- recombinants were recovered following HO induction, indicating that SSA was not playing a significant role in repair. Of the recombinants, 6% had the unstable Trp^+ phenotype indicative of a reciprocal exchange. The reasons for these conflicting results are unclear but could include the length of the repeats, the distance between the repeats, and the location (chromosomal versus plasmid). Deletions are the primary products recovered when the HO-cut site is within unique sequences between the repeats and appear to result from SSA (347, 362) (Fig. 1D).

One concern about the use of direct repeats to study recombination is that deletions can occur by a variety of mechanisms (177). Furthermore, direct repeat recombination still occurs at high frequency in most of the *rad52* group mutants (199, 233). In efforts to avoid SSA and replication-associated events, a number of studies have used repeats in inverted orientation (Fig. 4). These generally contain additional sequences between the repeats because perfect palindromes are unstable (118, 313). In most cases the repeats consist of heteroalleles or truncated genes, and in one study inversions were selected by increased expression of a reporter gene present between the repeats (4, 5, 421). To facilitate the detection of recombination events by a colony color assay, Rattray and Symington generated an inverted-repeat substrate from *ade2* heteroalleles (299). *ade2* mutants accumulate a red pigment, resulting in the formation of red colonies; recombination events that restore a functional *ADE2* gene result in white sectors within the red colony. Recombination events within the *ade2* reporter can occur by gene conversion or by inversion, and these events occur with equal frequency in wild-type strains (299). Although inversions between inverted repeats could conceivably occur by an intrachromatid crossover, recent studies suggest other mechanisms. Chen and Jinks-Robertson (56) presented evidence in support of long conversion tracts between misaligned sister chromatids to generate inversions (Fig. 4B), as originally suggested by Rothstein et al. (308). Another model suggests that BIR followed by SSA could generate inversions between inverted repeats (166).

Repeats present on nonhomologous chromosomes (ectopic), usually as heteroalleles, have also been used to study recombination (156). If the repeats have the same orientation with respect to the centromeres, reciprocal crossovers can occur to form viable products. The association of crossing over with gene conversion is about 20%, similar to that observed for recombination between homologous chromosomes (156). The advantage of using an ectopic recombination assay is that simple SSA events cannot give rise to recombinants; the disadvantage is the low frequency at which ectopic recombination occurs, compared with direct or inverted repeats.

Mating-Type Switching

The budding yeast mating-type switching system provides a useful tool to study intermediates in DSBR and the genetic control of this process (124) (Fig. 5). The *HO* gene is normally expressed in late G_1, but expression can be regulated using a *GAL1-HO* cassette so that HO endonuclease can be synchronously induced in a population of cells by addition of galactose to the growth medium (132). After the formation of a DSB at the *MAT* locus by the HO endonuclease, the ends are processed to form 3′ single-stranded tails (419). The 3′ single-stranded tail *CEN* distal to the cut site invades the donor cassette to initiate DNA synthesis by using components of both leading- and lagging-strand synthesis (137). The initial strand invasion and extension step can be monitored by PCR, and the formation of completed products can be detected by using novel restriction fragments (419). Branched DNA molecules have not been reported as intermediates during mating-type switching, suggesting that these intermediates are less stable than intermediates in meiotic recombination. The ability of mutant strains to switch mating type can also be determined by

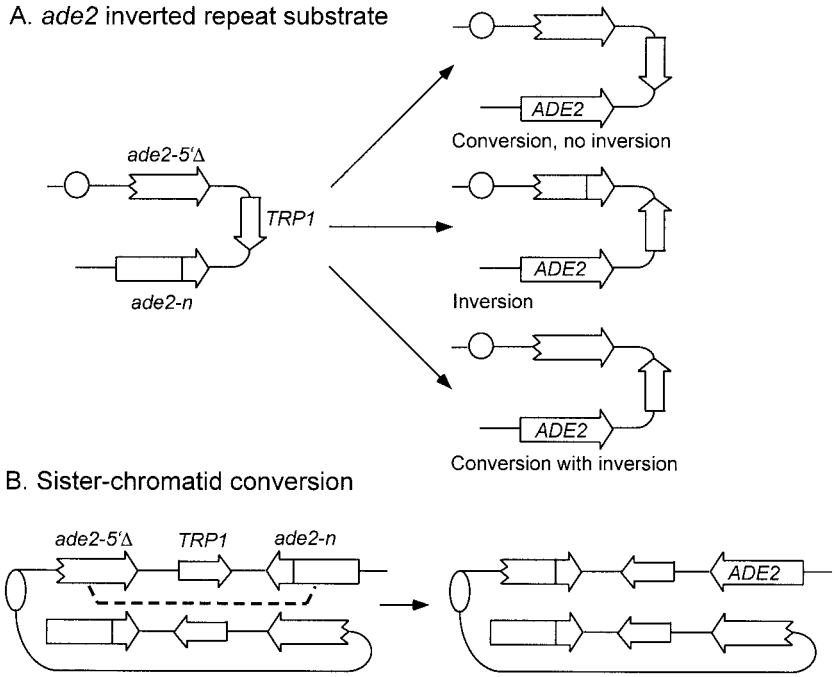

FIG. 4. Inverted-repeat substrates. (A) The *ade2* heteroalleles can recombine to Ade^+ by conversion or inversion (apparent crossover) of the intervening DNA. (B) A long-tract conversion event between sister chromatids can result in an Ade^+ product with the intervening DNA inverted.

monitoring the survival of cells following HO expression and analyzing the survivors for mating phenotype (221, 414).

As described above, the HO cut site can be inserted at other locations to create an initiation site for recombination. The advantage of this system over inducing events by irradiation is that the precise location of the initiating lesion is known. Furthermore, the high efficiency of cutting by HO endonuclease allows physical monitoring of recombination, as described for mating-type switching, and recovery of all the products of a recombination event. The Haber laboratory has used the HO system extensively to characterize the mechanisms of direct- and inverted-repeat recombination, as well as allelic recombination in diploids (271). The high efficiency of cleavage by HO endonuclease is currently being exploited to identify proteins that associate with DSBs in vivo by the chromatin immunoprecipitation method (91).

To study DSB-induced recombination in mammalian cells, several groups have made use of the rare-cutting HO and I-*Sce*I endonucleases. I-*Sce*I is a site-specific endonuclease responsible for intron mobility in mitochondria of yeast and initiates a gene conversion event that resembles HO-induced gene conversion (292). Adenoviruses expressing the HO gene or containing the HO cut site can be used to infect human cells in culture (262). Coinfection with the two viruses results in efficient cutting of viral genomes containing the cut site, and these are substrates for end-joining repair (262). Direct-repeat recombination substrates containing a cleavage site for I-*Sce*I have been stably transfected into several mammalian cell lines (157, 310). The I-*Sce*I endonuclease was expressed by transient transfection using the constitutive cytomegalovirus promoter. Although the efficiency of cleavage was lower than that found for I-*Sce*I or HO endonucleases in yeast, the frequency of homologous recombination was induced more than 100-fold in the presence of I-*Sce*I.

Ends-In and Ends-Out Recombination

The early studies by Orr-Weaver et al. demonstrated efficient repair of DSBs and gaps in plasmids by homologous recombination during yeast transformation (268, 269). The repair of breaks and gaps on plasmids is referred to as "ends-in" recombination. Subsequent studies showed that homologous linear DNA fragments could be used to replace chromosomal sequences during transformation (309). This method of gene replacement or gene targeting is routinely used to knock out genes. Since the orientation of the two ends is opposite to that for plasmid gap repair, gene replacement is sometimes referred to as "ends-out" recombination. The major difference between ends-in and ends-out events is that during ends-in recombination one invading end primes DNA synthesis toward the other end of the broken plasmid, much the same as in a chromosomal DSB, whereas replication is primed away from the fragment during ends-out recombination (Fig. 6). There is also evidence that ends-out recombination can occur by assimilation of a single strand from the linear transforming fragment into the genome to form a displacement loop (D-loop) (194). Subsequent repair of the D-loop restores the original marker or incorporates information from the transforming fragment into the chromosomal locus.

Genetic and Physical Assays for Meiotic Recombination

Analysis of the segregation of heterozygous markers during meiosis is used to measure recombination in sporulation-pro-

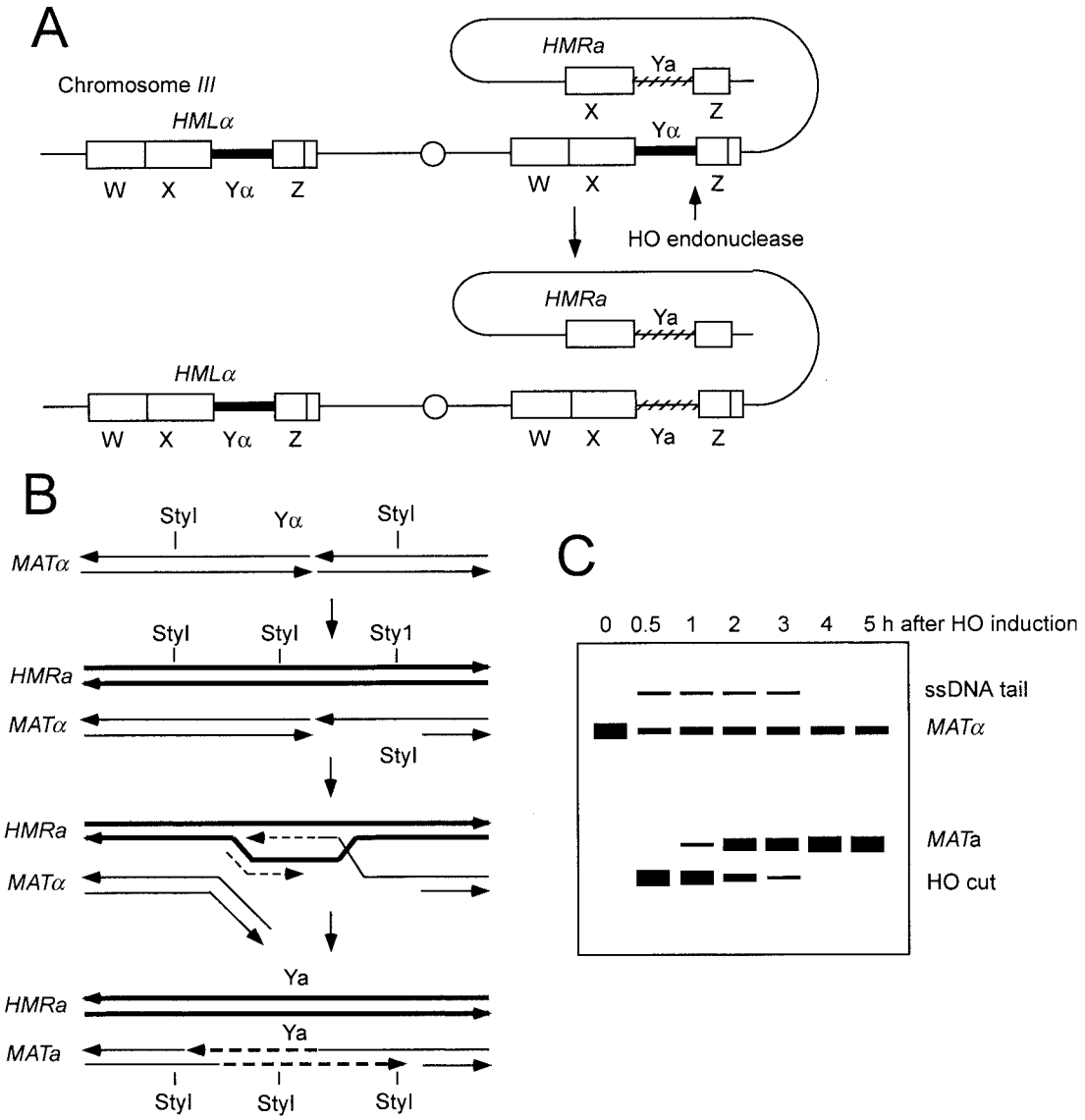

FIG. 5. Mating-type switching. (A) Cartoon of chromosome *III* showing the silent cassettes, *HML*α and *HMR***a** and the expressed *MAT*α locus. HO endonuclease cleaves between the Y and Z sequences, and repair using the *HMR***a** donor results in switching to *MAT***a**. Regions of homology flanking Yα and Y**a** are indicated by W, X, and Z. (B) After HO cleavage, the 5′ end on the distal side of the break is resected and the resulting 3′ single-stranded tail invades the *HMR***a** locus. DNA synthesis is primed from the invading strand, duplicating Y**a** sequences. Lagging-strand synthesis initiated from the D-loop results in synthesis of the other strand. The mechanism for removal of the Yα strands is currently unknown. (C) Schematic representation of a Southern blot showing the kinetics of mating-type switching. Switching to Y**a** results in transfer of a novel *Sty*I site and therefore can be monitored by Southern blot analysis of DNA extracted after HO induction and digested with *Sty*I. Resection of the 5′ strands beyond the distal *Sty*I site results in resistance to digestion to *Sty*I because the site is within ssDNA.

ficient strains. Gene conversion is detected as a departure from the normal 2:2 segregation of heterozygous markers and crossing over by new linkage arrangements between linked heterozygous markers. However, this method is not very useful for strains that fail to sporulate or that give rise to inviable spores. Diploid cells induced to undergo sporulation can return to vegetative growth when plated on rich medium (87, 334). The time at which cells commit to high levels of recombination precedes the time when they commit to completion of the sporulation program; therefore, when diploids are plated on rich medium after several hours of growth in sporulation medium, they experience high levels of recombination but retain the diploid state. The frequency of heteroallelic recombination increases 100- to 1,000-fold when cells initiate meiotic recombination and are returned to vegetative growth. Because the return-to-growth protocol does not rely on the formation of viable spores, it can be used to measure the induction of meiotic recombination in sporulation-defective mutants.

The development of physical assays to monitor the kinetics of formation and processing of DNA intermediates has been invaluable for understanding the mechanisms of recombination (Fig. 7). Also, physical methods can be used to detect recombination intermediates in strains that fail to produce viable spores. In wild-type cells, DSBs are formed about 1 h

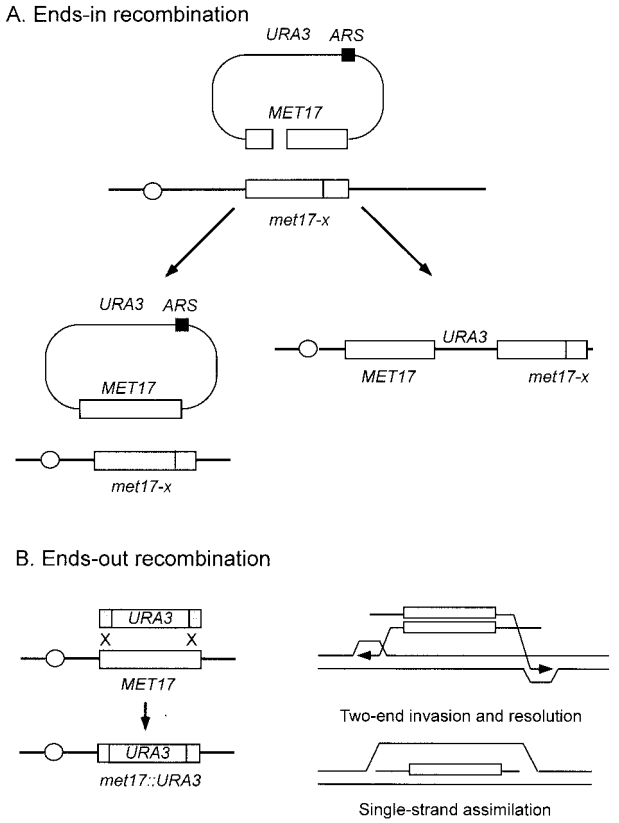

FIG. 6. Gene targeting. (A) When yeast cells are transformed with a replicating plasmid (*ARS* plasmid) containing a double-strand break or gap within a region of the plasmid with homology to the yeast genome, the break or gap is repaired by gene conversion from the chromosomal locus. The plasmid remains episomal if gene conversion without an associated crossover occurs but is integrated into the genome if conversion is associated with crossing over. If the plasmid contains a *CEN* sequence, only conversion events can give rise to viable transformants. If the plasmid contains no origin of replication (*ARS*), only integration events are observed. (B) Ends-out gene targeting refers to replacement of chromosomal sequences with sequences present on a linear DNA fragment introduced into cells by transformation. Gene targeting is thought to occur by invasion of the two ends into the chromosomal locus followed by resolution of the resulting Holliday junctions. Extensive DNA synthesis could be primed from the invading 3′ ends prior to Holliday junction resolution, or resolution could occur by replication to the end of the chromosome. Gene targeting could also result from integration of a single strand of the targeting fragment followed by trimming the D-loop and mismatch repair.

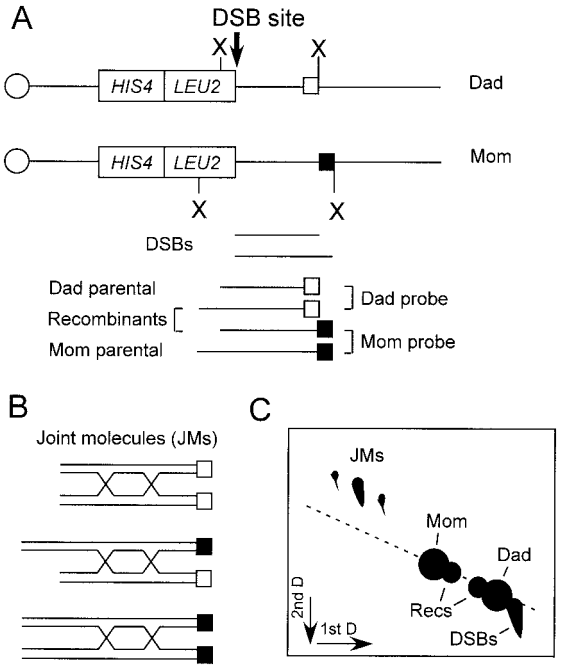

FIG. 7. Physical assays for meiotic recombination. (A) The hot spot created by insertion of the *LEU2* gene at *HIS4* in the SK1 strain background is shown. The end of the *LEU2* gene contains a site for meiosis-specific DSBs flanked by restriction sites that can be used to distinguish between DSB fragments, and the two parental and two recombinant fragments. The white and black boxes indicate insertions of heterologous sequences used to distinguish the parental molecules by using strand-specific probes. (B) Three types of joint molecules are expected: Mom-Mom intersister joint molecules, Dad-Dad intersister joint molecules, and Mom-Dad interhomologue joint molecules. Size and hybridization to strand specific probes distinguish the three types of joint molecules. (C) Cartoon of a neutral-neutral two-dimensional gel showing separation of joint molecules from the parental and recombinant fragments. The interhomologue joint molecules are more abundant than the intersister joint molecules in wild-type cells. (Adapted from Fig. 1 of reference 329 with permission)

after DNA synthesis at recombination initiation sites (defined by genetic methods) (46, 369). The ends are processed to form 3′ single-stranded tails of about 600 nucleotides that are the substrates for strand invasion to form heteroduplex DNA (370). Heteroduplex DNA can be detected when the mismatches formed are poorly recognized by the mismatch repair system and therefore persist in DNA (198, 255). Restriction site polymorphisms flanking an initiation site can be used to detect branched intermediates by two-dimensional gel electrophoresis and the formation of crossover products (36, 46, 61, 328). Two types of branched-DNA intermediates have been detected during meiosis: SEIs and dHJ intermediates (11, 144). Kinetic studies suggest that SEIs arise before dHJ intermediates and could be precursors to them or could be processed independently to form noncrossover products (11, 144). The formation of crossover products detected by novel restriction fragments occurs almost simultaneously with the meiosis I division, suggesting a tight coupling between resolution of recombination intermediates, exit from pachytene, and chromosome segregation.

GENETIC AND BIOCHEMICAL PROPERTIES OF THE *RAD52* GROUP GENES AND PROTEINS

The *RAD52* group genes can be broadly grouped into the *MRE11, RAD50, XRS2* (*NBS1*) subgroup and the *RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RDH54/TID1* subgroup. *MRE11, RAD50,* and *XRS2* are implicated in the formation and processing of DSBs during meiotic recombination and also function in the end-joining pathway of repair, telomere maintenance, in DNA replication-associated repair, and in the DNA damage checkpoint in mitotic cells. The *RAD51* subgroup appears to function only in homologous recombination and is described separately.

Mre11-Rad50-Xrs2 (Nbs1) Complex

Yeast strains with null mutations of *MRE11, RAD50,* or *XRS2* have very similar phenotypes. All of the mutants show poor vegetative growth, high sensitivity to IR, and defects in meiosis (7, 110, 149). The three proteins interact in the two-hybrid system, and coimmunoprecipitation studies have confirmed that they form a stable complex (160, 407). Although all three proteins can be coimmunoprecipitated from wild-type cells with antibodies directed against any one of the components, Rad50 and Xrs2 fail to interact in the absence of Mre11, suggesting a central role for Mre11 in complex formation (407). The Mre11 and Rad50 proteins are conserved in all kingdoms of life, whereas Xrs2 appears to be weakly conserved and is present only in eukaryotes. The human gene, Hs*MRE11,* was fortuitously recovered in a two-hybrid screen for DNA ligase I-interacting proteins (282). Using antibodies against HsMre11, Petrini and coworkers identified a large protein complex containing HsRad50 and a 95-kDa protein (78). The 95-kDa protein was subsequently shown to be mutated in humans with the rare autosomal recessive trait NBS (47, 411). Consequently, p95 is referred to as Nbs1 or Nibrin. Nbs1 is considered functionally analagous to Xrs2. Hypomorphic alleles of *MRE11* are found in persons with a different human chromosomal instability syndrome, A-TLD (358). At the cellular level, ataxia-telangiectasia (A-T), A-TLD, and NBS are very similar and are characterized by sensitivity to IR, radioresistant-DNA synthesis, and chromosome instability (primarily translocations) (280).

Although *mre11, rad50,* and *xrs2* null mutants of yeast are viable, *MRE11, RAD50,* and *NBS1* are all essential for the viability of vertebrate cell lines and for mouse early embryonic development (215, 426, 428, 431). An *mre11* conditional chicken cell line was generated by deleting both copies of *MRE11* in the DT40 cell line, in the presence of a transgene expressing *MRE11* from a tetracycline-repressible promoter (428). Down regulation of the gene resulted in rapid depletion of Mre11 concomitant with increased radiosensitivity, increased levels of spontaneous chromosome breaks, arrest in the G_2 phase of the cell cycle, and eventual cell death. Similarly, antibody depletion of Mre11 from *Xenopus* extracts resulted in aberrant DNA synthesis accompanied by the formation of DSBs (67). These studies suggest an essential role of the Mre11 complex in the repair of lesions generated during S phase in vertebrates.

Localization of the MRN complex to DSBs in vivo. Immunofluorescence has been used to localize the Mre11-Rad50-Nbs1 (MRN) complex in response to DNA damage in vertebrate cells. In unirradiated cells, Mre11 and Rad50 are uniformly distributed throughout the nucleus, but after exposure to IR, both proteins form discrete nuclear foci (226). Foci that are microscopically visible are thought to represent sites of repair. Discrete foci were not detected after UV irradiation, suggesting that Mre11 and Rad50 associate specifically with DSBs. Formation of Mre11 and Rad50 IR-induced foci was eliminated in cells from NBS and A-TLD patients, consistent with a defect in the DNA damage response (47, 358). In a highly innovative study, soft X rays were used to form discrete areas of DNA damage within the nuclei of fibroblasts and areas of repair localized by incorporation of bromodeoxyuridine (BrdU) (assayed using an anti-bromodeoxyuridine monoclonal antibody) by terminal deoxynucleotidyltransferase (258). This study suggests that DSBs are held in a relatively fixed position during the early stages of DNA repair. At 30 min after irradiation, Mre11 colocalized with BrdU, suggesting that the MRN complex migrates to the sites of DSBs within the nucleus. Discrete areas of DNA damage have also been generated using a laser scissor method (279). The phosphorylated form of H2AX (γ-H2AX), a nonessential member of the histone H2A family (48), associated with the sites of damage within 30 min, and HsRad50 was found to colocalize extensively with γ-H2AX. Treatment of cells with IR also resulted in the formation of γ-H2AX foci, but in this case Rad50 did not colocalize until 6 to 8 h after irradiation. Preirradiation exposure of cells to the fungal metabolite wortmannin, which inhibits phosphatidylinositol 3-kinases, prevented the formation of γ-H2AX and Rad50 foci, suggesting that γ-H2AX is required to recruit Rad50 to sites of damage (279).

Mre11 is associated with chromatin in replicating *Xenopus* extracts and appears to be important either for prevention of replication-induced breaks or for their repair (67). Immunofluorescence studies of detergent extracted cells also show association of Mre11 with sites of replication based on colocalization of Mre11 with proliferating-cell nuclear antigen during S phase (225). The Mre11 complex could play an important role during replication or could be associated with the fork in preparation for damage signaling or repair.

Structural and biochemical studies. The 83-kDa Mre11 protein is highly conserved among eukaryotes, and the N-terminal region has several sequence motifs shared by a large family of phosphodiesterases, including the *Escherichia coli* SbcD and bacteriophage T4 gp46 nucleases and protein phosphatases (333) (Fig. 8). Yeast and human Mre11 proteins have single-stranded DNA (ssDNA) endonuclease and weak 3′-to-5′ exonuclease activities (108, 242, 277, 397, 407). Like SbcD (62, 63), the Mre11 nuclease activities are dependent on manganese as a cofactor. An allele of *MRE11, mre11-58 (rad58),* originally thought to represent a new gene in the *RAD52* epistasis group, has a point mutation within the fourth phosphodiesterase motif (H213Y), and the encoded protein is devoid of nuclease activity in vitro (400, 407). Several other mutations have been made at conserved residues within the other phosphodiesterase motifs (*mre11-D16A, mre11-D56N,* and *mre11-H125N*), and all abolish the endonuclease and 3′-to-5′ exonuclease activities in vitro (108, 241). The aspartic acid residues at positions 16 and 56 are directly involved in coordination of two Mn^{2+} ions at the active site, and His125 stabilizes the transition state intermediate (139). Two-hybrid and size exclusion chromatography analyses indicate that Mre11 forms a dimer (108, 160). Consistent with these findings, diploid strains expressing an N-terminal *mre11* mutation (*mre11S*) and a C-terminal insertion mutation showed intragenic complementation for meiosis and methyl methanesulfonate (MMS) resistance (256).

Rad50, like Mre11, is conserved in all kingdoms of life. In bacteria, the Rad50 homologue, SbcC, forms a complex with SbcD, the homologue of Mre11. The SbcCD complex has ATP-dependent 3′-to-5′ exonuclease and ATP-independent single-stranded endonuclease activity (62, 63). The 150-kDa Rad50 protein is related to the SMC proteins, which have the

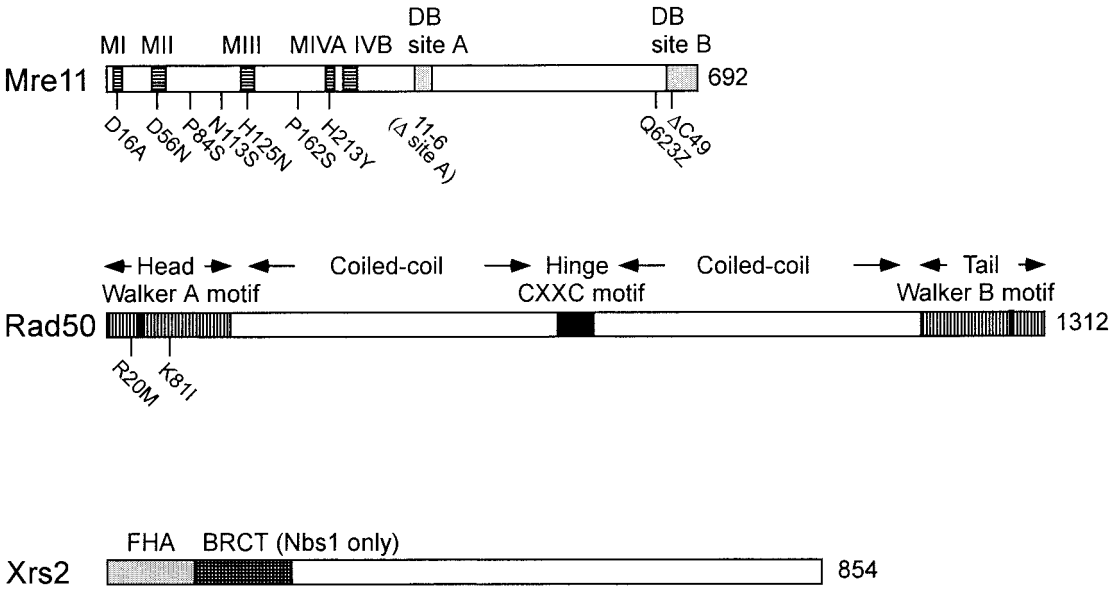

FIG. 8. Schematic representation of Mre11, Rad50, and Xrs2 (Nbs1). The phosphodiesterase motifs of Mre11 are labeled MI through MIV, and DNA binding sites are labeled DB site A and B. The Mre11-D16A, Mre11-D56N, Mre11-H125N, Mre11-H213Y, and Mre11-6 mutants are nuclease defective in vitro. Residue Pro84 is mutated in the *mre11-S* allele, and Pro162 is mutated in the *mre11-1* temperature-sensitive allele. The *mre11-N113S* and *mre11-Q623Z* alleles correspond to the mutations in the A-TLD patients. Rad50 contains two coiled-coil domains separating the Walker A and B motifs for NTP binding and hydrolysis. The hook domain, containing the conserved CXXC motif, is located between the two coiled-coil domains. The positions of the *rad50S* alleles, *rad50-R20M* and *rad50-K81I,* are shown.

Walker A and B motifs characteristic of nucleotide triphosphate (NTP)-binding proteins separated by a long coiled-coil region (9) (Fig. 8). The SMC proteins, including the Rad50 subgroup, have a conserved hinge region within the coiled region. The hinge region of the Rad50 subfamily is distinct from that of other SMC proteins and contains a conserved Cys-X-X-Cys motif (140). Electron microscopy studies of Rad50 suggest a dimeric structure to bring together the Walker A and B motifs, forming two catalytic sites. The dimer could result from two protomers in an antiparallel configuration or by hinge-mediated interactions between two intramolecularly coiled protomers (12, 139) (Fig. 9). Atomic force microscopy of the human Rad50-Mre11 complex identified two highly flexible intramolecular coiled coils emanating from the globular Rad50-Mre11 DNA binding domain (73). The intramolecular coiled coil places the Walker A and B motifs of one Rad50 monomer together juxtaposed to Mre11. The size of the globular domain is consistent with a dimer of Mre11 and a dimer of the Rad50 ATPase domain. Dimerization of Rad50 is mediated by the CXXC motif within the hinge region (140). Structural analysis of the hinge region revealed a hook structure at the apex of the coiled coil, creating half of a composite metal binding site. Two hook regions coordinate a single Zn^{2+} ion with the two coiled-coil regions extending in almost opposite directions. This arrangement could potentially bridge sister chromatids because the distance between the head domains of the $Mre11_2Rad50_2$ complex is approximately equal to the distance between sister chromatids (1,200 Å) (140). Another class of SMC proteins, called cohesins, is known to mediate sister chromatid cohesion (128). The Mre11-Rad50 complex could function specifically in sister chromatid interactions during DSBR, as suggested by genetic studies. Electron microscopy studies have provided direct evidence for end binding by the human and yeast Mre11-Rad50 complex and bridging of different DNA molecules (53, 73).

Structural studies of the *Pyrococcus furiosis* Rad50 catalytic domain (Rad50cd) indicate a structure similar to the ATP binding cassette transporter family of ATPases (142). Binding of the Rad50cd to the ATP analog AMP-PNP induces a structural change to align the Walker A and B motifs. The ATP-bound form of the Rad50cd binds DNA more tightly, suggesting that ATP regulates DNA binding by conformational switching. Mutation of the conserved lysine residue within the Walker A box confers a null phenotype in yeast, indicating the importance of ATP binding and/or hydrolysis to function (8). The DNA binding activity of yeast Rad50 is stimulated by ATP, but no ATPase activity has been observed for the purified protein (301).

Rad50 stimulates the nuclease activities of yeast and human Mre11. Unlike the EcSbcC-SbcD complex and the PfMre11-Rad50 complex, the complex of HsMre11 with HsRad50, or HsMre11, HsRad50, and Nbs1 has no demonstrated ATP-dependent exonuclease activity (63, 141). ATP stimulates a weak DNA unwinding activity of the HsMre11-Rad50-Nbs1 complex, and hairpin cleavage has also been observed for the complex (278). However, the yeast Mre11-Rad50 complex cleaves hairpin structures in the absence of Xrs2 (396).

In mammals, Nbs1 (p95) appears to be the functional homologue of Xrs2 in that it is tightly associated with Mre11 and Rad50, but sequence similarity to Xrs2 is limited to the N-terminal 115 amino acids (47, 411). Xrs2 and Nbs1 have no obvious sequence motifs indicative of function. Nbs1 contains two domains in the N-terminal region that are found in DNA damage-responsive cell cycle checkpoint proteins (95). A fork-

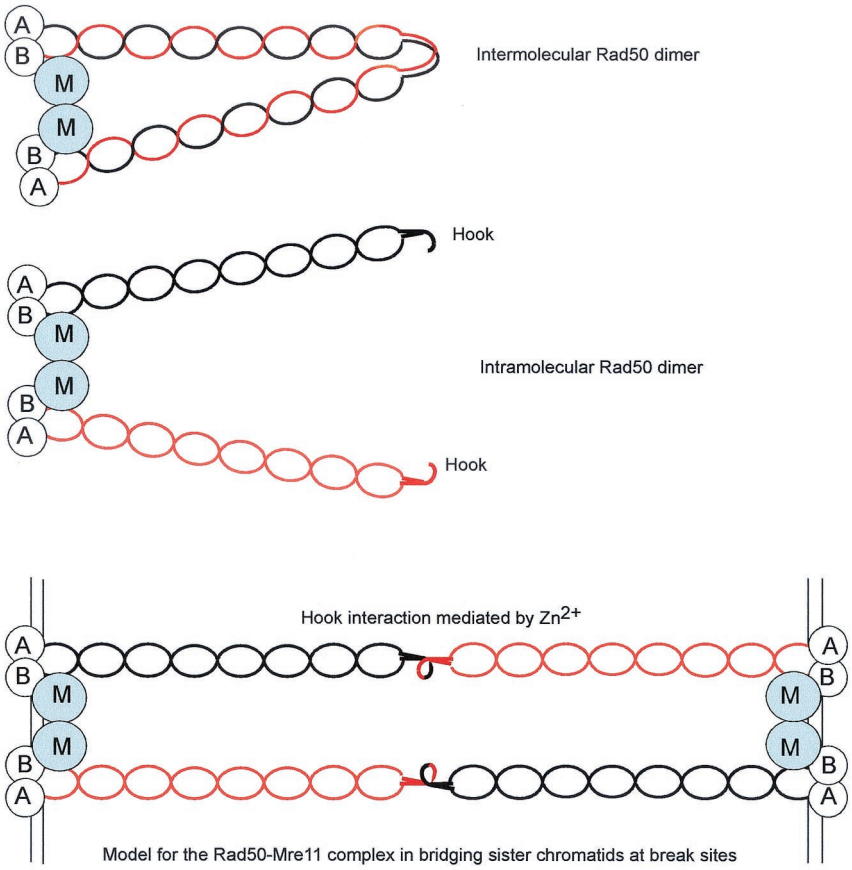

FIG. 9. Models for the Rad50-Mre11 complex. A dimer of Rad50 could form by antiparallel intermolecular interaction to position the Walker A motif from one monomer next to the Walker B motif of the other. A dimer of Mre11 binds adjacent to the head-tail region of Rad50. Recent results are more consistent with an intramolecular interaction between the coiled-coil domains of Rad50, with the dimer held together by a dimer of Mre11. Interactions between the hinge domains could connect two dimers of Rad50 and two dimers of Mre11. The length of the Rad50 tetramer is consistent with the distance between sister chromatids in eukaryotes. The Mre11 and Rad50 head-tail domains are envisioned to interact with DNA.

head-associated (FHA) domain, which is thought to be important for interactions between phosphorylated proteins, is found at the N terminus of Nbs1. The BRCT (breast cancer carboxy-terminal) domain, which is adjacent to the FHA domain in the N-terminal region of Nbs1, is found in a variety of proteins that participate in DNA damage-responsive cell cycle checkpoints. To date, the only biochemical activity assigned to Nbs1 is stimulation of the unwinding and hairpin cleavage activities of HsMre11 and HsRad50 (278).

Meiotic phenotype of *mre11, rad50,* and *xrs2* mutants. Diploid strains homozygous for *mre11, rad50,* or *xrs2* fail to form meiosis-specific DSBs and thus are unable to initiate meiotic recombination (8, 160). This results in the formation of aneuploid (inviable) spores in some yeast strain backgrounds (e.g., SK1) or a complete block of the sporulation pathway in others (e.g., W303). The *spo13* mutation causes cells to bypass the first meiotic division, and thus *spo13* strains no longer require recombination for segregation of homologous chromosomes. *spo13* suppresses the sporulation and spore viability defects of mutant strains unable to initiate DSB formation, including *mre11, rad50,* and *xrs2* (7, 149, 222). Because of the failure to induce meiosis-specific DSBs, *mre11, rad50,* and *xrs2* null mutants show no induction of meiotic heteroallelic recombination in the return-to-growth protocol or recombinants among viable dyads derived from *spo13* meiosis (7, 149, 222). Several separation-of-function alleles of *RAD50* have been identified that are proficient for DNA repair in mitotic cells but sporulation defective (8). The sporulation defect conferred by *rad50S* alleles cannot be suppressed by *spo13* but can be suppressed by *spo13* plus *spo11* (defective for initiation of recombination). Meiotic DSBs are still formed in *rad50S* mutants, but the ends are not processed to generate 3′ single-stranded tails and are stable rather than transient (8, 46, 370).

The role of *MRE11, RAD50,* and *XRS2* in DSB formation is unclear. *MRE11* is required for a meiosis-specific alteration of chromatin structure at recombination hot spots, and the chromatin alteration and DSB formation functions of Mre11 are eliminated by a deletion removing the C-terminal 49 residues (108, 266). This region of Mre11 contains one of the two DNA binding sites of Mre11 defined by in vitro studies (407). At least 10 genes are required for DSB formation; one of these, *SPO11,* is known to play a direct role since it encodes the catalytic subunit of the DSB-forming complex (170). Spo11 is an atypical type II topoisomerase that catalyzes DSB formation

by a transesterification mechanism. *MRE11* and *RAD50* also appear to function after the formation of meiosis-specific DSBs as evidenced by several separation-of-function alleles that are still proficient for DSB formation but are defective in DSB processing. In *rad50S* mutants, Spo11 remains covalently associated with the 5′ ends at break sites (170). Several alleles of *MRE11* have been identified that confer a similar phenotype to *rad50S* in meiosis (*mre11S, mre11-58, mre11-6, mre11-D16A,* and *mre11-H125N*) (108, 241, 256, 400, 407). Although covalent attachment of Spo11 at break sites has not been demonstrated for all of these mutants, it is assumed to occur because they all result in the accumulation of unprocessed breaks during meiosis. *mre11S* was isolated in a screen for mutants that showed *RAD50*-dependent spore inviability (256). From the same genetic screen, the *SAE2*/*COM1* gene was identified (295), as well as from a screen for *SPO11*-dependent spore inviability (235). Null mutations in *SAE2*/*COM1* confer a phenotype very similar to that of *rad50S* and *mre11S* mutants; accumulation of unprocessed DSBs in meiosis.

Two models have been proposed for the removal of Spo11 from break sites. First, Spo11 could be removed while still covalently attached to the 5′ strand by the endonucleolytic activity of the Mre11-Rad50-Xrs2 (MRX) (Sae2?) complex. Second, Mre11, Rad50, and Sae2 could be required for the reversal of the Spo11 transesterification reaction. The first model predicts that resection of the 5′ strand is intrinsic to removal of Spo11, whereas the second model predicts that resection of the 5′ strand occurs after Spo11 removal. Support for the first model comes from the observation that strains containing nuclease-defective alleles of *MRE11* (*mre11-D16A, mre11-H125N* and *mre11-58*) have unprocessed DSBs.

Role of the MRX complex in mitotic recombination. The complex phenotype of *mre11, rad50,* and *xrs2* mutants is even more apparent in vegetative cells. Although it is generally assumed that yeast cells repair IR-induced DNA damage by homologous recombination, *mre11, rad50,* and *xrs2* mutants show little or no defect in spontaneous mitotic recombination. Spontaneous heteroallelic recombination is elevated about 10-fold in *mre11, rad50,* and *xrs2* diploids and still shows some induction by DNA-damaging agents, but not to the extent observed in wild-type cells (7, 149, 314). Because diploids in the G_2 stage of the cell cycle preferentially repair lesions from a sister chromatid instead of a homologue, it has been suggested that *MRE11, RAD50,* and *XRS2* are specifically involved in sister chromatid recombination (8, 42, 149). In support of this hypothesis, *mre11* diploids are more resistant to irradiation than are haploids; G_1 diploids show the same sensitivity to IR as do wild-type strains, and mutant G_2 haploids and diploids both show high IR sensitivity (42). In a genetic assay designed specifically to detect sister chromatid recombination (94), *mre11* mutants showed a slight decrease in the rate of recombination but not to the extent expected if *MRE11* was essential for this process (42). *rad52* mutants show a 50-fold reduction in sister chromatid recombination in the same assay system. The hyperrecombination phenotype exhibited by *mre11, rad50,* and *xrs2* mutants for heteroallelic recombination in diploids could be due to channeling lesions from the normal sister chromatid repair pathway into interactions between homologues (8, 149).

Spontaneous deletion between direct repeats occurs at wild-type frequency in *rad50* mutants (119, 233). However, the recombination products were not studied in sufficient detail to determine whether there were defects in sister chromatid events using this system. Using the *ade2* inverted-repeat assay (Fig. 4), a threefold reduction in spontaneous recombination was reported for *rad50* and *xrs2* mutants, with the same distribution of events as found in the wild-type strain (298). Ectopic recombination between *ura3* heteroalleles on chromosomes II and V was unaffected by a *rad50* mutation in haploids but showed a threefold increase in *rad50* homozygous diploids (357). As suggested above for heteroallelic recombination, the hyperrecombination phenotype could be due to channeling lesions from sisters to interchromosomal interactions.

Studies of HO-induced recombination have also revealed only modest defects in these mutants (151, 400). The most striking finding that emerged from studies of mating-type switching is the decreased extent of processing of the 5′ strand at the HO-cut site in the null mutants (151, 362, 400). This result, in combination with the defect in processing of meiosis-specific breaks in certain *mre11* and *rad50* mutants, led to the suggestion that the MRX complex resects ends to produce 3′ single-stranded tails. However, even in *mre11, rad50,* or *xrs2* null mutants, processing does occur, and while there is a delay in mating-type switching, most cells are able to complete the process with fairly high efficiency. HO-induced SSA between chromosomal direct repeats is also delayed by 1 to 2 h in *rad50* mutants, but cells are able to repair the break with only a twofold decrease in viability (362).

Role of the Mre11 nuclease in processing DSBs in mitotic cells. The observation that HO-induced DSBs are processed more slowly in *mre11, rad50,* and *xrs2* null mutants suggests that the MRX complex is directly involved in end processing or regulates the activity of a nuclease. Because the exonuclease activity of Mre11 is of the opposite polarity to that expected for resection of DSBs, the endonuclease activity is thought to be targeted to the 5′ strand by an as yet unknown mechanism. The role of the Mre11 nuclease in resection has been investigated by generating nuclease-defective alleles of *MRE11* for analysis of end processing in vivo. The phenotype of the *mre11-58* (*rad58*) strain is due to mutation of a conserved residue in phosphodiesterase motif IV (H213Y) (400). The Mre11-58 mutant protein is proficient for DNA binding but lacks exonuclease and endonuclease activities in vitro and fails to interact with Rad50 and Xrs2 when immunoprecipitated from yeast extracts (407). The phenotype of the *mre11-58* strain is very similar to that of the *mre11* null mutant: high sensitivity to MMS, delayed kinetics of mating-type switching, and elevated rates of mitotic heteroallelic recombination (400). The Mre11-D56N and Mre11-H125N mutant proteins also lack nuclease activity (241), and Mre11-H125N retains interaction with Xrs2 and Rad50 (S. Moreau and L. S. Symington, unpublished data). These two mutations confer much less severe mitotic phenotypes than does the *mre11*Δ allele. Strains containing either the *mre11-D56N* or *mre11-H125N* allele show intermediate sensitivity to IR and normal levels of spontaneous mitotic recombination and plasmid gap repair, and the kinetics of mating-type switching are indistinguishable from those in the wild type (241, 379). These observations suggest that the Mre11 nuclease may not be involved in the resection of mitotic DSBs or is redundant with another nuclease. The more severe phenotype conferred by the *mre11-58* allele could be a conse-

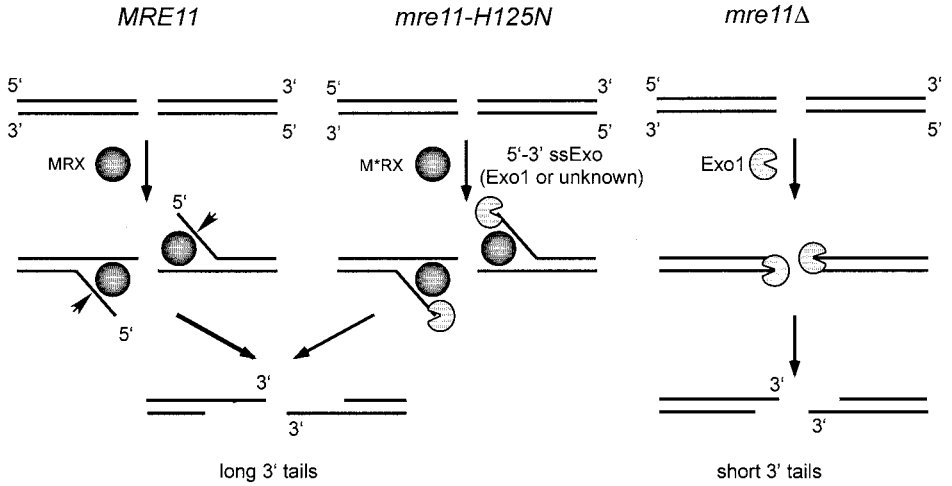

FIG. 10. Models for DSB processing by the MRX complex and Exo1. In wild-type cells, ends are processed by unwinding of ends and endonucleolytic cleavage of the 5′ strand by the Mre11 nuclease. Unwinding could be mediated by the weak unwinding activity of the Mre11 complex or by association with a DNA helicase. In the absence of the MRX complex, Exo1 inefficiently processes the ends. The M*RX complex in cells expressing the *mre11-H125N* allele is still able to unwind ends, and other nucleases remove the 5′ single-stranded tails.

quence of defective complex formation rather than the nuclease defect. Bressan et al. (43) generated mutations within all four phosphodiesterase motifs. The *mre11-2* and *mre11-4* alleles conferred phenotypes indistinguishable from those conferred by the null mutation for radiation sensitivity and spontaneous mitotic recombination. These two proteins both contain nonconservative substitutions of two amino acids. Both mutants failed to interact with Rad50 in the yeast two-hybrid system, suggesting that the severity of the mitotic DNA repair defect could be due to lack of the MRX complex rather than just loss of the Mre11 nuclease. The *mre11-11* and *mre11-3* alleles, containing substitutions within motifs I and III, respectively, conferred less severe phenotypes for sensitivity to IR than did the *mre11*Δ mutation, and both retained interaction with Rad50. Thus, the severity of the phenotype conferred by these alleles is directly correlated with the ability of the mutant proteins to form complexes. The Mre11-D16A protein lacks exo- and endonuclease activities but retains DNA binding; interaction with Rad50 and Xrs2 has not been determined (108). The *mre11-D16A* mutant shows intermediate sensitivity to MMS, intermediate-length telomeres, and normal rates of spontaneous mitotic recombination, but has not been tested for processing of HO-induced DSBs in vivo (108).

Because *mre11-H125N* strains are unable to process meiosis-specific DSBs but are proficient at repair of HO-induced breaks, we have suggested that the complex unwinds ends and that nucleases redundant with Mre11 can process free 5′ ends but not ends bound by Spo11 (241). A redundant endonuclease would be expected to substitute for Mre11 in both mitotic and meiotic cells, suggesting that it is a 5′-to-3′ exonuclease that processes ends in the absence of Mre11 in mitotic cells. Alternatively, the redundant activity could be an endonuclease that either is not expressed during meiosis or is excluded from the meiotic DSB-processing complex. The *EXO1* gene, which encodes a 5′-to-3′ exonuclease with a twofold preference for double-stranded over ssDNA (98), was found to suppress the mitotic DNA repair defect of *mre11* strains when present at high copy number (195, 242, 399). This suppression was also observed for *rad50* and *xrs2* mutants, suggesting that *EXO1* in more than one copy can bypass the requirement for the MRX complex in DNA repair. Furthermore, *exo1 mre11* double mutants have a severe growth defect (only about 30% plating efficiency), higher sensitivity to IR and MMS, and a longer delay in mating-type switching compared with *mre11* single mutants (242, 399). The *exo1* null mutation alone confers no significant sensitivity to IR or mating-type switching defects, and the *exo1 mre11-H125N* strain has normal kinetics of mating-type switching (98, 242). This contrasts with the severe defect observed for the *exo1 mre11*Δ double mutant. We envision inefficient processing of duplex ends by Exo1 in the absence of the MRX complex, but the efficiency of processing is greater when *EXO1* is present on a high-copy-number plasmid. In *mre11-H125N* cells there must be a single-strand-specific nuclease activity that can substitute for either the Mre11 or Exo1 nuclease activity but is unable to process duplex ends (Fig. 10).

An alternative hypothesis to explain the normal processing of HO-induced breaks in the *mre11-H125N* strain is that the Mre11 nuclease plays no role in resection and the role of the MRX complex is to recruit the resection nuclease to break sites or to clean up ends for the resection nuclease. The *mre11-H125N* diploid is unable to process DSBs with Spo11 covalently bound to the 5′ ends and does show sensitivity to high doses of IR. IR causes base and sugar damage in addition to strand breaks, and termini frequently have phosphate or phosphoglycolate groups. One attractive model is for the endonuclease activity of Mre11 to remove damaged nucleotides or protein-DNA covalent adducts (such as Spo11) from ends to provide the substrate for the resection nuclease. Further support for this model comes from recent studies suggesting that Mre11 removes the terminal protein of adenonvirus during infection (359). Adenoviruses use a protein-priming mechanism for replication, resulting in linear duplexes with a protein covalently bound to the 5′ ends. Viruses in which the E4 region

is deleted fail to package viruses due to concatemerization of viral genomes (413). Concatemerization of E4-deleted viral genomes was found to require DNA-PKcs, ligase IV, Mre11, and Nbs1, suggesting that concatemers are formed by the end-joining pathway (359). Concatemer formation was restored to the ATLD3 cell line by transfection with wild-type Hs *MRE11* but not with the Hs *mre11-3* nuclease-defective allele. Infection of cells with wild-type virus results in depletion of Mre11, and this is dependent on the E4 region. Together, these results suggest that one function of E4 is to promote the degradation of Mre11, thus preventing cleavage of the terminal protein from 5′ ends and subsequent concatemerization by end joining.

Recent studies suggest the Mre11 nuclease is important for processing unusual DNA structures, such as hairpins. Insertion of a 323-bp quasipalindrome derived from human Alu elements into the yeast *LYS2* gene stimulates the rate of ectopic recombination (with a different *lys2* allele) 1,000-fold. This stimulation is dependent on *MRE11, RAD50,* and *XRS2* (207). Interestingly, *mre11-D56N, mre11-H125N, rad50S,* and *sae2*Δ mutations also completely eliminate the hyperrecombination induced by the palindrome. The palindrome appears to be extruded to form a cruciform structure, which is cleaved at the base by an unknown activity to form two hairpin ends. These hairpins fail to be resolved in the mutant strains and are replicated, resulting in the formation of an inverted duplication. The failure of the *mre11-D56N, mre11-H125N, rad50S,* and *sae2*Δ mutants to resolve the hairpin intermediate directly implicates the nuclease activity of Mre11, the Rad50 function compromised by the K81I mutation, and Sae2 in cleaving these structures in vivo. *rad32* (*MRE11*)- and *rad50*-dependent stimulation of mitotic recombination by a 160-bp palindrome is also observed in *Schizosaccharomyces pombe* (93).

An allele of *SAE2* was isolated in a screen for mutants that aberrantly process DSBs within an inverted repeat (297). In the *sae2* mutant, DSB-stimulated events occurred normally 46% of the time but the other 54% of the events were characterized by a duplication of part of the inverted repeat. These events were hypothesized to occur by break-induced replication through the inverted repeat followed by an end-joining event to form a palindrome structure. These aberrant events were not detected in the wild-type strain, suggesting that they do not occur, or that the palindrome is not stably maintained, in wild-type cells. The *mre11-H125N* and *rad50S* mutations conferred the same phenotype as did the *sae2* mutation in this assay, again suggesting that the Mre11 nuclease and Sae2 resolve palindromes. The results of these two studies are consistent with the observation that palindromes are stabilized in *sbcC* and *sbcD* mutants of *E. coli* (49, 115).

The *RAD27* gene encodes a flap endonuclease that removes RNA primers from Okazaki fragments during DNA synthesis. *rad27* mutants are viable but depend on homologous recombination functions (*RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, MRE11,* and *XRS2*) for viability (378, 393). The *sae2*Δ, *mre11S, mre11-H125N,* and *rad50S* mutations all cause death in a *rad27* strain (72, 241). The Mre11 nuclease could be partially redundant with the Rad27 nuclease or could be required to process aberrant DNA structures that accumulate in *rad27* mutants. An alternative explanation is that the large number of lesions generated in a *rad27* mutant overloads the homologous recombination system so that mutants with subtle DNA repair defects are unable to repair all of them. The similarity in the phenotypes of *mre11S, mre11-H125N, rad50S,* and *sae2*Δ mutants suggests that the nuclease activity of Mre11 is absent in these mutants. This has clearly been shown for the Mre11-H125N protein, but the Mre11S protein retains endo- and exonuclease activities (E. A. Morgan and L. S. Symington, unpublished data). This suggests the possibility that the Mre11 nuclease is active in vivo only in the presence of Sae2 and when Rad50 is fully functional. An attractive model is for Sae2 to interact with the MRX complex to activate the Mre11 nuclease and for the Sae2-MRX complex to be disrupted by *rad50S* and *mre11S* mutations.

Role of the MRX complex in nonhomologous end joining. The end-joining pathway of repair requires Ku70 and Ku80, encoded by the *YKU70* (*HDF1*) and *YKU80* (*HDF2*) genes, respectively, a specialized DNA ligase encoded by the *DNL4* gene, and a ligase stimulatory factor, Lif1 (XRCC4 in mammals) (271). In mammals, the Ku heterodimer associates with a kinase (DNA-PKcs) to form the DNA-dependent protein kinase (DNA-PK), but to date a similar kinase has not been identified in yeast (83, 120). An additional factor, Lif2/Nej1, which interacts with Lif1 and is regulated by mating type, is required for end joining in yeast (104, 171, 267, 408). Defects in any of the components of this pathway, with the exception of *MRE11, RAD50,* and *XRS2,* do not cause IR sensitivity but do increase the IR sensitivity of a *rad52* strain in stationary phase. This suggests that the homologous pathway is the primary means of repairing IR-induced damage and that end joining can be used as a backup pathway.

Several assays have been used to measure end joining in yeast. The transformation efficiency of autonomously replicating plasmid DNA that has been linearized with a restriction enzyme to produce cohesive ends is used to measure precise rejoining (38). In wild-type cells, ligation occurs with high fidelity, with no loss or gain of nucleotides at the junction most of the time (38). A similar assay to monitor the repair of chromosomal breaks measures cell survival in response to induction of *Eco*RI endonuclease in a strain containing a *GAL1*-regulated *Eco*RI gene (196). Imprecise end joining can be assayed by survival in response to HO endonuclease induction of a strain that cannot repair the break at the *MAT* locus by homologous recombination (either by deletion of the donor cassettes or by deletion of *RAD52*) (240). In all three assays, *mre11, rad50, xrs2,* and *yku70* strains have similar phenotypes and appear to be epistatic (37, 240). Although *mre11* and *yku70* mutations cause a similar reduction in the efficiency of joining cohesive ends of plasmids, the types of products recovered are different. Repaired plasmids recovered from *yku70* and *yku80* strains have large deletions flanking the break site and rejoin through short sequence homologies, whereas plasmids recovered from *mre11, rad50,* and *xrs2* strains show mainly faithful repair (37, 38). In the HO assay, faithful repair restores the HO-cut site, which can then be recut by HO. Survivors of continuous HO expression have small deletions or insertions at the HO cut site, which prevent further cutting by HO. The frequency of survivors is reduced in *mre11, rad50,* and *xrs2* mutants, but the survivors have large deletions (240). Although it has been suggested that the Mre11 nuclease could function in processing ends for the end-joining pathway, the characterization of junctions produced in *mre11* mutants in yeast is

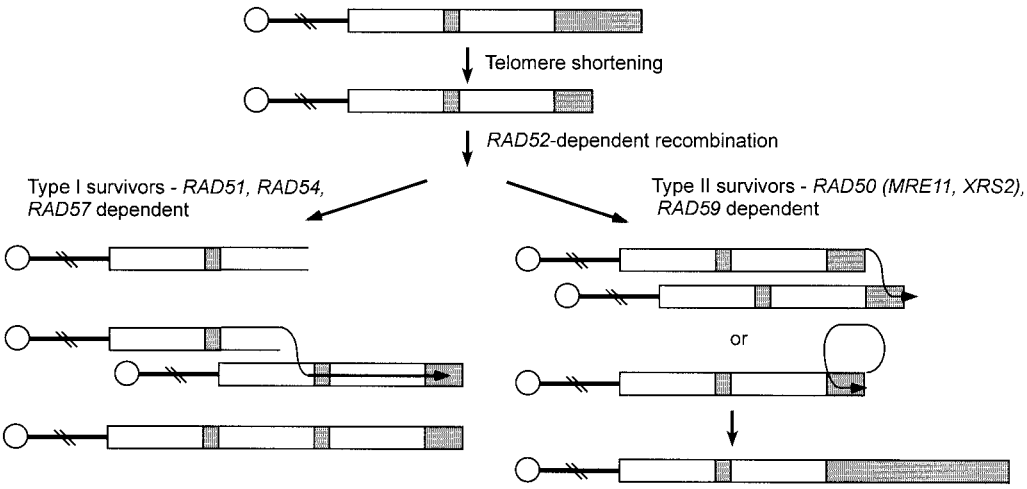

FIG. 11. Telomere maintenance by recombination in the absence of telomerase. In the absence of telomerase, telomeres become progressively shorter and are maintained by *RAD52*-dependent recombination. Two types of recombination events give rise to survivors: *RAD51*-, *RAD54*-, and *RAD57*-dependent recombination between Y′ sequences, and *RAD50*- and *RAD59*-dependent recombination between T G_{1-3} tracts. Y′ elements are marked by open boxes, and tracts of TG_{1-3} are indicated by shaded boxes.

inconsistent with this hypothesis. Furthermore, the *mre11-H125N* strain is proficient for end joining and *EXO1* present on a high-copy-number plasmid is unable to suppress the end-joining defect of *mre11* strains (195, 241, 242). *S. pombe rad32* (*mre11*) and *rad50* mutants show no defect in a plasmid recircularization assay, raising the issue of how general the *S. cerevisiae* findings are (223, 422).

The purified MRX complex stimulates intermolecular DNA joining by the Dnl4-Lif1 complex (53). Atomic force microscopy analysis revealed juxtaposition of DNA ends by the MRX complex to form linear concatemers, suggesting that the MRX complex can align DNA molecules for ligation. Interaction between the MRX complex and Dnl4-Lif1 appears to be mediated by Xrs2, suggesting that Xrs2 might function to recruit Dnl4 to ends held together by Mre11 and Rad50. Intermolecular end joining promoted by the human DNLIV-XRCC4 complex and DNA-PK is also stimulated by the addition of the MRN complex (143).

Role of the MRX complex in telomere maintenance. Strains with a mutation of *MRE11, RAD50,* or *XRS2* have short, but stable telomeres (37, 174). The length of the telomeric repeat tracts in these strains is similar to that of strains with a mutation of *TEL1,* the yeast homologue of the human ATM gene (121, 216, 249). The *tel1* mutation is epistatic to *mre11, rad50,* and *xrs2* for telomere length, and, like *tel1,* a *rad50* mutation confers senescence to *mec1* strains (305, 306). Thus, Tel1 and the MRX complex work in the same pathway to maintain normal telomere clength. One attractive hypothesis for the role of the MRX complex in telomere maintenance is to produce a substrate for telomerase, perhaps by generation of a single-stranded tail. However, the *mre11-D56N* and *mre11-H125N* strains, defective for the Mre11 nuclease, have normal length telomeres (241; Morgan and Symington, unpublished). Furthermore, the *mre11-D56N* and *mre11-H125N* mutations do not cause senescence in a *mec1* strain (401). As suggested for processing of HO-induced breaks, there may be other nucleases that can substitute for the Mre11 nuclease as long as the complex is present. Interestingly, overexpression of *EXO1* is unable to suppress the short-telomere defect of *mre11, rad50,* or *xrs2* strains, suggesting that the nuclease activity is not important for the function of MRX at telomeres (50, 242, 399). The length of the single-stranded tail at native telomeres in *mre11, rad50,* and *xrs2* strains is sufficient for binding of the TG_{1-3} DNA binding protein, Cdc13, in asynchronous cells (401). Targeting of the telomerase catalytic subunit (Est2) to telomeres by using a Cdc13-Est2 chimera suppresses the telomere length and senescence phenotype of *mre11* and *mre11 mec1* strains, respectively, suggesting that one function of the MRX complex at telomeres is to recruit telomerase (401).

In the absence of telomerase, telomeres become progressively shorter, which leads to cellular senescence (214). Suppressors of this growth defect arise at high frequency due to *RAD52*-dependent recombination to restore telomere length either by amplification of the subtelomeric Y′ elements (type I survivors) or by formation of very long telomeric tracts (type II survivors) (213, 389) (Fig. 11). The type I survivors have multiple copies of Y′ elements but still have very short TG_{1-3} tracts. Both types of survivors are generated in a *tlc1* strain (deficient for the RNA component of telomerase). Generation of type I survivors requires *RAD51, RAD54, RAD55,* and *RAD57* (55, 190, 388). The type I survivors eventually convert to the type II survivor pattern after several hundred divisions due to the improved growth of type II survivors compared with type I survivors. In *rad50* mutants, type I survivors are predominantly recovered, indicating a role for the MRX complex in the generation of long telomeric tracts in the absence of telomerase (55, 388). The type II survivors are thought to arise by inter- or intramolecular recombination between TG_{1-3} tracts (Fig. 11). The requirement for *RAD50* for this process suggests that Rad50 could be important for pairing between short homologies.

Role of the MRX complex in suppression of gross chromosome rearrangements. Chen et al. described a genetic assay to detect gross chromosome rearrangements (GCR) by measur-

ing the simultaneous loss of two linked subtelomeric markers in haploid yeast strains (52). In wild-type cells such events are rare (3×10^{-10}/cell/generation) and are due primarily to loss of the markers followed by telomere addition. The rate of GCRs is increased by 600-fold in *mre11, rad50,* and *xrs2* strains. One-third of the events represent telomere additions after loss of the markers, indicating that de novo telomere formation can still occur in *mre11, rad50,* and *xrs2* mutants. The other 70% of the events recovered from *mre11* mutants are chromosome translocations, most of which have no significant homology at the breakpoints (52). The increased rate of GCRs observed in the *mre11, rad50,* and *xrs2* mutants could be due to increased accumulation of lesions and/or channeling of lesions from the normal nonmutagenic repair pathway to mutagenic repair. *mec1* mutants, defective for the S-phase and damage checkpoints, show a 200-fold increase in GCRs, and these are due primarily to telomere addition (254). The *tel1* mutation by itself confers no increase in the rate of chromosome rearrangements in wild-type or *mre11* strains, but the *tel1 mec1* and *mre11 mec1* double mutants show a synergistic increase (≈10,000-fold) in GCR events (254). The high rates of GCRs observed in the *mec1 tel1* and *mec1 mre11* double mutants are most probably due to the combination of checkpoint, telomere addition, and DNA repair defects and are consistent with Tel1 and MRX functioning in the same pathway.

Defects in *MRE11* and *NBS1* are causes of human chromosomal instability syndromes. The human chromosomal instability syndrome A-T is caused by mutation of the ATM gene, which encodes a protein kinase homologous to the yeast Tel1 and Mec1 proteins (335). Cells established from A-T patients show sensitivity to IR, chromosomal instability, and radioresistant-DNA synthesis (the failure to suppress replication initiation in the presence of DNA DSBs). The cellular features of A-T are shared by two related chromosomal instability syndromes, NBS and A-TLD (335, 358). Although the clinical presentations of these syndromes are different, individuals with A-T and NBS show growth retardation, immunodeficiency, and cancer predisposition. Of individuals with NBS, 90% are homozygous for the 657del5 allele, a truncating mutation of *NBS1* that causes premature termination at codon 219 (411). This was originally assumed to be a null mutation. However, recent studies provide evidence for a 70-kDa Nbs1 protein that is produced by internal translation initiation within the *NBS1* mRNA (227). The 70-kDa Nbs1 protein is present at reduced levels but retains association with Mre11 and Rad50. Thus, the most common *NBS1* allele encodes a partially functional protein, consistent with recent studies showing cell death for a *NBS1* null mutation (227, 431). The N-terminal truncation of Nbs1, which removes the FHA domain, results in defects in checkpoint signaling and localization of the complex but retains the essential function of Nbs1 (47, 227).

Two mutations within the *MRE11* gene have been found for the two families with A-TLD-affected members (358). In one family, the mutation is a stop codon at position 633, producing a truncated protein. The truncated form of Mre11 produced by these patients is less abundant, and the levels of Rad50 and Nbs1 are also reduced. The mutation in the other family is a change of Asn117 to Ser. Both of these mutations have been generated in the Sc *MRE11* gene. As expected based on analysis of other yeast *mre11* mutations, the truncating mutation confers a meiotic defect but no effect on survival to IR whereas the missense allele confers a slight increase in sensitivity to IR and reduced spore viability. Interestingly, the *mre11-N113S* strain has short telomeres and this defect is partially complemented by the *mre11-58* allele, suggesting intragenic complementation between N-terminal alleles (191). The integrity of the intra-S phase checkpoint has not been determined for the yeast *mre11-Q623Z* or *mre11-N113S* strains.

Although no human cancer-prone syndrome has been assigned to a defect in Hs *RAD50,* a *rad50* hypomorphic mutant mouse has been generated (26). The *rad50-K22M*/*rad50-K22M* mutation corresponding to one of the *rad50S* alleles identified by Alani et al. (8) supports the viability of mouse embryonic stem cells. The *rad50-K22M* allele conferred no obvious sensitivity to clastogens and no apparent growth defect, and the intra-S-phase checkpoint was unaffected. However, the *rad50-K22M* allele caused a profound defect at the organismal level. Mice homozygous for this allele showed decreased birth weight, and most died with complete bone marrow depletion due to progressive hematopoietic stem cell failure. Rare long-term survivors were highly predisposed to malignancy, and cell lines derived from these tumors showed chromosome aberrations. Surprisingly, both male and female mice were fertile and there were no obvious defects in ovarian or testicular development.

Function of the MRX complex in the DNA damage checkpoint. DNA damage and stalled replication forks cause temporary arrest of the cell cycle to allow time for DNA repair to occur before progression through DNA synthesis or mitosis (415). Defects in the signaling pathway result in sensitivity to DNA-damaging agents and replication inhibitors, such as hydroxyurea (HU), and genome instability. The failure of cells from NBS and A-TLD patients to arrest DNA synthesis in response to IR was the first evidence suggesting a role for the MRN complex in the DNA damage checkpoint (281). ATM phosphorylates the Nbs1 protein in response to IR, and mutation of the Nbs1 residue that is phosphorylated results in loss of the S-phase checkpoint (114, 201). However, Nbs1 is still phosphorylated in ATLD3 cells, which are checkpoint defective, suggesting that Nbs1 phosphorylation is not sufficient for checkpoint activation.

Recent studies with yeast also implicate the MRX complex in the DNA damage response (68, 122, 192, 406). The presence of a single, unrepairable DSB induces a long G_2/M arrest, but cells adapt to the damage and resume the cell cycle (192, 315). *mre11* mutants arrest normally in response to the DSB but adapt faster than do wild-type cells (192). Conditions that result in increased amounts of ssDNA (from processing the break) correlate with inability to adapt, leading to the suggestion that ssDNA or a ssDNA-protein complex is recognized as the damage signal. Strains containing the nuclease-defective *mre11-3* allele show normal adaptation, consistent with studies by Moreau et al. showing normal resection of HO-induced breaks in the *mre11-H125N* strain (191, 241). The MRX complex is required for phosphorylation of the Rad53 kinase in response to IR but not to UV irradiation in G_1-phase cells, and G_1-arrested *mre11* mutants fail to delay DNA synthesis in response to IR (68, 122). *mre11, rad50,* and *xrs2* mutants are hypersensitive to HU and show no delay in DNA synthesis in the presence of HU or bleomycin, indicating a defect in the

intra-S checkpoint (68). One attractive model is for the MRX complex to recognize unusual structures that arise during replication and to signal through Rad53 to arrest S phase. D'Amours and Jackson suggested that the nuclease activity of Mre11 is required for the intra-S checkpoint, but the *mre11-58* and *mre11-P162S* strains utilized in their study are defective for MRX complex formation (50, 407). In contrast, the *mre11-H125N* strain is not hypersensitive to HU and is proficient for complex formation (S. Moreau, B. Krogh, and L. S. Symington, unpublished data). The Mre11 and Xrs2 proteins are also phosphorylated in response to IR, and this phosphorylation is dependent on Tel1 (68, 406). The physiological relevance of the Tel1-mediated phosphorylation is unknown. Studies in other systems, however, are not consistent with the MRX complex functioning in the S-phase checkpoint. *S. pombe rad50* mutants delay DNA synthesis in the presence of HU and MMS, suggesting that the replication checkpoint is still functional (129). Similarly, initiation of DNA replication is still delayed in the presence of DNA damage in *Xenopus* cell extracts following depletion of Mre11 (67).

RAD51, RAD52, RAD54, RDH54/TID1, RAD55, RAD57, RAD59, and *RFA1* Subgroup.

Genetic studies place the *RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RDH54,* and *RFA1* genes in the homologous recombination pathway. Within this group, the *RAD51, RAD52, RAD54, RAD55,* and *RAD57* genes are essential for conservative DSB repair, resulting in gene conversion (and associated crossing over), and *RAD52* and *RAD59* have additional functions in the nonconservative BIR and SSA pathways. The requirement for replication protein A (RPA) has been more difficult to address in vivo because of the essential function of this complex in DNA replication. However, several non-null alleles of *RFA1* exhibit recombination and repair deficiencies. *RDH54/TID1* is discussed with *RAD54* (see below) because it encodes a protein with homology to Rad54 and shows redundancy with *RAD54* in some assays. In this section, the biochemical activities of each of the proteins are described, followed by characterization of mutant phenotypes and implications for the mechanism of recombination.

Rad51. Sc *RAD51* encodes a 43-kDa protein with 30% identity to bacterial RecA proteins; the highest homology is with the catalytic domain of RecA, encompassing the Walker A and B motifs for nucleotide binding and/or hydrolysis (1, 22, 338). In *E. coli, recA* is the most important gene for homologous recombination and the purified RecA protein promotes homologous pairing and strand exchange in vitro (184). Rad51 is conserved in all eukaryotes for which sequence information is available, and the mouse and human proteins are 59% identical to ScRad51 (246, 337). Alignment of the RecA and Rad51 amino acid sequences reveals an N-terminal extension of 100 amino acids in the eukaryotic Rad51 proteins that is absent from RecA (337). This region is well conserved among eukaryotic Rad51 proteins, suggesting that it is important, and recent studies implicate the N-terminal domain in DNA binding and protein-protein interactions (6, 185).

Yeast *rad51* null mutants are viable but show high sensitivity to IR and meiotic inviability. Surprisingly, deletion of *RAD51* in vertebrates results in cell inviability and early embryonic death in mice (200, 404). Trophoblast-like cells derived from mouse *rad51*$^{-/-}$ blastocysts are sensitive to IR, suggesting that the normal function of MmRad51 is to repair DSBs. A *RAD51* conditional cell line has been made by deleting both copies of *RAD51* in DT40 chicken cells in the presence of Hs *RAD51* regulated by a tetracycline-repressible promoter (352). Down regulation of the gene, resulting in depletion of HsRad51, is concomitant with a G_2/M-phase arrest, accumulation of cytologically visible chromosomal breaks, and eventual cell death. These data suggest that the essential role of *RAD51* in vertebrates is to repair breaks generated during DNA replication. In support of this hypothesis, Hs *RAD51* is expressed primarily during S phase and forms foci in proliferating cells (387). The *RAD51* transcript is also highly induced during meiosis and following treatment of cells with DNA-damaging agents such as MMS (22, 30, 338). Rad51 foci are detected during meiosis coincident with the timing of meiotic recombination and colocalize with the meiosis-specific RecA homologue, Dmc1 (31). The formation of Rad51 foci is dependent on Spo11 and can be induced in *spo11* mutants by treatment of meiotic cells with IR (113). Meiosis-induced Rad51 foci are not formed in *rad52, rad55,* or *rad57* mutants, consistent with biochemical studies implicating these factors in assembly of the Rad51 presynaptic filament (113). Radiation-induced Rad51 foci form at reduced levels in *rad55* and *rad52* mutants but are absent from *rad52 rad55* double mutants, suggesting some redundancy for presynaptic complex formation between these factors in mitotic cells (112a).

(i) Biochemical studies. Purified Rad51 forms right-handed helical filaments on double-stranded DNA with structural similarity to those formed by RecA (265). Formation of filaments on ssDNA is stimulated in the presence of the heterotrimeric DNA binding protein RPA, although short filaments of HsRad51 have been observed on tailed duplex molecules in the absence of RPA (234, 374). Rad51 binds with higher affinity to DNA duplexes with single-stranded tails than to duplex or single-stranded oligonucleotides (232). Although Rad51 is expected to bind to any DNA sequence independent of base composition, preferred binding sites have been identified. Rad51, RecA, and the *Sulfolobus solfataricus* RadA protein all bind to GT-rich sequences with higher affinity than to random sequences (331, 394, 395). Binding of HsRad51 to DNA can occur in the absence of ATP, but ATP is required for DNA binding by ScRad51 (76). Binding to ssDNA activates the Rad51 ATPase, but the k_{cat} for the ATPase is about 30-fold lower than found for the RecA protein (371). Mutation of the conserved lysine residue within the Walker A motif to alanine (Rad51-K191A) abolishes DNA binding and ATPase activities of ScRad51 (375). When the same lysine residue is replaced by arginine (Rad51-K191R), the protein retains ATP-dependent DNA binding, but no significant hydrolysis of ATP (375). The ATPase activity of Rad51 is higher when poly(dT) is used as a substrate compared with M13 DNA, and RPA stimulates the ATPase activity of Rad51 only when M13 is used as a cofactor, not poly(dT) (368). *E. coli* single-strand binding protein can substitute for RPA in stimulation of the Rad51 ATPase, indicating that Rad51 and RPA are unlikely to interact. These observations suggest that the role of RPA in presynapsis is to remove secondary structures from ssDNA to allow the formation of a continuous Rad51 nucleoprotein filament.

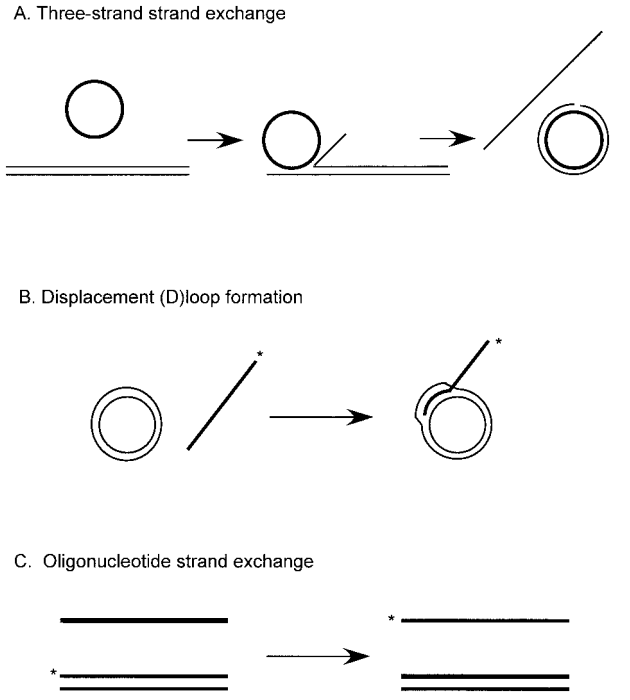

FIG. 12. Substrates used for in vitro strand exchange assays. (A) Pairing of a circular single-stranded molecule with linear duplex results in the formation of a nicked circular duplex product and a displaced linear single strand. (B) Invasion of a linear single-stranded molecule into supercoiled circular DNA results in the formation of a joint molecule with a D-loop. (C) Strand exchange between an unlabeled single-stranded oligonucleotide and labeled double-stranded oligonucleotides is detected by displacement of a labeled single-stranded oligonucleotide.

Once assembled, the Rad51 nucleoprotein filament is capable of interacting with a second DNA molecule, either ssDNA or dsDNA, to initiate strand exchange. Synapsis entails alignment of the nucleoprotein filament with homologous sequences within the second molecule. Although the details of this step of the reaction have not been well characterized for Rad51, studies with RecA protein suggest that the homology search is rapid and involves random collisions of the two molecules. Both RecA and HsRad51 form coaggregates of ssDNA and duplex DNA that are independent of sequence homology during the homology search (23, 398). Once homology is found, strand exchange occurs between the two aligned molecules if one of the partners has a free end to allow stable intertwining of the complementary strands.

Strand exchange can be measured *in vitro* using a variety of DNA substrates (Fig. 12). The three-strand exchange reaction using circular ssDNA and homologous linear duplex has been used extensively to characterize Rad51-promoted strand exchange. The reaction requires ATP but is also supported by nonhydrolyzable analogs of ATP (375). Furthermore, the Rad51-K191R mutant protein catalyzes extensive strand exchange when present at high concentration. The polarity of strand exchange is 5′ to 3′ with respect to the complementary strand of the DNA duplex, opposite to the polarity observed for RecA but the same as for the *U. maydis* Rec2 protein (27, 181, 374). The strand exchange activity of Rad51 is greatly stimulated by the addition of RPA to the reaction mixture if RPA is added after Rad51 has already nucleated onto the initiating ssDNA substrate (373). However, addition of RPA simultaneously with or prior to Rad51, to mimic the likely in vivo situation, results in a severe reduction in strand exchange products. The inhibition of Rad51-mediated strand exchange imposed by RPA can be overcome by the addition of Rad52 or the Rad55-Rad57 heterodimer to the reaction mixture (28, 259, 339, 372, 373). Both Rad52 and Rad55-Rad57 are thought to mediate the assembly of the Rad51 presynaptic filament, although most biochemical studies have focussed on the effects of these proteins in the strand exchange assay.

Oligonucleotide substrates have been used in fluorometric assays to distinguish between the pairing and strand displacement phases of strand exchange (123). When the G+C content of the oligonucleotides is very low (16%), HsRad51 promotes efficient pairing and strand exchange, but when the G+C content is increased to 40%, pairing still occurs but strand displacement does not (123). These results suggest that the initial homology recognition step requires partial base pairing between the incoming and outgoing strands prior to the full base-pairing interactions necessary for strand exchange. The use of single-stranded tailed duplex oligonucleotides improves the yield of strand exchange products; however, the effects of G+C content have not been determined with these substrates (232).

The D-loop assay, which requires the invasion of intact duplex DNA by ssDNA, appears to most closely model the expected situation in vivo (Fig. 12) (136). Rad51 inefficiently promotes D-loop formation between single-stranded oligonucleotides and homologous supercoiled DNA (232). HsRad51 promotes efficient D-loop formation between large linear single-stranded molecules or duplex molecules with single-stranded tails of either 5′ or 3′ polarity and supercoiled plasmid DNA (234). This reaction does not require stimulatory factors, whereas the equivalent reaction catalyzed by ScRad51 is inefficient in the absence of RPA and Rad54 (283). The reason for this difference is unclear.

(ii) Behavior of *rad51* mutants. Comparison of the sequences of RecA and Rad51 shows that several residues are invariant, notably in the regions assigned to ATP binding or hydrolysis (1, 22, 338). Site-directed mutation of the conserved lysine present in the Walker A-site for NTP binding to alanine results in a protein that is unable to bind DNA or promote strand exchange, while the Rad51-K191R protein is proficient for DNA binding and strand exchange (375). When the *rad51-K191A* allele is present in a *rad51*Δ mutant on a low-copy-number vector or overexpressed, the phenotype is indistinguishable from that due to a *rad51* null mutation, but this allele confers a semidominant phenotype when expressed in wild-type cells (79, 338, 375). In vivo, the *rad51-K191R* allele suppresses the MMS sensitivity of a *rad51*Δ strain when overexpressed, but when present on a low-copy-number plasmid it shows only partial activity (338, 375). Constitutive high-level expression of the Hs *rad51-K133R* allele supports growth and radiation resistance of chicken DT40 *rad51*$^{-/-}$ cells but not gene targeting, leading to the suggestion that ATP hydrolysis is not important for the essential function of Rad51 but is required for some homologous recombination functions (247). Expression of the Hs *rad51-K133R* allele in wild-type mouse

embryonic stem cells causes increased sensitivity to IR and cross-linking agents and reduced efficiency of homologous recombination (355). These dominant negative effects support the hypothesis that ATP hydrolysis is important for Rad51 function in vivo.

In contrast, when the *rad51-K191R* allele is expressed as the only chromosomal allele of *RAD51* in budding yeast, the phenotype conferred is quite similar to that conferred by the null allele with respect to radiation sensitivity, mating-type switching and spontaneous mitotic recombination (244). However, diploids homozygous for the *rad51-K191R* allele show normal levels of sporulation and high spore viability and are much more resistant to IR than is the *rad51-K191R* haploid. The suppression of the DNA repair defect conferred by the *rad51-K191R* allele in diploids is due to heterozygosity at the *MAT* locus. One attractive hypothesis to explain these results is that the *rad51-K191R* mutant protein has a defect in recycling and repair can occur when there is sufficient mutant protein in the cell. Based on this idea, it would be expected that other factors that promote recycling of Rad51 should be able to suppress the *rad51-K191R* phenotype when overexpressed and might be regulated by mating type and/or during meiosis. Alternatively, the Rad51-K191R nucleoprotein filament might be less stable than the filament formed from wild-type Rad51 and suppression by *MAT***a**/α could occur by stabilization of the mutant protein filament by an unknown factor. *RAD54* present at high copy number also suppresses the radiation sensitivity of the *rad51-K191R* strain (244). The partial suppression conferred by *RAD54* is consistent with either of the proposed models because Rad54 could function to stabilize the Rad51-DNA interaction by binding to the Rad51 nucleoprotein filament or could function to displace Rad51 from DNA by translocation activity (231). The HsRad51K133R protein forms more stable D-loops between a 90-mer oligonucleotide and supercoiled plasmid DNA than does wild-type HsRad51; this is attributed to decreased turnover of Rad51 in the absence of ATP hydrolysis (346).

Chanet and coworkers isolated a number of *rad51* alleles that suppress the MMS sensitivity of *srs2* diploids (1, 51). *SRS2* encodes a DNA helicase that is suggested to limit heteroduplex extension and/or reverse abnormal recombination intermediates (51, 307). Mutations in *SRS2* suppress the UV sensitivity of *rad6* and *rad18* mutants, and this suppression is thought to occur by channeling lesions from error-prone repair into the recombinational repair pathway (1, 189, 321). The *srs2* diploids show elevated rates of spontaneous and induced mitotic recombination and sensitivity to both ionizing and UV irradiation. The UV sensitivity of *srs2* diploids is suppressed by homozygous mutations in *RAD51*, *RAD52*, *RAD55*, and *RAD57*. The *rad51* alleles isolated as suppressors of the MMS sensitivity of *srs2* diploids were all semidominant, requiring one functional allele of *RAD51* for suppression. When present in haploids or as hemizygous diploids, all of the alleles were as defective in DNA repair and recombination as was a *rad51* null allele. Thus, the screen required decreased recombination to suppress lethal recombination events in the *srs2* cells but, at the same time, required residual recombination activity to survive the lethal effects of MMS. Sequencing of the 26 *rad51* mutations recovered revealed substitutions of 18 different amino acids, most of which are conserved in the RecA family of proteins. Ten of the mutations correspond to regions of RecA directly involved in ATP binding, ATP hydrolysis, or ATP-induced allosteric changes; six are in putative monomer-monomer interaction domains. Presumably, the mutant monomers can be assembled with wild-type monomers to yield a partially functional filament.

Fortin and Symington isolated a novel class of *RAD51* gain-of-function alleles that partially suppress the requirement for *RAD55* and *RAD57* in DNA repair (102). Five of the six mutations map to the region of Rad51 that, on the basis of modeling with RecA, corresponds to one of the DNA binding sites. The other mutation is in the N terminus of Rad51, in a domain implicated in protein-protein interactions or DNA binding (6, 185). These *rad51* alleles were unable to suppress the DNA repair defect conferred by a partially functional *rad52* allele, demonstrating genetic separation of the mediator functions. The Rad51-I345T mutant protein showed increased binding to ssDNA and dsDNA and was proficient in displacement of RPA from ssDNA, suggesting that the normal function of Rad55-Rad57 is to promote and stabilize Rad51-ssDNA complexes.

(iii) Rad51-interacting proteins. The yeast two-hybrid system has been used to identify Rad51-interacting proteins and to map domains of Rad51 involved in protein-protein interactions. Rad51 self-interacts and interacts with Rad52, Rad54, Rdh54/Tid1, and Rad55 (57, 79, 82, 130, 155, 159, 237). Rad51 self-association, as well as Rad51-Rad52 interaction, is mediated via a domain in the amino terminus of Rad51 (79). However, single-amino-acid substitutions within Rad51 that disrupt the interaction with Rad52 map to the C terminus of Rad51 (185). Some of the mutations that disrupt the Rad51-Rad52 interaction also disrupt the interaction between Rad51 and Rad54, suggesting that interactions between these proteins and Rad51 are likely to be dynamic. Mutations within the N terminus of Rad51 that reduce Rad51 homotypic interactions also disrupt the interaction with Rad55 (185). The *rad51* mutations that disrupt interaction with Rad52 and Rad54 confer sensitivity to MMS, confirming the importance of these interactions (185).

Most of the interactions determined by the two-hybrid system have been verified using biochemical methods (57, 117, 283, 285, 338). However, the interaction between Rad51 and Rad55 is extremely weak in vitro (373), and the interaction between MmRad51 and MmRad54 by coimmunoprecipitation is detected only following exposure to IR (384). HsRad51 associates directly with two important tumor suppressor proteins, p53 and Brca2 (54, 361, 424). Colocalization of HsRad51 and Brca1 has been detected on meiotic chromosomes, but this is probably due to the interaction between Brca1 and Brca2 rather than a direct interaction between HsRad51 and Brca1 (330). Rad51 interacts with the BRC repeats of Brca2, which are important for resistance to MMS (54), indicating the functional significance of this interaction. Furthermore, cell lines expressing hypomorphic alleles of *BRCA2* exhibit chromosome instability, radiation sensitivity, and defective homologous recombination (252, 332, 412, 430). HsRad51 also interacts directly with BLM, a member of the RecQ family of DNA helicases (425). The *BLM* gene is mutated in individuals with Bloom's syndrome, a cancer predisposition syndrome.

To analyze the dynamics of Rad51, Rad52, and Rad54 in

living cells, Essers et al. introduced green fluorescent protein (GFP)-tagged versions of all three genes into CHO cells (89). On treatment with IR, the proteins assembled into discrete nuclear foci. Rad51, Rad52, and Rad54 colocalized, as demonstrated previously by immunofluorescence of fixed cells (89, 384). The mobility of these proteins was determined in irradiated cells by using fluorescence redistribution after photobleaching (FRAP). While Rad51 was stably associated with damage-induced structures, Rad52 and Rad54 rapidly and reversibly interacted with these structures. These results suggest that interactions between Rad51, Rad52, and Rad54 are dynamic and argue against a stable complex of all three proteins (89). In yeast, most of the Rad52 present in extracts is associated with Rad51, suggesting that this subcomplex is quite stable (372).

Rad51 paralogs. (i) Fungal Rad51 paralogs. The *RAD55* and *RAD57* genes of *S. cerevisiae* encode proteins with sequence similarity to RecA and Rad51 and are considered to be Rad51 paralogs (167, 210). Null mutations of either *RAD55* or *RAD57* cause cold sensitivity for DNA repair (130, 159, 211). At 23°C, *rad57* mutants show the same sensitivity to γ-irradiation as *rad51* mutants, and *rad51 rad57* double mutants have the same sensitivity as the single mutants, whereas *rad57* mutants are 10- to 100-fold more resistant than *rad51* or the *rad51 rad57* double mutant at 30°C (102). Cold sensitivity is usually indicative of proteins that act as components, or stabilizers, of protein complexes. Consistent with this idea, Rad55 and Rad57 form a stable heterodimer and Rad55 also interacts with Rad51 (130, 159, 373). Unlike Rad51, neither Rad55 nor Rad57 exhibits self-interaction in the two-hybrid system, but the Rad55-Rad57 heterodimer has the anticipated molecular weight as determined by molecular sieve chromatography (130, 159, 373). *RAD51* present on a high-copy-number plasmid partially suppressed the radiation sensitivity of *rad55* and *rad57* mutants, and further suppression occurred when *RAD52* was also present in more than one copy (130, 159). These studies are suggestive of an accessory role for Rad55 and Rad57 in DNA repair, consistent with results of in vitro studies showing a stimulation of the Rad51-promoted strand exchange reaction by the Rad55-Rad57 heterodimer. Meiosis-specific Rad51 foci are not observed in *rad55* or *rad57* mutants, supporting the idea that Rad55 and Rad57 act in the formation or stabilization of the Rad51 nucleoprotein filament. Mutation of the invariant lysine residue of the Walker A box of Rad57 confers no defect in DNA repair or sporulation, but mutation of the corresponding residue in Rad55 does cause sensitivity to IR and prevents sporulation (159). The Rad55 Walker A-box mutants have not been tested for mediator function either in vitro or in vivo; therefore, it is not clear whether ATP hydrolysis is important for this function of Rad55.

Diploids homozygous for *rad55* or *rad57* mutations are more resistant to IR than are haploids (159, 211, 314). This suppression is due to mating-type heterozygosity and can be recapitulated in haploids by coexpression of *MAT***a** and *MAT*α alleles (by a *sir* mutation or introduction of *MAT* alleles on plasmids) (211). Because overexpression of *RAD51* also suppresses the radiation sensitivity of *rad55* and *rad57* mutants, it seemed possible that *RAD51* might be regulated by mating-type heterozygosity. However, there is no evidence from microarray analyses for increased expression of *RAD51* in diploids, and Western blot analysis indicates that there does not appear to be an increase in the Rad51 protein level in haploids expressing both *MAT* alleles (244). *RAD54* present at high copy number also partially suppresses the radiation sensitivity of *rad57* mutants, but, like Rad51, Rad54 protein levels do not appear to be affected by *MAT* heterozygosity (58, 244).

Rad55 is phosphorylated in response to DNA-damaging agents. This phosphorylation is dependent on Mec1 and partially dependent on Rad53 but not on other checkpoint functions (21). *rad55* mutants show normal responses to DNA damage, indicating that Rad55 is not required for the damage checkpoint. Interestingly, *mec1* mutants are defective in both spontaneous and MMS-induced heteroallelic recombination (21). This reduction is greater than reported for *rad55* mutants, suggesting that there might be *RAD55*-dependent and independent pathways for recombination, both of which require *MEC1* (211, 314).

The *S. pombe* Rad51 paralogs are encoded by the *rhp55* and *rhp57* genes (172, 403). Like their counterparts in *S. cerevisiae,* deletion mutations of *rhp55* and *rhp57* confer higher sensitivity to DNA-damaging agents at low temperatures than at 30°C and their DNA repair defects can be suppressed by *rhp51* present on a high-copy-number plasmid. Replacement of the conserved lysine residue of the Walker A motif by alanine of either Rhp55 or Rhp57 confers a modest DNA repair defect, but a strain containing the Lys-to-Ala substitutions in both genes is as sensitive to DNA-damaging agents as are the deletion mutants (402). This result suggests that the heterodimer may require only one active ATP binding site.

The fungus *U. maydis* encodes two Rad51-like proteins. One is orthologous to Rad51, and the other, encoded by the *REC2* gene, is much more divergent, is more than twice the size of Rad51, and has no clear structural equivalent in databases (97, 311). Unlike the *rad55* and *rad57* mutants of *S. cerevisiae,* the DNA repair defect of *rec2* strains is not cold sensitive, is not suppressed by Um *RAD51* on a multicopy plasmid, and is not suppressed in the diploid state (97, 182). The *rec2-1* mutant was the first eukaryotic recombination-defective mutant identified and shows decreased radiation-induced recombination, defective plasmid gap repair, reduced levels of gene targeting, and meiotic death (97, 134). A proteolytic form of Rec2 purified by conventional methods from *U. maydis* extracts and referred to as the rec1 protein was shown to promote homologous pairing and strand exchange in vitro (180). The full-length product of the recombinant *REC2* gene catalyzes similar reactions *in vitro* and has the same polarity and preference for tailed duplex molecules as shown for Rad51 (27, 232). The pairing and strand exchange activities of Rec2 require ATP but are not dependent on ATP hydrolysis. Consistent with this observation, replacement of the conserved lysine residue of the Walker A site by alanine results in a null mutant phenotype whereas the *rec2-K257R* allele confers no apparent DNA repair defect (311).

(ii) Characterization of vertebrate Rad51 paralogs. In addition to Rad51, vertebrates appear to encode five Rad51 paralogs, Xrcc2, Xrcc3, Rad51B/Rad51L1, Rad51C/Rad51L2, and Rad51D/Rad51L3 (392). These proteins have 20 to 30% sequence identity to Rad51 and to each other. The XRCC2 and XRCC3 genes were identified by complementation of the mitomycin C sensitivity of the *irs1* and *irs1SF* hamster cell lines

(161, 204). The *irs1* and *irs1SF* cell lines show high sensitivity to DNA-cross-linking agents and are about twofold more sensitive to IR than are the parental cell lines (161). Rad51B, Rad51C, and Rad51D were identified through database analysis or by PCR amplification on the basis of their similarity to Rad51 (10, 81, 290). Multiple protein alignments suggest that Xrcc2 is most similar to yeast Rad55 and Rad51D and that Xrcc3 is closest to yeast Rad57. The *irs3* and CL-V4B Chinese hamster cell lines have recently been shown to have mutations in the *RAD51C* gene, and the mitomycin C sensitivity of these cell lines was complemented by expression of the Hs *RAD51C* gene (106, 116). Two- and three-hybrid studies have shown interactions among the Rad51 paralogs but no evidence for self-associations (324). Rad51 interacts with Xrcc3 and weakly with Rad51C; the Rad51-Rad51C interaction is improved in the presence of Xrcc3. Rad51C interacts with Xrcc3, Rad51B, and Rad51D; and Rad51D interacts with Xrcc2. Many of these interactions have been confirmed by coimmunoprecipitation of the Rad51 paralogs from HeLa cells or following expression of recombinant proteins in *E. coli* or baculovirus-infected insect cells (40, 188, 229, 324). Recent studies have identified two discrete complexes of Rad51 paralogs, one containing Rad51C and Xrcc3 and the other containing Rad51B, Rad51C, Rad51D, and Xrcc2 (205, 230, 420). Although Rad51 was found to interact with Xrcc3 in the two-hybrid system, recent biochemical studies have failed to detect this interaction (205, 230, 420).

The Rad51D protein has been purified following expression in *E. coli* and exhibits preferential binding to ssDNA over dsDNA and a weak DNA-stimulated ATPase activity (40). The complex of Rad51C and Xrcc3 has also been purified (188, 229). The two proteins form a heterodimer with 1:1 stoichiometry, similar to that observed for Rad55-Rad57 and Rad51D-Xrcc2 (40, 188, 229, 373). The Rad51C-Xrcc3 complex binds to ssDNA but only weakly to dsDNA and aggregates ssDNA to form large networks (188, 229). The DNA binding and aggregation activities of the complex are independent of ATP (229). The Rad51C-Xrcc3 complex also promotes ATP-independent D-loop formation and strand exchange between short oligonucleotides (188). The Rad51B-Rad51C complex was purified from insect cells and shown to bind to ssDNA with higher affinity than dsDNA. Although DNA binding is ATP independent, the complex exhibits a weak ssDNA-stimulated ATPase activity. The Rad51B-Rad51C complex has mediator activity in the HsRad51-promoted strand exchange reaction. HsRad51 can efficiently make joint molecules between circular ssDNA and homologous linear duplex DNA in the absence of RPA (24), but the formation of complete strand exchange products requires RPA (344). As described for the yeast system, when RPA is added to the reaction mixture at the same time as Rad51, the formation of products in inhibited. The addition of Rad51B-Rad51C to the reaction mixture partially alleviates the RPA inhibition (345). The Rad51B, Rad51C, Rad51D, and Xrcc2 proteins form a stoichiometric complex that has similar DNA binding and ATPase activities to those of the constituent subcomplexes (230). The Rad51B-Rad51C-Rad51D-Xrcc2 complex has the interesting property of specific binding to nicked duplex DNA (230), which could be relevant to the function of these proteins in repair.

Knockout mutants of each of the Rad51 paralog-encoding genes have been generated in the DT40 chicken cell line (382, 383). All of the cell lines are viable but show higher spontaneous cell death, a large increase in spontaneous chromosomal aberrations (chromosome and chromatid breaks), and increased sensitivity to IR and cross-linking agents compared with the parental cell line (382, 383). The sensitivity of each of the mutant cell lines to cross-linking agents can be suppressed by overexpression of Hs *RAD51*. This result illustrates the similarity in phenotype between the chicken and yeast *rad51* paralog mutants. The number of cells containing γ-radiation-induced Rad51 foci and the number of foci produced are reduced in the mutant cell lines, again consistent with a mediator function for the Rad51 paralogs (382, 383). The hamster cell lines deficient for Rad51C show a reduction in spontaneous and mitomycin C-induced sister chromatid exchanges, in addition to sensitivity to DNA cross-linking agents and chromosome aberrations (106, 116). Interestingly, a reduced level of sister chromatid cohesion was observed in the CL-V4B cell line, which could contribute to chromosome instability (116). Although Rad51C is a component of both paralog complexes, the *rad51c*$^{-/-}$ DT40 cell line exhibits less severe defects in gene targeting and formation of spontaneous chromosome aberrations than do the other Rad51 paralog-defective cell lines (383). This could potentially be due to redundancy between Rad51C and Rad52 (107). Chicken DT40 cell lines deficient in Xrcc2, Rad51B, and Rad51D are viable; however, homozygous mutant mice undergo embryonic death (71, 289, 342). *Xrcc2*$^{-/-}$ embryos are recovered from early embryonic stages (before day 8.5), with embryonic death occurring from midgestation. Embryos that survive to late stages of development exhibit developmental abnormalities and neurological defects (71). The large increase in the number of apoptotic cells in neural tissues from *xrcc2*$^{-/-}$ mice is similar to that observed for ligase 4 and Xrcc4-deficient mice (103, 112).

The role of the Rad51 paralogs in homologous recombination has been assessed using recombination reporters containing two nonfunctional copies of the neomycin phosphotransferase (*neo*) gene or the GFP gene (41, 158, 287). These reporters contain an insertion of an I-*Sce*I-cut site to inactivate one copy of the gene, and the other copy has inactivating 5′ and/or 3′ truncations. In the presence of I-*Sce*I, a DSB is made within the reporter and can be repaired by homologous recombination or end joining. In the *irs1* (*xrcc2*$^{-}$) and *irs1*SF (*xrcc3*$^{-}$) cell lines, 100- and 25-fold decreases, respectively, in the efficiency of repair by homologous recombination were observed (158, 287). Analysis of the recombinants derived from the *irs1*SF (*xrcc3*$^{-}$) cell line revealed altered produce spectra compared with the *XRCC3*-complemented cell line (41). These alterations included increased gene conversion tract lengths, discontinuities of the tracts, and frequent rearrangements. Presumably, recombination still initiates in the cell line but occurs with lower fidelity. This could be due to Rad51-independent events, such as the BIR events seen in yeast that would be expected to be associated with longer tracts, or could be due to a late role of Xrcc3 in stabilizing Rad51-promoted recombination intermediates.

(iii) Dmc1. *DMC1* is not considered to be a member of the *RAD52* epistasis group because mutants are resistant to IR, but *DMC1* is essential for the repair of DSBs during meiotic recombination. *DMC1* was identified in a screen for meiosis-

specific prophase-induced genes that, when disrupted, resulted in meiotic prophase arrest (33). Mutation of *DMC1* leads to the accumulation of DSBs in meiosis and a reduction in the level of reciprocal recombinants, as measured by a physical assay. *DMC1* encodes a 334-amino-acid polypeptide with significant similarity to RecA and Rad51. Dmc1 is 45% identical to Rad51 along its entire length, and biochemical studies indicate a higher functional conservation than the other Rad51 paralogs to RecA-Rad51. Both Dmc1 and Rad51 form foci during meiosis, and the foci indicate significant colocalization of the two proteins (31). *RAD51* and *DMC1* are both required for high levels of meiotic recombination and play overlapping but nonidentical roles in meiosis (336). Homologues of Dmc1 have been identified in a number of eukaryotes, including mouse and human, and *dmc1*$^{-/-}$ knockout mice show the expected sterile phenotype (127, 288, 429).

The purified HsDmc1 protein binds to both ssDNA and dsDNA DNA, has a weak ATPase activity, and catalyzes strand exchange between oligonucleotides or from linear DNA to a single-stranded circular molecule (197, 228). The yeast Dmc1 protein also promotes annealing of complementary ssDNA (138). Unlike Rad51, Dmc1 does not appear to form helical filaments on DNA and instead forms octameric rings with DNA in the central channel (228, 276).

Rad52. Deletion of *RAD52* in budding yeast results in severe defects in homology-dependent DSBR and meiosis. *rad52* mutants are defective in BIR and SSA in addition to the *RAD51*-dependent gene conversion pathway; consequently, they show the most severe recombination defects of all the *rad52* group mutants. However, chicken and human cell lines lacking *RAD52* are viable, and, in contrast to *RAD51,* the deletion of *RAD52* does not cause embryonic death in mice (303, 427). Furthermore, mutant cell lines show no increase in sensitivity to DNA-damaging agents and the efficiency of gene targeting is only marginally reduced. *S. pombe* encodes two Rad52-like proteins, Rad22 and Rti1 (376, 409). The *rad22* mutant has more severe defects in mitotic recombination and repair of IR-induced damage than does the *rti1* mutant, but the double mutant is even more defective than the *rad22* single mutant (409). This suggests some redundancy between these genes in fission yeast and raises the possibility that another Rad52-like protein exists in vertebrates or that vertebrates have another function that is redundant with Rad52. Evidence for the latter comes from recent experiments showing inviability of a *rad52 xrcc3* DT40 chicken cell line even though the single mutants are able to proliferate (107, 353). The death of the double-mutant cell line is overcome by overexpression of *RAD51,* consistent with defects in two mediator functions. This result suggests that the Rad52 and Xrcc3 proteins are redundant or that they function in two different homologous recombination pathways that contribute to survival.

RAD52 of budding yeast is expressed throughout the cell cycle and is induced 2- to 3-fold by DNA-damaging agents and about 10-fold during meiosis (59, 60). Rad52 is nuclear and forms discrete foci in response to IR and during S phase of unirradiated cells (203). Surprisingly, only one or two foci are observed using a Rad52-GFP fusion, independent of the dose of IR, suggesting that damaged DNA is sequestered at one or two sites within the nucleus, perhaps at replication factories. Rad52 foci are also observed during meiosis, are dependent on *SPO11,* and show extensive colocalization with RPA (113, 203).

(i) Structural and biochemical studies. The published Sc *RAD52* open reading frame encodes a protein of 504 amino acids (3). The actual length is likely to be shorter because there are five putative start codons near the N terminus of the *RAD52* gene and mutation of either of the first two methionine residues confers no obvious DNA repair defect (251). Furthermore, the homologues of Rad52 identified in *S. pombe* and vertebrates lack the 34-amino-acid N-terminal extension present in ScRad52, suggesting that the start site is likely to be the third putative initiating codon (251). There is less sequence identity between ScRad52 and other family members than for other Rad52 epistasis group proteins, and most of the homology is restricted to the N-terminal 200 residues. The C terminus, which includes the Rad51 interaction domain, is less highly conserved. *RAD51* present on a high-copy-number plasmid suppresses the MMS sensitivity of strains expressing a deletion of Rad52 C terminus, suggesting that the primary function of this domain is to recruit Rad51 (237).

Both yeast and human Rad52 are multimeric and form ring structures as visualized by electron microscopy (296, 340, 356). Using both conventional and scanning transmission electron microscopy, the HsRad52 protein was observed to form heptameric rings with a strong pinwheel appearance and a central channel (356). It is generally accepted that DNA is bound within the central channel of hexameric helicases, but to date there is no evidence that DNA lies within the central channel of the Rad52 heptamer. Rad52 appears to have two modes of self-association. Assembly of monomers into rings requires sequences in the conserved N-terminal domain of Rad52, whereas the formation of higher-order multimers is mediated by the C terminus (296). The N-terminal 192 residues of Rad52 form rings with an average mass of 277 kDa, consistent with an average of 10 subunits, while the full-length protein forms a ring with an average mass of 298 kDa, consistent with the heptameric ring form (296). Formation of multimers is consistent with genetic studies showing intragenic complementation between N- and C-terminal mutations of *rad52* (39). The crystal structure of the N-terminal domain (residues 1 to 212) of the HsRad52 protein has been determined and reveals an 11-member ring (163). The overall structure resembles a mushroom, consisting of a stem containing highly conserved hydrophobic residues and a domed cap. A positively charged groove was identified under the domed cap, and mutational analysis was consistent with this region comprising a DNA binding surface (163). One of the residues identified as important for DNA binding, Arg55, is equivalent to Arg70 of ScRad52, previously shown to be important for Rad52 function in vivo (16). Surprisingly, several of the residues identified as being important for DNA binding do not appear to be important in vivo in the context of the full-length ScRad52 (251).

The purified Rad52 protein binds preferentially to ssDNA and promotes annealing of complementary ssDNA (250). Rad52-promoted annealing of long molecules is stimulated by RPA, whereas Rad52 efficiently anneals oligonucleotides in the absence of RPA (250, 340, 367). The probable role of RPA in strand annealing is to remove secondary structures from ssDNA to allow annealing by Rad52, but the stimulation of annealing also requires a species-specific interaction between

Rad52 and RPA. RPA alone inhibits the annealing of oligonucleotides; this inhibition is overcome by Rad52 (367). HsRad52 binds directly to HsRPA, and a similar interaction is thought to occur in yeast based on two-hybrid and genetic studies (99, 131). Because Rad52 interacts with both Rad51 and RPA, the contemporary models hold that Rad52 replaces RPA from ssDNA with Rad51 or that Rad52 provides a seeding site within the RPA-bound ssDNA for subsequent cooperative binding by Rad51 (351). Rad52 forms a complex with RPA-coated ssDNA, but does not displace RPA (366). Rad51 can displace RPA from ssDNA following interaction with Rad52 bound to RPA-coated DNA (366).

Studies with the HsRad52 protein have shown preferential binding to the ends of ssDNA of tailed duplex molecules (275). The terminal nucleotide is protected, and the region of ssDNA bound shows highly regular sensitivity to hydroxyl radicals. The periodicity of the hydroxyl radical sensitivity of DNA within Rad52-DNA complexes is thought to be due to wrapping of ssDNA on the outside of the Rad52 ring (275, 356). Genetic studies with yeast suggest a *RAD52*-dependent, *RAD51*-independent pathway for strand invasion, and recent studies show the formation of D-loops by HsRad52 (162). This activity, as well as DNA binding, is retained in a truncated form of the protein comprising the first 237 residues (162). The formation of D-loops by Rad52 probably occurs by annealing between the incoming ssDNA and transiently ssDNA present in the supercoiled plasmid.

(ii) Protein interactions. As described above, Rad52 self-associates to form a ring structure. It also interacts with Rad51 via the C-terminal domain of Rad52, and deletion of residues 409 to 412 eliminates Rad51 binding (186, 237). The Rad51 interaction domain of Rad52 is necessary for overcoming the RPA inhibition to strand exchange in vitro, consistent with the model that the mediator function of Rad52 requires interaction between Rad52 and Rad51 (186, 339). Although ScRad52 shows genetic and two-hybrid interaction with the large subunit of RPA (Rfa1) (99, 131), studies with the human proteins suggest a direct interaction between the 34-kDa subunit of RPA and Rad52 (274). The central domain of HsRad52 is required for interaction with RPA (131, 274). Rad52 also interacts with Rad59, raising the possibility of formation of a heteromeric Rad52-Rad59 ring (70).

(iii) Behavior of *rad52* mutants. Some point mutations in the C-terminal region of *RAD52* and deletions that encode only the first 210, 252, or 327 residues of Rad52 can be partially suppressed by *RAD51* present on a high-copy-number plasmid (15, 39, 237, 323). However, *rad52* null alleles, and most inactivating point mutations within the N-terminal region of *RAD52,* cannot be suppressed by *RAD51* at high copy number. No extragenic or high-copy-number suppressors of the *rad52*Δ allele have been identified. These observations suggest that the N-terminal region of Rad52 comprises a core domain with discrete functions in DSB repair that are independent of Rad51 (15). The first two alleles of *RAD52* identified, *rad52-1* (A90V) and *rad52-2* (P64L), both cause high sensitivity to IR and meiotic death, but *rad52-2* strains show increased levels of spontaneous heteroallelic recombination compared with *RAD52* strains. This suggests that spontaneous lesions might have a less stringent requirement for *RAD52* than do radiation-induced lesions. A non-null allele of *RAD52, rad52-R70K,* was identified in a screen for mutants defective in *RAD51*-independent recombination of inverted repeats (16). The *rad52-R70K* strain showed only a 4-fold reduction in inverted-repeat recombination, compared with a 3,000-fold decrease observed for the *rad52* null strain and a 1,300-fold decrease found for the *rad51 rad52-R70K* strain. The *rad52-R70K* mutation conferred partial sensitivity to γ-irradiation and was synergistic with a *rad59* null mutation for inverted-repeat recombination, SSA, mating-type switching, and sporulation, suggesting that some weak alleles of *rad52* are highly dependent on *RAD59* function.

In a systematic analysis, positively charged, aromatic, and hydrophobic residues within the N-terminal region of Rad52 were replaced by alanine and tested for radiation resistance and spontaneous mitotic recombination (251). From this analysis, mutations that conferred a phenotype similar to that for a null mutation were identified, along with several classes of separation-of-function mutations. One class, defined by a single allele, conferred intermediate sensitivity to IR but was completely defective for heteroallelic recombination. Another class, represented by several alleles, showed intermediate sensitivity to IR but wild-type or higher rates of heteroallelic recombination. The last class was partially defective for heteroallelic recombination but showed only mild sensitivity to IR. To date, there is no information on the biochemical activities of these Rad52 mutants.

Rad59. The *RAD59* gene was identified in a screen for mutants defective for *RAD51*-independent spontaneous mitotic recombination between inverted repeats (17). The *rad59* mutation was shown to cause modest defects in several mitotic recombination assays and moderate sensitivity to IR (16). In the chromosomal inverted-repeat recombination assay, *rad52* was epistatic to *rad51* and *rad59* and *rad59* was synergistic with *rad51.* The level of recombination in a *rad51 rad59* double mutant is higher than in a *rad52* mutant, indicating either that there is an additional pathway or that Rad52 is able to carry out some recombination functions by itself. *RAD59* is important for SSA between chromosomal direct repeats, and the requirement for *RAD59* increases as the repeat length decreases (16, 363). This suggests that Rad52 becomes more dependent on Rad59 when the repeats to be annealed are short or when short homologies are embedded within extensive regions of heterology.

The *RAD59* gene encodes a 238-residue protein with significant homology to the N-terminal half of Rad52 (17). The *RAD52* gene, when present on a *CEN*-based plasmid (one to three copies/cell), partially suppressed the radiation sensitivity of the *rad59* strain, suggesting that Rad52 and Rad59 have overlapping functions and/or that they interact. Interaction between Rad52 and Rad59 was demonstrated using the two-hybrid system and by coimmunoprecipitation of the two proteins from yeast extracts (70). A complex of Rad51, Rad52, and Rad59 can be immunoprecipitated from yeast extracts, but Rad51 and Rad59 fail to interact in the absence of Rad52 (A. P. Davis and L. S. Symington, unpublished data). In addition, Rad52 and Rad59 share some biochemical activities. Rad59 binds to DNA, with a preference for ssDNA, and anneals complementary ssDNA (70, 284). Annealing of long molecules is not stimulated by RPA, but Rad59 can overcome the inhibitory effect of RPA on annealing of oligonucleotides. Despite the similarity of their biochemical activities, *RAD59* can-

not substitute for *RAD52* in vivo, even in *RAD51*-independent recombination events such as SSA (70).

Rad54 and Rdh54/Tid1 (Rad54B). Rad54 and the Rad54 homologue Rdh54 (Tid1) have sequence motifs characteristic of DNA helicases and are members of the SNF2/SW12 family of DNA-dependent ATPases (84). As with the other *RAD52* group genes, *RAD54* is not essential for viability in yeast but is required for resistance to IR. Rdh54 was found by database analysis and independently identified in a two-hybrid screen as Tid1, a Dmc1-interacting protein (82, 179, 341). Diploid yeast cells disrupted for *RDH54*/*TID1* show slight sensitivity to high levels of MMS, but haploid mutants show no significant sensitivity to DNA-damaging agents (179). The *rad54 rdh54* haploid strains have similar growth rates and MMS sensitivities to *rad54* haploids, but homozygous *rad54 rdh54* diploids grow slowly and are more sensitive to MMS than are *rad54* diploids. Interestingly, the growth defect of the diploid double mutant can be suppressed by mutation of *RAD51,* suggesting that the poor growth is due to inappropriate recombination (179). A *rad54* mutation causes death in *srs2* strains; this is suppressed by mutation of *RAD51, RAD52, RAD55,* or *RAD57* or by elimination of the checkpoint functions, *RAD17, RAD24,* or *MEC3* (178, 270, 323). Although *srs2 rdh54* haploids are viable, the homozygous diploids are not; again, this is suppressed by mutation or checkpoint or homologous recombination functions (178, 179). The suppression of the *srs2 rad54* or *srs2 rdh54* lethality by *rad51, rad52, rad55,* and *rad57* suggests that Rad54 and Rdh54 function later in recombination than Rad51, Rad52, Rad55, and Rad57. The intermediates that accumulate in *rad54 srs2* strains presumably trigger the damage checkpoint, leading to cell cycle arrest.

Homologues of Rad54 have been identified in vertebrates by PCR amplification on the basis of their similarity to ScRad54 and SpRad54 (165). The mouse and human Rad54 proteins have 48% identity with ScRad54. A second Rad54 homologue, called Rad54B, has been identified in humans and is thought to be more closely related to ScRdh54/Tid1 than to ScRad54 (385, 386). Mouse and chicken *rad54*$^{-/-}$ cells are sensitive to IR, MMS, and mitomycin C, but not to UV light, and they show reduced frequency of gene targeting (29, 88). Human *rad54B*$^{-/-}$ cell lines are defective for gene targeting but do not show sensitivity to DNA-damaging agents (238). In contrast to *RAD51,* the absence of *RAD54* is compatible with normal mouse development. B- and T-cell development and immunoglobulin class switch recombination all occur normally in *rad54*$^{-/-}$ mice, indicating that these recombination events are independent of homologous recombination; however, immunoglobulin light-chain gene conversion is reduced in chicken bursal cells (29, 88). Although Mm *rad54*$^{-/-}$ embryonic cells are sensitive to IR, adult *rad54*$^{-/-}$ mice show high survival following IR (90). Survival of adult mice following IR is dependent on DNA PKcs, suggesting that the relative contribution of homologous recombination and end joining changes during development of the animal (90).

(i) Biochemical characterization. Both Rad54 and Rdh54/Tid1 proteins show dsDNA-dependent ATPase activity and promote a conformational change of closed-circular duplex due to the creation of positive and negative writhe (231, 286, 377, 384, 410). However, neither protein shows helicase activity in the standard strand displacement assay. ATP-dependent translocation of Rad54 along duplex DNA generates both negative and positive supercoiled domains and is stimulated by Rad51-ssDNA filaments (231, 304, 410). DNA remodeled by Rad54 becomes more sensitive to the ssDNA-specific nuclease, P1, indicating transient strand separation (410). Rad54 and Rdh54/Tid1 both stimulate Rad51-promoted D-loop formation in the presence of either oligonucleotides or long ssDNA and supercoiled plasmid DNA (283, 285). Presumably, the change in writhe by Rad54 facilitates the invasion of duplex DNA by the Rad51 nucleoprotein filament by creating transiently unwound DNA. Rad54 also stimulates Rad51-promoted strand exchange between circular ssDNA and linear duplex DNA (350). The stimulation is greatest at the initial stage of synapsis, and the rate of heteroduplex extension is increased three- to fourfold by Rad54. All of the biochemical activities of Rad54 and Rdh54, except binding to dsDNA, are dependent on ATP hydrolysis and are eliminated by mutation of the invariant lysine residue within the Walker A motif to either arginine or alanine (284, 285, 350). However, *rad54-K341A* and *rad54-K191R* homozygous diploids retain high levels of interchromosomal, but not intrachromosomal, gene conversion (286).

Replication protein A. RPA was discovered by the requirement for an ssDNA binding protein in the simian virus 40 in vitro replication assay (423). Yeast RPA was found to substitute for human RPA in stabilization of unwound DNA by T antigen, providing an assay for purification (45). The RPA complex consists of three subunits of 70, 34, and 14 kDa, encoded by the *RFA1, RFA2,* and *RFA3* genes, respectively. All three subunits of the heterotrimeric complex are essential for viability in yeast, confirming the requirement for RPA in DNA replication (44, 133). Several alleles of *RFA1* have been identified in screens for altered rates of recombination. Firmenich et al. recovered the *rfa1-44* allele in a screen for mutants defective in DSB-induced mitotic recombination (99). This allele of *RFA1* failed to complement certain *rad52* alleles, and the DNA repair defects of the *rfa1-44* strain were suppressed by *RAD52* expressed from a high-copy-number plasmid. These results suggest that RPA and Rad52 are likely to interact, a prediction subsequently verified by experiments with the two-hybrid system (131). The temperature-sensitive *rfa1-M2* allele confers sensitivity to UV and displays an increased spontaneous mutation rate and decreased rates of direct repeat recombination (209).

Another allele of *RFA1, rfa1-D228Y,* was identified as a suppressor of the spontaneous direct-repeat recombination defect of a *rad1 rad52* strain (348). The *rfa1-228Y* strain by itself conferred a hyperrecombination phenotype for deletions between direct repeats that was independent of *RAD52* and partially dependent on *RAD1.* Physical studies showed an increased level of deletion product by SSA in the *rad52 rfa1-D228Y* strain and the disappearance of long single-stranded intermediates that are characteristic of *rad52* strains (347). By cell fractionation, there appeared to be less of the RPA complex from the *rfa1-D228Y* strain compared with a wild-type strain (348). These results suggest that Rad52 is required to displace RPA from ssDNA to promote strand annealing; in the absence of Rad52, RPA is inhibitory to spontaneous annealing or to another factor that is unable to displace RPA. Rad59 does not appear to be this factor because the *rad59 rad52*

rfa1-D228Y strain exhibits equivalent levels of SSA to those for the *rad52 rfa1-D228Y* strain (70).

Random mutagenesis of the RFA1 gene resulted in the identification of 19 recessive alleles of *RFA1* that confer sensitivity to UV and MMS (405). Of these, the *rfa1-t11* (*rfa1-K45E*) mutation was characterized in detail and shown to be defective in mating-type switching and HO-induced SSA. The *rfa1-t11* and *rfa1-t48* homozygous diploids also showed greatly reduced meiotic recombination and spore viability (354). These studies confirm an important role for RPA in homologous recombination, presumably to remove secondary structure from ssDNA in order to allow more efficient binding by Rad51.

Role of the Rad51 subgroup in meiotic recombination. Diploids homozygous for *rad51, rad52, rad55,* or *rad57* mutations either fail to sporulate or give rise to inviable spores (111, 294, 338). The sporulation defect is not rescued by *spo13,* but the sporulation and spore viability of *rad52* diploids are restored by *rad50* and *spo13* mutations (219, 222). This suggests that the defect is subsequent to DSB formation and can be bypassed by preventing the initiation of recombination. Suppression of the spore inviability of *spo13 rad52* strains has been used to identify genes required the initiation of meiotic recombination (220). Induction of meiotic recombination in sporulation-defective strains can be measured by using a return-to-mitotic-growth protocol. By this assay, *rad51, rad52, rad57,* and *rfa1* mutants show a reduction in the level of recombinants, but significant induction over mitotic levels is observed (111, 338, 354). In *rad52* mutants, recombinants appear to be unstable and the yield is reduced if the cells are held in rich growth medium before being plated onto selective medium (302). By physical analysis, *rad51, rad52, rad55, rad57,* and *rfa1* mutants form normal levels of DSBs, but the ends are hyperresected due to the defect in strand invasion (264, 338, 354). Levels of crossover products, monitored using restriction fragment length polymorphisms flanking the initiating DSB, are reduced about fivefold in both *rad51* and *rad52* mutants (36, 264, 338). Mutation of the gene encoding the meiosis-specific RecA homologue, *DMC1,* results in a similar defect to that observed in *rad51* mutants; however, the double mutant shows a greater reduction in the level of crossover products (33, 336). In a *rad51 rad55 rad57* triple mutant, levels of interhomologue dHJ intermediates are greatly reduced indicating an important role for the Rad51 pathway in the generation of these intermediates (329).

Unlike other members of this subgroup, *rad54* and *rdh54* homozygous diploids exhibit only about a twofold decrease in sporulation efficiency and spore viability compared with wild-type strains (179, 325, 341). However, the *rad54 rdh54* double mutant is severely defective for sporulation and spore viability, indicating functional redundancy between these proteins in meiosis. The results of physical and genetic assays to measure meiotic recombination are also consistent with redundancy between Rad54 and Rdh54. Rdh54 was identified in a two-hybrid screen for Dmc1-interacting proteins, and the phenotype of the mutants is consistent with a role in interhomologue recombination (82). Although *RAD54* does not appear to play an important role in meiotic recombination, the high viability of *dmc1* strains when returned to vegetative growth medium during meiosis is dependent on *RAD54* (14). Furthermore, the pachytene arrest of *dmc1* mutants can be suppressed by *RAD54* on a high-copy-number plasmid (32). The interpretation of these results is that *RAD54* is required for sister chromatid interactions; hence the important role in mitotic but not meiotic recombination. In *dmc1* mutants, cells retain high viability when returned to vegetative growth because sister interactions can still occur; however, in the absence of *RAD54,* meiosis-induced DSBs cannot be repaired from the sister. High-copy-number expression of *RAD54* is envisioned to direct some meiosis-specific DSBs from the interhomologue to the intersister pathway.

RAD51*-dependent mitotic recombination.** The observation that *rad51, rad54, rad55,* and *rad57* mutants show high sensitivity to IR indicates an important role for these genes in mitotic DSBR. However, in several assays for spontaneous and DSB-induced recombination, *rad51, rad54, rad55,* and *rad57* mutants show high levels of recombination. *RAD52* is required in the same assays, leading to the suggestion of *RAD51*-dependent and -independent pathways for mitotic recombination (the *RAD51* pathway includes *RAD54, RAD55,* and *RAD57*), both of which are dependent on *RAD52* (199, 233, 299, 364). *RAD51, RAD52, RAD54,* and *RDH54* are required for high levels of spontaneous heteroallelic recombination in diploids, which is thought to occur primarily by gene conversion, and there is no induction of recombination by DNA-damaging agents in the corresponding mutants (179, 314). Diploids homozygous for *rad55* or *rad57* show wild-type levels of spontaneous heteroallelic recombination but show a reduction in UV-induced recombination at 23°C (211, 314). Because the DNA repair defect of *rad55* mutants is suppressed by mating-type heterozygosity, the natural state of diploids, it is possible that the recombination defect in *rad55* diploids is suppressed (211). Consistent with this hypothesis, ectopic gene conversion between heteroalleles in haploids is slightly reduced in *rad55* and *rad57* mutants (105, 199). However, *rad51* and *rad54* mutants show a much greater decrease in the rate of ectopic heteroallelic gene conversion in haploids (10- to 30-fold), and in *rad52* mutants the rate is reduced more than 50-fold (105, 199). Thus, the requirement for *RAD55* and *RAD57* in spontaneous mitotic gene conversion is less stringent than for *RAD51, RAD52,* and *RAD54.* HO-induced gene conversion between allelic *MAT* sequences in diploids is dependent on *RAD51, RAD52,* and *RAD54* but not on *RDH54* (217, 343). As observed for radiation sensitivity, *rad55* and *rad57* diploids are more defective in HO-induced gene conversion at 23 than at 30°C, and the recombination defect at 23°C is comparable to that in *rad51* mutants (343). Diploids homozygous for *rad59* are not defective for HO-induced gene conversion between allelic *MAT* sequences, and they show elevated rates of spontaneous heteroallelic recombination (17, 343). In contrast, ectopic gene conversion in haploids is slightly reduced by mutation of *RAD59* (105, 152). Because the radiation sensitivity of *rad59* mutants is suppressed by mating-type heterozygosity, the recombination proficiency of *rad59* diploids could be due to suppression of a mild defect by *MATa**/α (Y. Bai and L. Symington, unpublished data).

Very few studies have addressed the role of the *RAD52* group genes in spontaneous mitotic crossing over. Because half of the crossover-associated conversion events are masked by chromatid segregation, it is difficult to distinguish between true

reciprocal recombination and BIR (Fig. 2). Furthermore, mitotic crossover events that are selected by homozygosis of a recessive drug resistance marker are indistinguishable from BIR events. One assay system that avoids some of these problems is ectopic recombination in haploids. If the repeats are arranged in the same orientation relative to the centromeres of the two chromosomes, then viable reciprocal recombination products can be recovered. When essential genes are distal to the repeats, BIR events cannot give rise to viable recombinants. The ectopic repeats can be manipulated so that only crossover or only conversion products can be recovered as recombinants (105). Using such a system, Freedman and Jinks-Robertson showed a 10- to 20-fold decrease in crossovers in *rad51, rad52,* and *rad54* mutants, a 3-fold decrease in crossovers in *rad55, rad57,* and *rad59* mutants, and a 3-fold elevation in crossovers in *rad50* mutants (105). These results confirm that true reciprocal exchanges occur during mitotic recombination and require *RAD51, RAD52,* and *RAD54* for their formation. The mitotic crossovers that accompany rare gene conversions in *rad52* diploids are aberrant and usually associated with the loss of one of the two chromosomes that participated in the recombination event (125).

HO-induced recombination occurs at a high enough frequency to monitor unselected events and therefore to determine whether crossovers are reciprocal. Using a chromosome *VII* disome, Galgoczy and Toczyski showed that repair of an HO-induced break was accompanied by reciprocal exchange in 27% of recombinants in wild-type strains, but fewer than 1% of the reciprocal products were recovered from *rad51* and *rad52* mutants (109).

Mating-type switching in *S. cerevisiae* occurs by a DSB-induced gene conversion event that is rarely associated with crossing over. Early studies using diploids heterozygous for *HO* and *RAD52* demonstrated that *RAD52* is essential for mating-type switching (221, 414). *RAD51* is also essential, but some switched products are recovered from *rad54, rad55,* and *rad57* mutants (1, 102, 159, 326). Physical monitoring of DNA during mating-type switching has shown more extensive resection of sequences distal to the HO break site in *rad51, rad52, rad54, rad55,* and *rad57* mutants, and no strand invasion products are detected by PCR or Southern blot analysis (364, 419). The disparity between the physical monitoring and genetic assays for mating-type switching in *rad54, rad55,* and *rad57* mutants may be due to the decreased sensitivity of physical assays compared with the genetic assay.

Gene conversion between heteroalleles oriented as a direct repeat in haploid strains is reduced 100-fold by mutation of *RAD52* and 20- to 90-fold by mutation of *RAD51* or *RAD54* (153, 176, 286, 338). The rate of gene conversion between direct repeats has not been determined for *rad55, rad57,* and *rad59* mutants. *RAD52* is also required for mitotic recombination between the cluster of delta sequences oriented as direct and inverted repeats around the *SUP4* locus (308). Spontaneous gene conversion between heteroalleles oriented as an inverted repeat is reduced more than 5,000-fold in *rad52* mutants (299). However, the rate of gene conversion (unassociated with inversion) is reduced only 19-fold in *rad51* mutants, and inversion events occur at 50% of the wild-type rate (299). The rate of gene conversion between inverted repeats is reduced about 60-fold in *rad54* and *rad57* mutants, but the *rad51 rad54* and *rad51 rad57* double mutants show the same rate as the *rad51* single mutant (298). This epistasis could reflect channeling of recombinogenic lesions into an alternate pathway that is used less effectively when Rad51 is present and the mediator proteins are absent, perhaps because of unproductive association of Rad51 with ssDNA. When Rad51 is also removed, the alternate pathway functions more efficiently. As described above, *RAD59* was identified as a component of the alternate pathway because recombination of inverted repeats was reduced more than 1,000-fold in the *rad51 rad59* double mutant (17).

***RAD51*-independent mitotic recombination.** When HO is used to create a DSB in one of the two *MAT* alleles in wild-type diploids (in strains with *HMR* and *HML* deleted to prevent intramolecular recombination), repair occurs by gene conversion (217). As described above, gene conversion is eliminated in *rad51* mutants but repair of the broken chromosome can still occur by a mechanism that results in homozygosis of all markers distal to the DSB. This mechanism is known as BIR and is thought to occur by one-ended strand invasion followed by duplication of the sequences from the break site to the telomere. The process is inefficient in *rad51* mutants and appears to be preceded by cell cycle arrest and checkpoint adaptation (109, 217). BIR is observed in *rad51, rad54, rad55,* and *rad57* mutants but not in *rad52* mutants (343). BIR in *rad51* diploids requires *RAD50* (and presumably *MRE11* and *XRS2*), as well as *RAD59* and *RDH54.* Interestingly, *RDH54* is required for checkpoint adaptation; therefore, the requirement in *rad51*-independent BIR could be due to the failure of cells to adapt (193). Because BIR in *rad51* mutants involves the degradation or about 30 kb of DNA from the HO-cut site to a preferred site for BIR, it is possible that the requirement for *RAD50* is in DNA degradation rather than in the strand invasion step (218). The genetic requirements for BIR and telomere maintenance in the absence of telomerase are quite similar, leading to the suggestion that BIR plays an important role in recombination between telomeres. Telomerase-deficient survivors that arise by recombination between the subtelomeric repeated Y′ elements require the *RAD51* pathway genes, suggesting that this pathway does promote BIR, but when two ends are present, gene conversion is favored over BIR (388).

Ends-in and ends-out gene targeting involves the introduction of naked DNA into cells by transformation followed by homologous recombination with the chromosomal locus. Both types of events are dependent on *RAD52* (20, 269, 322). *RAD51* and *RAD57* are required for ends-in targeting but show only a 3- to 10-fold reduction in ends-out targeting (*rad54* and *rad55* mutants were not tested) (20, 322). Several models have been proposed for ends-out gene targeting; presumably, they are all dependent on *RAD52,* but only one of the proposed pathways may require *RAD51* function (194).

Analysis of recombination between repeated sequences has caused the greatest confusion with regard to the requirement for the *RAD52* group genes in homologous recombination. As described above, recombination between sequences oriented as direct repeats can occur by gene conversion, maintaining the structure of the duplication, or by deletion, removing one repeat and the intervening sequences. Some direct-repeat substrates are designed only to select for deletion events, whereas those employing heteroalleles can be used to measure gene

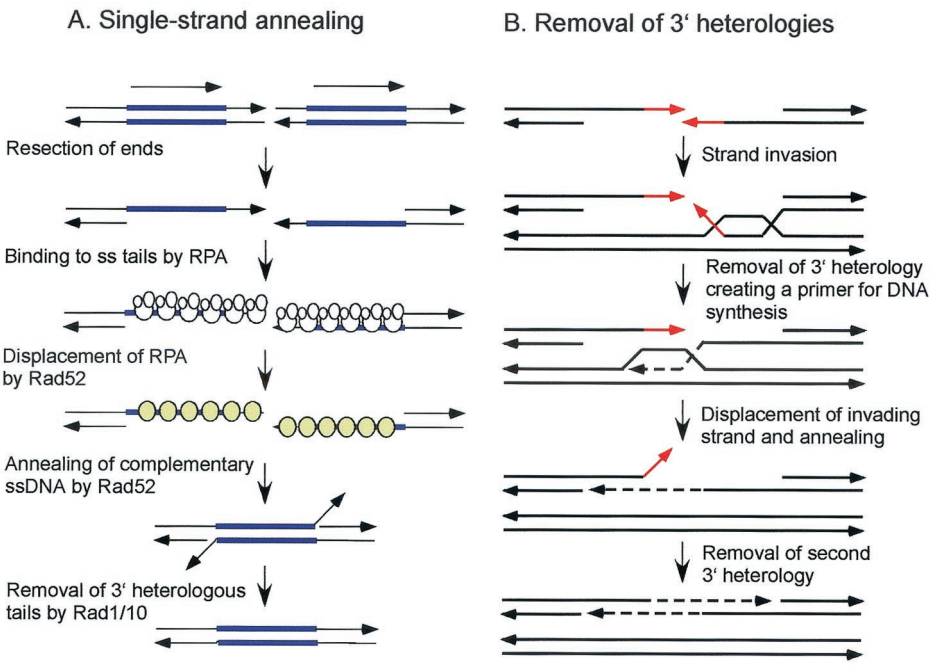

FIG. 13. Role of the Rad1-Rad10 nuclease in recombination. (A) In the SSA reaction, resected 3′ single-stranded tails are first coated with RPA; the RPA is then displaced by Rad52 to promote annealing of the complementary single strands. After annealing, 3′ heterologous tails are removed by the Rad1-Rad10 endonuclease, which cleaves at the junction between dsDNA and ssDNA. Gaps are filled by repair synthesis and ligated. (B) If the invading 3′ end is heterologous to the donor, then the 3′ end cannot be used to template DNA synthesis. The branched structure is cleaved by Rad1-Rad10, creating a primer for DNA synthesis. The second 3′ heterologous tail is also removed by Rad1-Rad10 for the second round of DNA synthesis predicted by the SDSA model (shown) or DSBR model.

conversion and/or deletion events. Most studies have focused on spontaneous deletion events between direct repeats, and these events are actually elevated in *rad51, rad54, rad55,* and *rad57* mutants (199, 233). The higher rate of deletions in *RAD51* pathway mutants is presumably due to channeling lesions from the favored gene conversion pathway to mutagenic pathways such as SSA, sister strand mispairing, and replication slippage (177). Studies in which HO endonuclease is used to create a DSB between repeats have shown that SSA occurs with high efficiency and with normal kinetics in *rad51, rad54, rad55,* and *rad57* mutants (150). These results are consistent with the hypothesis that spontaneous DSBs cannot be repaired by gene conversion in *RAD51* pathway mutants but can be efficiently repaired by SSA when direct repeats are available.

In contrast to *rad51, rad54, rad55,* and *rad57* mutants, *rad52* mutants show a 2- to 10-fold reduction in the rate of spontaneous deletions between direct repeats (176, 319, 390). DSB-induced deletion formation between plasmid-borne direct repeats is reduced about eightfold in *rad52* mutants; however, deletions are not detected by physical methods between chromosomal *ura3* or *leu2* repeats in *rad52* strains (101, 347, 362). This difference could be due to plasmid versus chromosomal sequences or to the location of the HO-cut site within one of the repeats in the plasmid study and between them in the chromosomal studies. These results suggest that *RAD52* is important for SSA, consistent with biochemical experiments demonstrating annealing of complementary ssDNA by Rad52 (250).

Studies of spontaneous direct-repeat recombination led to the discovery of a role for the nucleotide excision repair gene, *RAD1,* in this process. The effect of a *rad1* mutation by itself is quite variable in different direct-repeat assays, but when combined with *rad52, rad1* causes a synergistic decrease in the rate of direct-repeat recombination (176, 319, 391). Rad1 interacts with Rad10, and, as anticipated, *rad10* mutants have similar defects to *rad1* mutants in direct-repeat recombination (320). Although the *rad1* mutation confers a defect in deletion formation, there is no effect on spontaneous gene conversion (319). Studies of HO-induced recombination have demonstrated a role for Rad1 in SSA (148), and, consistent with the prediction from the genetic studies, the Rad1/10 heterodimer is a structure-specific nuclease that cleaves 3′ tails from branched intermediates (Fig. 13) (19, 100).

Inverted repeats have been used to study recombination with the expectation that viable recombinants cannot be generated by nonconservative pathways such as SSA (80, 299). Although recombination between inverted repeats is highly dependent on *RAD52,* the rate of recombination in the other *RAD52* group mutants is reduced by only 4- to 30-fold (4, 17, 80, 299, 421). Recombinants can be generated by gene conversion, inversion of the intervening DNA (apparent crossover), or gene conversion associated with inversion when the inverted-repeat substrate consists of heteroalleles. In *rad51* mutants, most of the recombinants recovered are inversions, suggesting that *RAD51* is more important for gene conversion than for crossing over (299). Using a plasmid containing inverted copies of the *lacZ* gene, one containing an HO-cut site, Sugawara et al. reported no effect of the *rad51* mutation on the efficiency of

HO-induced recombination (364). However, *RAD51* was required for repair of an HO-induced break within plasmid-borne inverted repeats when the donor was in heterochromatin. This interesting result suggests that the *RAD51* pathway can efficiently use donor DNA assembled into heterochromatin but that the *RAD51*-independent pathway, presumably mediated by Rad52, cannot. Analysis of the products generated from the *lacZ* inverted repeat plasmid in *rad51* strains revealed a higher proportion of inversions (crossovers), consistent with the study of chromosomal inverted repeats (166). This observation suggests that the mechanism of repair in *rad51* mutants is different from that in wild-type strains.

Although *RAD51* is not required for DSB-induced recombination of plasmid-borne inverted repeats that are transcribed, *rad51* mutants are severely deficient in the repair of gapped plasmids introduced into cells by transformation from either plasmid or chromosomal donor sequences (20). *RAD51* is also required for the repair of an HO-induced DSB present on a plasmid from a chromosomal donor (86). These results suggests that plasmid context is not the determinant of *RAD51*-independent recombination. Instead, the results are more consistent with intermolecular recombination events requiring *RAD51* function and intramolecular recombination events being largely independent of *RAD51.* HO-induced recombination between inverted repeats, like spontaneous events, requires *RAD52* (364). Because BIR and SSA can both occur in the absence of *RAD51* but are dependent on *RAD52,* we have suggested that recombination between inverted repeats could occur by a combination of these two processes (Fig. 14) (20, 166). The BIR-SSA model proposes that one end at the break site invades the homologous repeat and primes DNA synthesis to the end of the molecule. The small repeats generated by BIR could then be substrates for SSA, generating either inversion (crossover), or noninversion (conversion) products. One of the attractive features of this model is that apparent crossover products can be generated by a mechanism that does not involve the resolution of a dHJ intermediate. Experimental support for this model is derived from the observations that *rad1 rad51* double mutants show a lower efficiency of repair of inverted-repeat plasmids and that more inversion products are recovered from *rad51* strains (166). The *RAD51*-independent pathway for HO-induced inverted-repeat recombination is still efficient even when the homologies are short (30- bp) and requires *RAD50* and *RAD59* (147). Although this mechanism might be functionally active in *rad51* mutants, there is no evidence for these events in wild-type cells.

Because *RAD52* is the only known gene required for BIR and other *RAD51*-independent recombination events, it is assumed that the annealing activity of Rad52 can be used for strand invasion (217, 299). Presumably some donor sequences are transiently single stranded and can be annealed to ssDNA generated from a resected DSB by Rad52. Once this annealing step has occurred, the 3′ end could be extended by DNA synthesis to the end of the molecule. For plasmids, DNA synthesis would need to occur for only a few kilobases to complete replication of the molecule whereas synthesis might have to occur over several hundred kilobases to repair a chromosome arm.

In summary, the *RAD51* pathway is clearly important for gene conversion unassociated with crossing over and for true

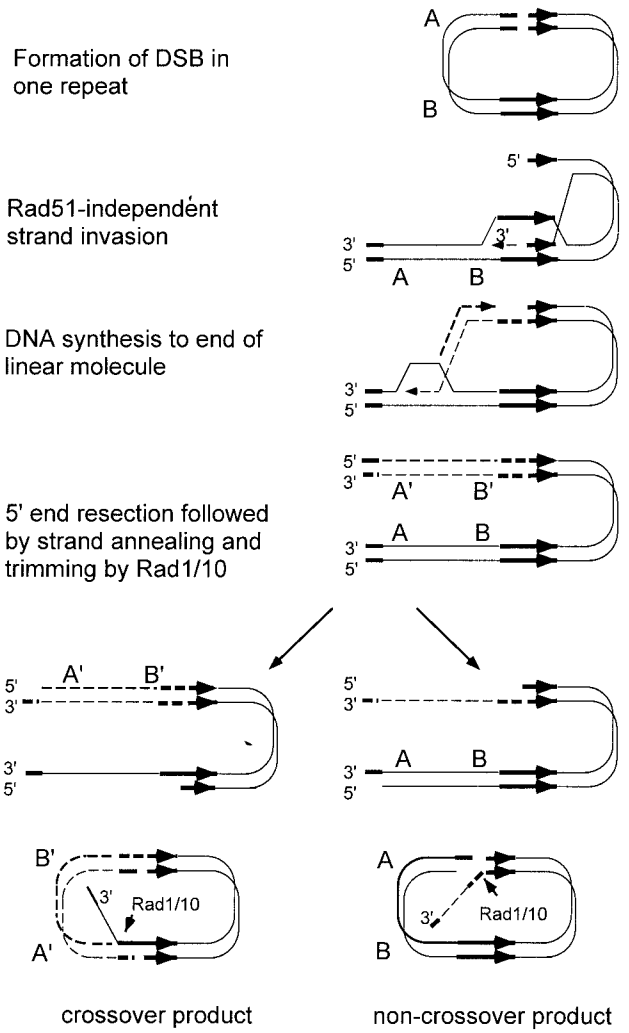

FIG. 14. The BIR/SSA model for repair of DSBs within inverted repeat plasmids. After formation of the DSB, ends are processed and one end invades the other repeat (drawn here as an intramolecular event, but it could also occur intermolecularly). If DNA synthesis extends to the end of the molecule, small repeats will be present at both ends of the linear molecule. Following resection of the ends, the repeats at either end of the molecule can pair with an internal repeat by SSA, generating either noncrossover or apparent crossover products.

reciprocal crossover recombination. These events probably occur as envisioned in the DSBR and SDSA models (Fig. 1), with the Rad51 protein playing a central role in homologous pairing and strand invasion. The mediator proteins, Rad52, Rad55, and Rad57, function with Rad51 to promote the assembly of the Rad51 nucleoprotein filament on ssDNA (Fig. 15). Rad54 is required during synapsis to promote unwinding of the donor duplex in order to allow pairing between the incoming ssDNA and the complementary strand of the donor duplex. Rad54 could play additional roles in heteroduplex extension and removal of Rad51 from the newly formed joint molecule to allow access of the 3′ end to the DNA replication apparatus (349, 350). In the absence of *RAD51, RAD54, RAD55,* and *RAD57,* some types of recombinational repair can still occur but are dependent on the context of the recombining sequences. SSA

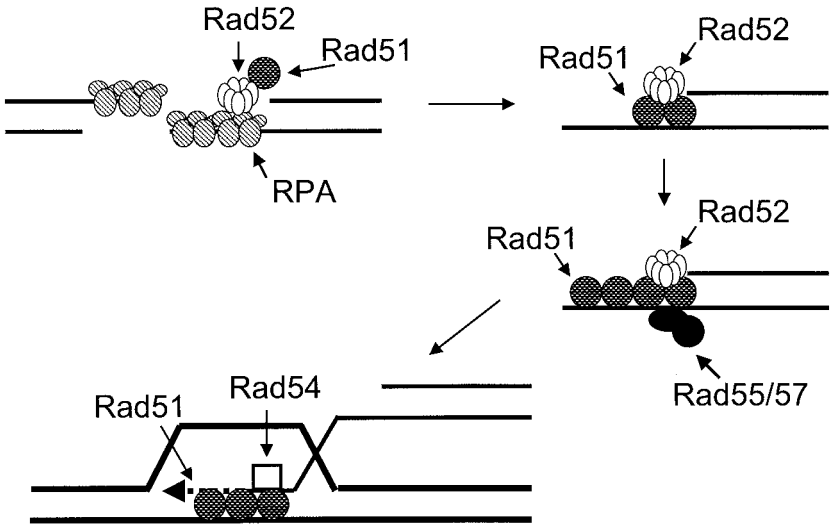

FIG. 15. Model for strand invasion by Rad51 and the mediator proteins, Rad52, Rad54, and Rad55-Rad57. Single-stranded tails produced at break sites are coated by RPA. Rad52 interacts with RPA targeting Rad51 to the ssDNA. Rad52 is thought to displace RPA and promote the binding of Rad51 to ssDNA. This initial interaction between Rad51 and ssDNA is thought to be stabilized by Rad55-Rad57 to allow cooperative binding by Rad51. Rad54 interacts with the Rad51 nucleoprotein filament and promotes the unwinding of duplex DNA for pairing between the donor DNA and the incoming single strand.

is extremely efficient in the absence of the *RAD51, RAD54, RAD55,* and *RAD57* but is a viable option only if direct repeats are available flanking the break site. *RAD52* is clearly important for SSA, and *RAD59* is required when the direct repeats are short (362, 363). *RAD51*-independent BIR may be restricted because certain sequences are required for strand invasion, and unscheduled replication through the centromere has not been documented (218, 248). Thus, *RAD51*-independent BIR may be an option only for cells that have undergone prolonged cell cycle arrest and adaptation to damage and that have a BIR facilitator sequence centromere proximal to the break site.

RAD51 is essential for S-phase progression in chicken cells and is proposed to function in restoration of collapsed replication forks (Fig. 16). Repair of collapsed forks involves the invasion of one broken end into an intact duplex. This configuration is similar to the BIR reaction modeled by Malkova et al. (217), except that the donor during replication restart is a sister chromatid instead of a homologue. Because BIR can occur in the absence of *RAD51* in yeast, why is *RAD51* essential for viability in vertebrates? There are several possible explanations for this conundrum. First, *RAD51*-independent BIR is inefficient in yeast, raising the possibility that this pathway would be unable to repair multiple replication-induced breaks in more complex genomes. Second, the genetic requirements for BIR between sister chromatids might be different from the requirement for BIR between homologues. Studies of telomere maintenance have demonstrated *RAD51*-dependent recombination between subtelomeric repeats, and this most probably occurs by BIR. It seems possible that *RAD51* could be required for BIR under most circumstances and that rare BIR events could occur in the absence of *RAD51,* but these would probably be inefficient and would only rarely contribute to cell survival.

HONORARY MEMBERS OF THE RECOMBINATIONAL REPAIR GROUP

Rad1/Rad10

The Rad1/10 heterodimer is a structure-specific nuclease that cleaves at the 5′ side of UV-induced photoproducts and bulky lesions during nucleotide excision repair (19, 74). As described above, the Rad1/10 nuclease is thought to remove 3′ flaps during SSA (Fig. 13). In addition, it is required to remove heterologies from the 3′ ends of DSB breaks to allow initiation of DNA synthesis during gene conversion. This activity was first discovered when a substrate containing an insertion of a 117-bp HO-cut site within one copy of the *lac*Z gene was used to monitor the efficiency of HO-induced gene conversion (100). *rad1* mutants were found to be defective for gene conversion using this substrate, but there was no defect in repair when the donor sequence contained a 117-bp insertion at the homologous site that differed from the recipient allele by a nucleotide change preventing cleaving by HO. Thus, the requirement for *RAD1* and *RAD10* in homologous recombination is specific for events that require removal of heterologies, either during strand invasion or during SSA (Fig. 13). The mismatch repair proteins Msh2 and Msh3 are also required for SSA and for removal of heterologies of more than 30 nucleotides from DSBs during gene conversion (272, 317, 365). Mutation of *MSH2* or *MSH3* is epistatic to *rad1* in these repair processes. These results have led to the suggestion that branched intermediates are stabilized by Msh2/Msh3 binding in preparation for cleavage by Rad1/Rad10 (365).

In mammalian cells, *XPF* and *ERCC1* encode the Rad1 and Rad10 homologues, respectively (74). Although *ERCC1* is not required for spontaneous recombination between repeated sequences (318), *ercc1*$^{-/-}$ cell lines do exhibit defects in ends-in

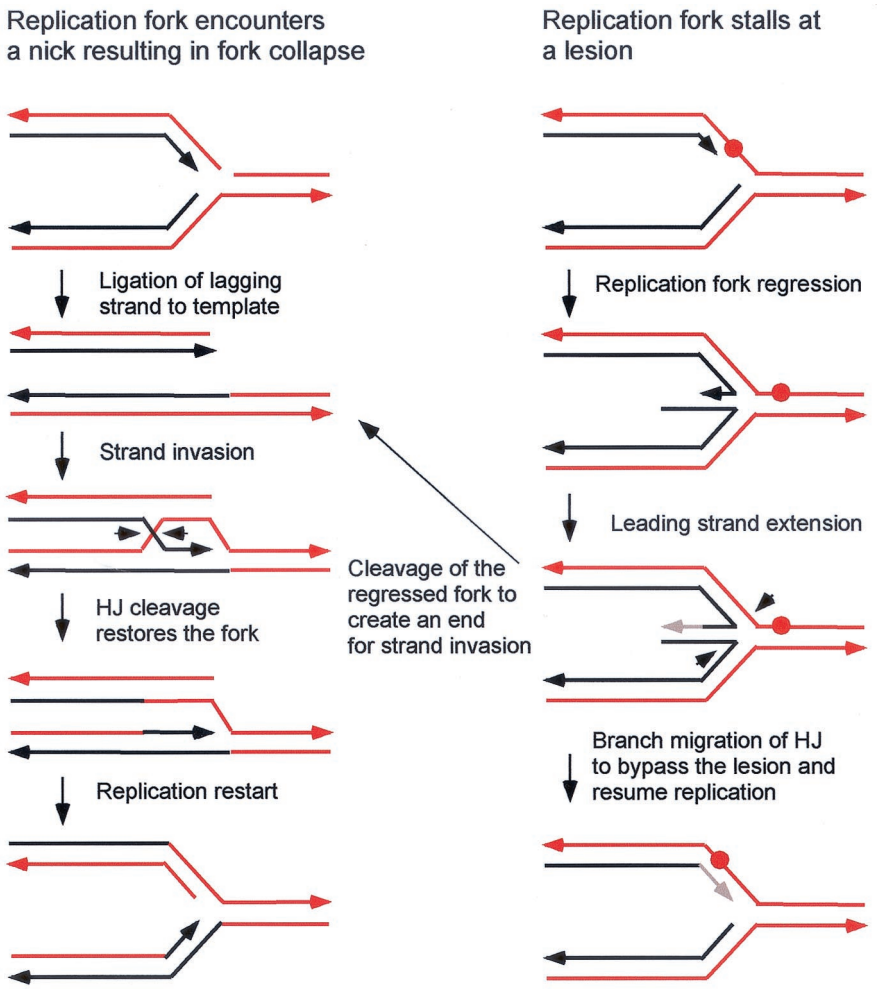

FIG. 16. Role of recombination in restoring collapsed or regressed replication forks. The replication fork collapses if it encounters a nick on the template strand. After ligation of the lagging strand to the template, the broken arm invades, forming a D-loop. Replication can then be primed from the invading strand. Resolution of the Holliday junction (HJ) restores the replication fork. A replication-blocking lesion stalls replication, and the fork regresses by pairing of the nascent strands. If the lagging strand has progressed ahead of the leading strand, the single-strand extension on the lagging strand can serve as a primer for synthesis of the leading strand. If the Holliday junction is cleaved, an end is made for strand invasion, as shown in the left panel. Alternatively, branch migration of the regressed fork can restore the replication fork.

and ends-out gene targeting (Fig. 6) (2, 263). *ERCC1* is required for ends-in gene targeting when the ends of the transformed DNA have long heterologies; this is consistent with studies from yeast (2). Surprisingly, *ercc1*$^{-/-}$ cell lines were found to be completely defective for ends-out gene targeting, even when the ends of the targeting fragment were homologous to the target sequence (263). Because gene-targeting constructs generally have an insertion of a selectable marker creating a large heterology, the defect in targeting could be due to the central heterology. Repair of large-loop heteroduplexes during meiotic recombination requires *RAD1* and *RAD10* in addition to *MSH2* (169, 173). Studies with yeast have shown a reduction in ends-out gene targeting in *rad1* mutants but of only 3- to 10-fold (319, 379). The reason for the disparity between yeast and vertebrates is unclear but could involve alternate mechanisms for targeting in yeast, which are unavailable in vertebrate cells.

Holliday Junction Resolution Activities

Molecular and biochemical studies of the Rad52 group proteins have shown that most are required at early steps during recombinational repair. The absence of a yeast X-ray-sensitive mutant defective for Holliday junction resolution is surprising and suggests that (i) redundant activities exist in eukaryotes, (ii) it is an essential activity, or (iii) Holliday junction resolution is not obligatory for recombinational repair in yeast. For example, the repair of DSBs by the SDSA model is predicted to occur without Holliday junction resolution (Fig. 1). However, some mitotic recombination events do result in reciprocal exchange, and integration of linearized plasmids into the genome is thought to occur by resolution of Holliday junctions as predicted by the DSBR model (Fig. 1). The identification of branched-DNA molecules with the expected topology for Holliday junctions during meiotic recombination is consistent with

the hypothesis that these structures are normal intermediates during meiotic recombination in yeast (61, 327, 328). Branched-DNA molecules have been detected during mitotic S phase within the tandemly repeated rDNA locus, and formation of these intermediates is dependent on *RAD52,* suggesting that they correspond to recombination intermediates (433). Postreplicative, DNA replication-dependent X-shaped molecules have also been detected between sister chromatids in *Physarum polycephalum* (25). The X-forms are readily detected, indicating that they form at high frequency; this suggests that resolution of Holliday junctions could in fact be essential for chromosome segregation in eukaryotes.

In *E. coli,* the RuvC nuclease promotes the resolution of Holliday junctions and works in concert with the RuvA/RuvB branch migration complex (417). The *ruv* genes are required for resistance of *E. coli* to UV, X rays, and mitomycin C and for plasmid and conjugal recombination. No homologues of RuvC or the bacteriophage-encoded resolvases have been identified among sequenced eukaryotic genomes, except for the mitochondrial activity, Cce1 (13). Biochemical approaches have identified three Holliday junction-resolving activities from fractionated extracts of mitotic yeast cells (154, 380, 418). Of these, Cce1 (Mgt1) is mitochondrial and is important for the segregation of mitochondrial genomes (92, 175, 208, 380). The identity of the other two yeast resolvase activities is still unknown. Holliday junction resolvases have also been identified from fractionated extracts of mammalian cells (65, 85). The resolvase identified by Constantinou et al. (65) was found to cofractionate with an ATP-dependent branch migration activity during several chromatographic steps. These activities were shown to be nuclear and independent of the BLM and WRN helicases, which had previously been shown to promote ATP-dependent branch migration of Holliday junctions (65, 66, 168). The mammalian activity promotes a concerted branch migration/resolution reaction similar to that catalyzed by RuvABC (65).

Recent studies have identified the Mus81-Mms4 (Eme1) heterodimer as a putative Holliday junction resolvase (126). Mus81 and Mms4 were identified in two-hybrid screens by interaction with Rad54, *S. pombe* Cdc1 (the orthologue of *S. cerevisiae* Rad53), and the meiosis-specific checkpoint kinase, Mek1 (35, 75, 146). The *MMS4* gene was originally identified by the sensitivity of mutants to MMS and rediscovered in a screen for mutations that cause synthetic lethality with *sgs1,* along with *mus81* (253, 293). *SGS1* encodes a helicase of the RecQ family and is homologous to the human BLM and WRN helicases, which play important roles in genome stability and life span (111a, 239). Mus81 has sequence similarity to the nuclease domain of Rad1, and Mms4 has limited similarity to Rad10, suggesting that they are components of a structure-specific nuclease (146, 253). Eme1 was identified by interaction with SpMus81 and is the presumptive orthologue of Mms4 (34). The Mus81-Mms4/Eme1 heterodimer cleaves a variety of branched molecules, including simple Y structures, duplex Y structures and X forms, with a strong preference for duplex Y structures (34, 164). The preferred substrate for Mus81-Mms4/Eme1 is structurally related to a stalled replication fork, leading to the hypothesis that Sgs1 and Mus81-Mms4 are redundant functions for processing stalled replication forks (Fig. 16) (77, 164). Alternatively, unusual structures may accumulate in *sgs1* mutants requiring cleavage by Mus81-Mms4.

MUS81 and *MMS4/EME1* are also required for meiotic DNA metabolism (34, 75, 146, 164, 253). In *S. pombe, mus81* and *eme1* diploids produce only 1% viable spores and the high inviability is partially suppressed by the *rec12* mutation, which prevents initiation of meiotic recombination (34). Most striking is the observation that expression of the *E. coli* Holliday junction resolvase, RusA, in *mus81* diploids restores spore viability to 35%. While these data provide a compelling argument for Mus81-Eme1 being the elusive Holliday junction resolvase, studies with *S. cerevisiae* are not fully compatible with this hypothesis. First, *mus81* mutants are resistant to IR (146). Although one could argue that IR-induced damage is repaired by mechanisms that do not require Holliday junction resolution, *mus81* mutants also do not show any defect in integration of plasmids (B. Llorente and L. S. Symington, unpublished data). Second, *mms4* and *mus81* diploids show quite high spore viability in some strain backgrounds and crossover products are readily detected by both genetic and physical analyses (75). Third, Holliday junctions cleaved by Mus81-Mms4/Eme1 cannot be ligated, indicating that the cleavage sites are not symmetrical (64). The bacterial and bacteriophage-encoded resolvases cleave Holliday junctions symmetrically to generate products that can be ligated. This result has led to the suggestion that Mus81-Mms4/Eme1 cleaves branched structures that form during SDSA, instead of the dHJ intermediates that are destined to become crossovers (75).

The discrepancy between the *S. pombe* and *S. cerevisiae* data suggests that budding yeast could have another activity for resolving Holliday junctions and that this activity might be absent or less effective during *S. pombe* meiosis. Support for the hypothesis of two resolvase activities comes from recent studies by Constantinou et al. (64). Fractionation of HeLa cell extracts revealed two discrete Holliday junction resolvase activities, one corresponding to Mus81 and the other corresponding to the previously described resolvase that cofractionates with a branch migration activity, referred to as resolvase A (64, 65). In addition to their distinct chromatographic properties, resolvase A activity was not depleted by anti-Mus81 polyclonal antibodies and the substrate specificities of the two activities were different (64). Resolvase A shows high specificity for Holliday junctions, whereas Mus81 cleaves 3′ flap and Y-shaped molecules more efficiently than Holliday junctions (64, 77, 164). Thus, genetic and biochemical data support the hypothesis that Mus81 cleaves stalled replication forks and 3′ flaps that form during meiotic SDSA. Resolvase A has the hallmarks of a true resolvase, and we eagerly await the identification of the gene encoding this activity.

CONCLUSIONS AND FUTURE DIRECTIONS

In summary, significant advances have been made in our understanding of the structure and biochemical functions of the Mre11-Rad50 complex, but the function of the complex in various aspects of DNA metabolism remains elusive. Recent studies support the hypothesis that the primary function of the Mre11-Rad50 complex is structural and serves to bridge sister chromatids and/or DNA ends (53, 73, 140). The nuclease ac-

tivity of Mre11 is required to remove covalent adducts from DNA ends and to process unusual DNA structures, but it may not play a significant role in resection of ends to produce long 3′ single-stranded tails (108, 207, 241, 359, 407). Xrs2/Nbs1 is restricted to eukaryotes and may function to recruit other factors for specialized functions within the cell, such as the Dn14-Lif1 complex for end joining (53). The *RAD51* pathway is clearly important for gene conversion unassociated with crossing over and for true reciprocal-crossover recombination. These events probably occur as envisioned in the DSBR or SDSA models, with the Rad51 protein playing a central role in homologous pairing and strand invasion with the assistance of the mediator proteins. In the absence of *RAD51, RAD54, RAD55,* and *RAD57,* some types of recombinational repair can still occur but are dependent on the context of the recombining sequences. SSA is only a viable option if direct repeats are available flanking the break site, and *RAD51*-independent BIR may be restricted because certain sequences are required for strand invasion. *RAD52* is the most important recombination gene in *S. cerevisiae,* and this is presumably due to the important role of Rad52 as a mediator for Rad51 and in strand annealing for *RAD51*-independent recombination. While many advances have been made in this field, we still know little about the nucleases that function in early and late stages of recombination, the roles of the Rad51 accessory proteins, and coordination of homologous recombination with other cellular processes. Understanding the intimate relationship between damage sensing, replication, and homologous recombination and the ways in which these processes function in tumor suppression is likely to keep the field busy for many years to come.

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