Double-Strand Break-Induced Recombination in Eukaryotes

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Genetic recombination is of fundamental importance for a wide variety of biological processes in eukaryotic cells. One of the major questions in recombination relates to the mechanism by which the exchange of genetic information is initiated. In recent years, DNA double strand breaks (DSBs) have emerged as an important lesion that can initiate and stimulate meiotic and mitotic homologous recombination. In this review, we examine the models by which DBSs induce recombination, describe the types of recombination events that DBSs stimulate, and compare the genetic control of DBS-induced mitotic recombination in budding and fission yeasts.

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Genetic recombination is ubiquitous in living organisms. It can be defined as the exchange of information between DNA sequences. Recombination can occur between DNA sequences on two or more DNA molecules, or within a single DNA molecule. For the purposes of this review, three types of recombination are relevant: homologous, site-specific, and illegitimate recombination. Homologous recombination typically occurs between DNA sequences with extended regions of homology. It can occur anywhere along the length of homology, but is not restricted to specific sites, although some sites may be preferentially used. Site-specific recombination typically occurs between precisely prescibed sites on two partner DNA sequences that otherwise bear no overall homology to each other. These sites typically comprise short recognition sequences for a particular DNA-binding protein(s) that acts on these binding sequences to catalyze recombination. Illegitimate or nonhomologous recombination occurs between DNA sequences with no prescribed sites and with no homology, or at best only a few base pairs of homology. Illegitimate events are typically nonconservative in that they result in the loss or gain of a small number of nucleotides at the site of recombination in the DNA. This review focuses mainly on homologous recombination.

Recombination is of fundamental importance for a wide variety of biological processes in eukaryotic cells, including meiosis, vegetative chromosome stability and segregation, antigenic variation and immunoglobulin gene rearrangements, maintenance of copy number and sequence homogeneity in repeated gene families, and control of gene expression. Recombination also plays a role in neoplastic transformation. In addition, in many organisms, the modification of specific chromosomal genes in a predetermined manner by gene targeting or gene replacement relies on homologous recombination between chromosomal and newly introduced DNA sequences.

Accumulating evidence highlights the central role of DNA double-strand breaks (DSBs) in these recombinational processes. DSBs are important DNA lesions that can arise in mitotic cells spontaneously or in response to certain DNA-damaging agents. Some DNA-damaging agents, such as x-rays, can produce DNA DSBs directly, whereas others generate DSBs or gaps following processing of initial lesions by repair enzymes. The repair of DSBs and gaps also occurs via recombinational mechanisms. The DNA molecular structures generated during meiotic and mitotic recombination of chromosomes are similar to those occurring during recombinational repair of DSBs. Given this overlap, it is therefore not surprising that the genes involved in DSB repair have important roles in mitotic and meiotic chromosome metabolism.

An understanding of recombination involves two aspects: (i) a description in molecular detail of the stepwise structural changes of DNA as parental sequences undergo recombination to give rise to recombinant products, and (ii) the elucidation of the precise roles of enzymes and other molecules that catalyze or facilitate these structural changes and regulate their stability, duration, and extent at each step.

In eukaryotes, the best studied organism with respect to recombination is the budding yeast *Saccharomyces cerevisiae*, though much has also been learned from studies in other fungi and from *Drosophila*, *Xenopus*, and mammalian cells. Several experimental approaches have been employed to elucidate the underlying molecular mechanisms and genetic control of recombination in yeast and other eukaryotes. The first approach has relied on classical genetic analysis techniques to examine the segregation of linked genes, or alleles within one gene, by the characterization of the products of genetically detectable exchange events resulting from recombination between DNA sequences during both meiotic and mitotic development. Such genetic studies have examined interchromosomal, intrachromosomal, and extrachromosomal recombination, as well as recombination between chromosomal and extrachromosomal sequences. These analyses have been aided by the ability to use *in vitro* molecular biological techniques to artificially engineer defined chromosomal and extrachromosomal recombination substrates.

Defined DSBs and double-strand gaps (DSGs) can be introduced *in vitro* within extrachromosomal sequences prior to introduction into cells. This has allowed the analysis of *in vivo* recombination events, both between extrachromosomal sequences and between chromosomal and extrachromosomal sequences in many eukaryotic organisms. In recent years, the genetic analysis of DSB-induced recombination has been aided by the use of biological tools that allow a single site-specific DSB to be induced *in vivo* within defined recombination substrates that are either chromosomal or extrachromosomal. Typically, the substrate includes a recognition site for a site-specific endonuclease, and the expression of the corresponding endonuclease is under the control of an inducible promotor.

The second major approach has been to analyze the genetic control of recombination by the isolation and characterization of mutants defective in some aspect of recombination. Using the recombination substrates in which *in vivo* site-specific DSBs are induced, existing mutants have been characterized and new mutants are now being isolated on the basis of a direct defect in DSB-induced recombination. The isolation and molecular characterization of the genes involved in recombination has played an important role in gaining insights into the recombination process and the role of DSBs. In most cases, recombination genes have been isolated by complementation of the mutant phenotype with DNA, particularly in lower eukaryotes. In recent years some of the corresponding genes from higher eukaryotes have been defined following the identification of conserved sequences of homologs of recombination genes from lower eukaryotes.

Biochemical studies are also having an increasingly important impact on

our understanding of recombination. *In vitro* recombination systems have been developed in S. *cerevisiae* (*1*) and mammalian cells (*2*). In addition, from what is known regarding biochemical activities of recombination enzymes in bacteria, and from genetic and molecular studies in eukaryotes, several enzymatic activities have been postulated to be required for eukaryotic recombination. A subset of these can be assayed *in vitro*, many using substrates with DSBs. These systems have been used to purify (partially or completely) specific recombination activities from eukaryotes. However, there has only been limited success in characterizing the precise biochemical activities of individual gene products involved in eukaryotic recombination.

A different biochemical approach in studying both the pathways and the role of the gene products of eukaryotic recombination has been the physical monitoring of DNA undergoing recombination following the introduction of a single *in vivo* DSB by the site-specific endonucleases mentioned earlier (reviewed in *3*). The correlation of mutant phenotype to the loss of a biochemical activity *in vivo*, and of gene product to biochemical activity *in vitro*, will be the critical criteria by which the underlying molecular mechanisms of recombination will be elucidated.

This review focuses on our knowledge of DSB-induced homologous recombination in *S. cerevisiae*, the best studied eukaryotic organism for this subject, and the one in which most of the more informative studies have been conducted. It also includes work on other eukaryotes, and our own work on DSB-induced intrachromosomal homologous recombination in the fission yeast *Schizosaccharomyces pombe*.

I. Models of Double-Strand Break-Induced Recombination

The extensive genetic studies on meiotic recombination in fungi have been reviewed by many authors (*4–7*). The properties of fungi make them particularly amenable for such analyses. Following a sexual cross between two strains differing in one or more marker genes, the isolation and analysis of the spores of intact tetrads and octads permits the genetic constitution of *each* DNA strand of each chromatid present after meiosis to be determined. As well as demonstrating reciprocal exchange, or crossing over, between homologous chromatids, these studies also established the occurrence of nonreciprocal exchanges, gene conversion, and postmeiotic segregation (PMS). PMS, the segregation of alleles at the division following meiosis, was taken as evidence that the chromatids that were segregated at the second division of meiosis consisted of heteroduplex DNA (hDNA), that is, a DNA duplex in which the two strands contain different information for

the segregating marker. These studies also showed that there was a definite correlation between nonreciprocal gene conversion within a gene and reciprocal crossing over of flanking markers. The existence of hot spots of meiotic recombination and the phenomenon of polarity of gene conversion (that gene conversion frequency along the length of a gene is polarized) led to the hypothesis that meiotic recombination events are initiated at preferred specific sites on the DNA.

Models were postulated to explain the genetic data on meiotic homologous recombination in fungi in molecular terms. Homologous recombination is envisaged as a multistep process catalyzed by many gene products. The models defined a mechanism for the association of gene conversion and crossing over. In the initial models, it was proposed that recombination was initiated by single-strand nicks (*8–10*; Fig. 1). It was postulated that, following a single-strand nick, a Holliday junction and hDNA intermediates were generated by strand exchange between homologous duplexes. In these models gene conversion is the result of the formation and mismatch repair of hDNA. A Holliday junction could be resolved into a crossover or noncrossover event according to which strands were cut at the junction. In Meselson and Radding's model (*9*), the molecule on which the initiating single-strand nick is made becomes the donor of genetic information. However, numerous pieces of evidence suggested that the initiating event occurred on the molecule that was ultimately the recipient of genetic information (*4–7*), and Radding (*10*) proposed a modified model (Fig. 1) to account for this. Although there is good evidence for the role of hDNA as a recombination intermediate, evidence for the role of single-strand nicks as initiators of eukaryotic recombination has not been forthcoming. Although attempts have been made (*11*), a problem in assessing the importance of single-strand nicks as initiators has been the absence of an efficient experimental system to study them. In contrast, the development of such systems for the study of DSBs has produced compelling evidence for their role as initiators of recombination.

Studies on x-ray-induced DNA DSB repair in *S. cerevisiae* led Resnick (*12*) to propose a model in which recombination was initiated by DSBs (Fig. 2). In Resnick's model (Fig. 2), exonucleolytic degradation of one strand on each side of the DSB gives rise to two 3′ single-strand tails, but only one recombines with homologous sequences of the intact duplex (a one-sided invasion). In the model, DNA synthesis primed from the 3′ end of the invading 3′ tail replaces the missing information at the break. As in the previous models, gene conversion is the result of the formation and mismatch repair of hDNA. Resnick's DSB repair model for recombination involves either no, or only one, Holliday junction.

Experiments on DSB repair and DSG filling of plasmids transformed into *S. cerevisiae* (*13, 14*) were, in part, responsible for the development of the

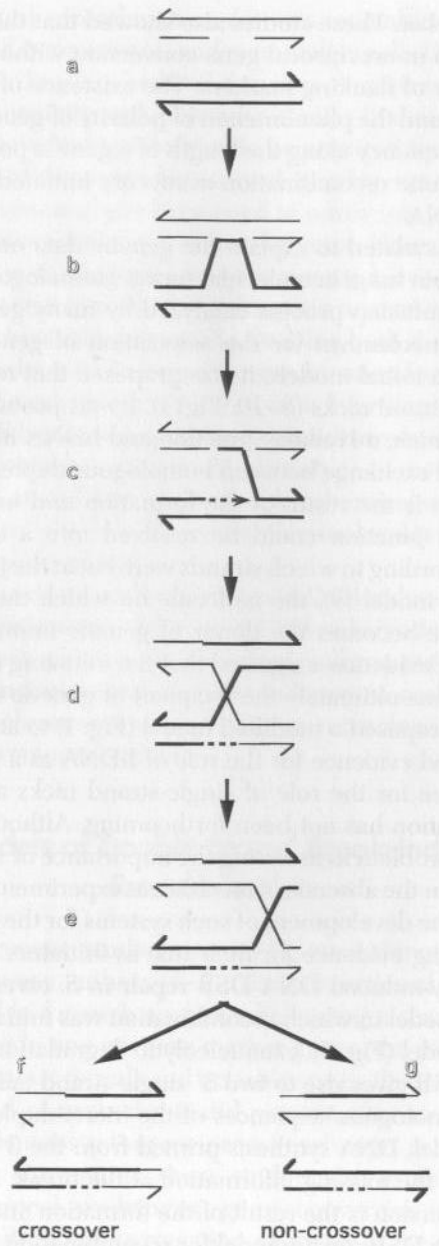


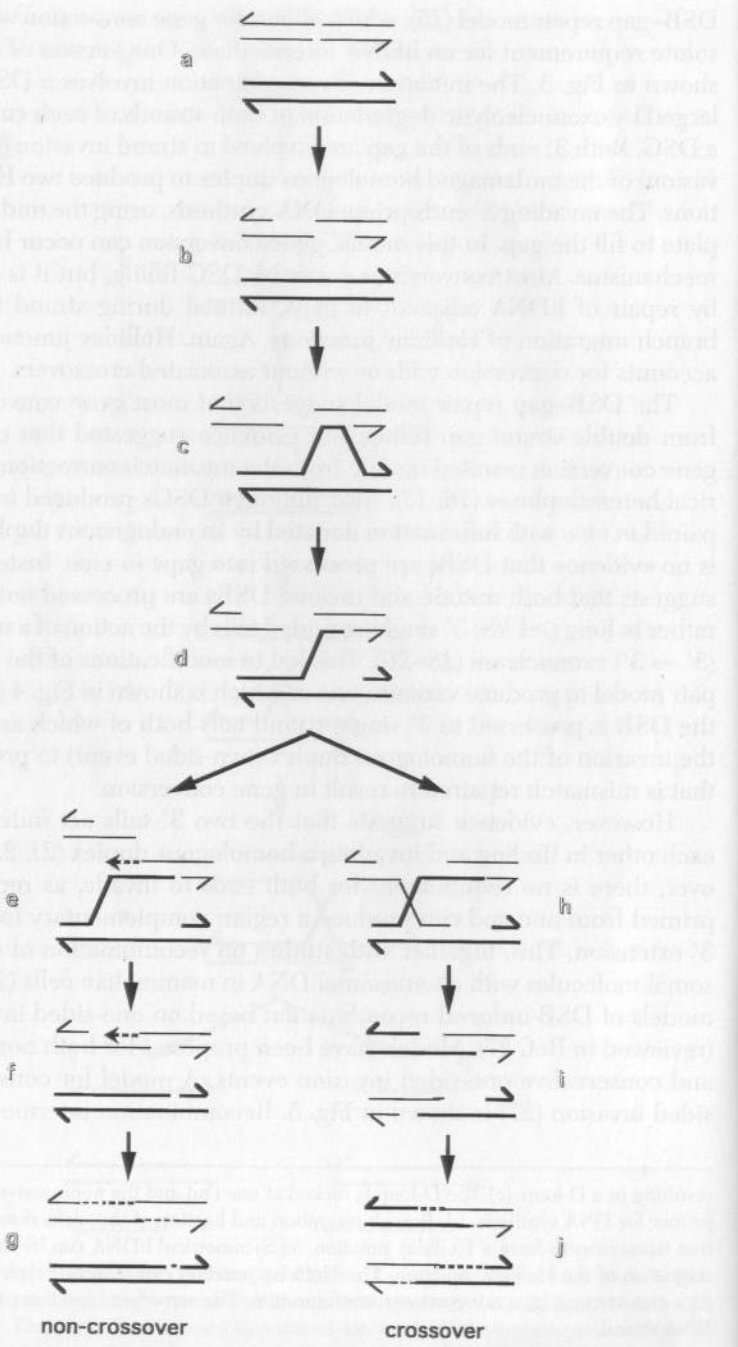
FIG. 1. Radding's model for recombination initiated by a single-strand nick (*10*). (a) Recombination is initiated by a single-strand nick in one of the duplexes that is extended into a single-strand gap. (b) The gap is then invaded by a strand derived from the homologous chromatid

DSB–gap repair model (*15*), which allows for gene conversion without an absolute requirement for an hDNA intermediate. One version of this model is shown in Fig. 3. The initiation of recombination involves a DSB that is enlarged by exonucleolytic degradation of both strands of each end to produce a DSG. Both 3′ ends of the gap are involved in strand invasion (two-sided invasion) of the undamaged homologous duplex to produce two Holliday junctions. The invading 3′ ends prime DNA synthesis, using the undamaged template to fill the gap. In this model, gene conversion can occur by one of two mechanisms. Most conversions occur by DSG filling, but it is also possible by repair of hDNA adjacent to gaps, formed during strand invasion and branch migration of Holliday junctions. Again, Holliday junction resolution accounts for conversion with or without associated crossovers.

The DSB–gap repair model suggests that most gene conversion results from double-strand gap filling, but evidence suggested that chromosomal gene conversion resulted mainly from the mismatch correction of asymmetrical heteroduplexes (*16, 17*). Also, although DSGs produced *in vitro* are repaired *in vivo* with information donated by an endogenous duplex (*13*), there is no evidence that DSBs are processed into gaps *in vivo*. Instead, evidence suggests that both mitotic and meiotic DSBs are processed not to a gap but rather to long (>1 kb) 3′ single-stranded tails by the action of a unidirectional (5′ → 3′) exonuclease (*18–20*). This led to modifications of the DSB–gap repair model to produce variants, one of which is shown in Fig. 4 (*18*), in which the DSB is processed to 3′ single-strand tails both of which are involved in the invasion of the homologous duplex (two-sided event) to produce hDNA that is mismatch repaired to result in gene conversion.

However, evidence suggests that the two 3′ tails act independently of each other in finding and invading a homologous duplex (*21, 22*) and, moreover, there is no requirement for both ends to invade, as repair synthesis primed from one end can produce a region complementary to the opposite 3′ extension. This, together with studies on recombination of extrachromosomal molecules with chomosomal DNA in mammalian cells (*23–26*), led to models of DSB-induced recombination based on one-sided invasion events (reviewed in Ref. 27). Models have been proposed for both nonconservative and conservative one-sided invasion events. A model for conservative one-sided invasion (*27*) is shown in Fig. 5. Recombination intermediates consis-

resulting in a D-loop. (c) The D-loop is nicked at one end and the noninvasive 3′ end acts as a primer for DNA synthesis. (d) Branch migration and ligation of the nicks results in a structure that isomerizes to form a Holliday junction. (e) Symmetrical hDNA can be formed by branch migration of the Holliday junction. The Holliday junction can then be resolved to give either (f) a crossover or (g) a noncrossover configuration. The arrowhead indicates the 3′ end of the DNA strand.



tent with one-sided invasion events have also been observed in DSB-induced recombination in *S. cerevisiae* (*1, 21, 28*). The model proposed that following strand invasion by one of the 3′ tails, DNA synthesis was primed from the 3′ end using the invaded strand as template. Annealing between the newly synthesized strand and the noninvading single-stranded end would lead to the formation of hDNA on one side of the DSB, and a short heteroduplex could also be formed on the other side. A Holliday junction could be generated by cutting at the front end of the D-loop with subsequent annealing with the noninvading end and DNA synthesis. As in previous models, associated crossovers would depend on Holliday junction resolution. These one-sided invasion models are similar to Resnick's model (Fig. 2), but the events leading to hDNA formation differ in the two sets of models. Detailed analysis has shown that processing of DSBs at the *MAT* locus in *S. cerevisiae* occurs at only one end, with asymmetrical strand transfer resulting in hDNA only in the recipient molecule, as predicted from one-sided models. However, Schwacha and Kleckner described double Holliday junctions in *S. cerevisiae* meiotic recombination intermediates, indicating that these events involve two-ended invasions (*29*).

Detailed genetic analysis of homologous recombination induced by transposable P-elements in *Drosophila melanogaster* has led to proposals for another model for DSB-induced recombination (*30, 31*). The model, referred to as the synthesis-dependent strand-annealing model, is shown in Fig. 6. It proposes that the DSB is processed to give two 3′ single-strand tails that behave independently of each other during homology search. It postulates that strand invasion and hDNA formation between invading 3′ single-strand tails and the template DNA strand of the homologous duplex are transient. Newly synthesized DNA, spanning the DSB and primed from the invading 3′ ends, is released from the templates and reanneals to form a duplex. Such transient formation of hDNA means that there is no Holliday junction intermediate. This accounts for P-element recombination only generating gene

FIG. 2. Resnick's model (*12*). (a) Recombination is initiated by a DSB in one duplex. (b) 3′ OH single-strand overhanging tails are exposed on either side of the break by 5′ → 3′ exonucleolytic digestion. (c) One of the two 3′ ends invades the intact homologous duplex. (d) The homologous duplex is now cut on one chain. There are two alternative pathways for processing the intermediate in (d). In one pathway (e) the invading 3′ end is extended by DNA synthesis using the intact homologous strand as template. (f) This is followed by release of the invading fragment and its annealing with the other fragment. (h) Repair synthesis and ligation results in one parental chromosome and one that has a patch of information derived from the homologous duplex. Alternatively, (h) the second 3′ end invades the intact homologous duplex as did the first, forming a Holliday junction. (i) The intact strand of the invaded duplex is cut, resolving the Holliday junction. (j) Repair synthesis and ligation restores the duplexes, giving crossover molecules, each with a segment of hDNA.

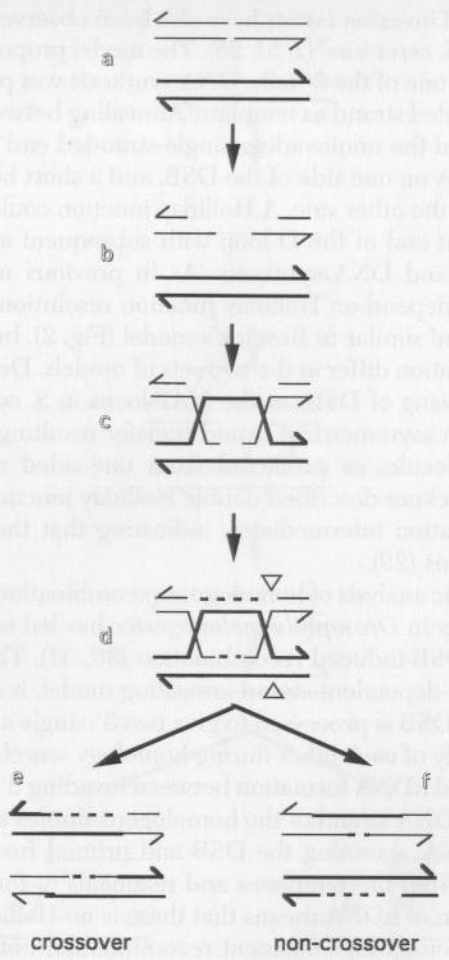


FIG. 3. DSB–gap repair model (*15*). (a) Recombination is initiated by a DSB in one duplex. (b) Both ends of the DSB are processed by exonucleases to form a double-strand gap with 5′ and 3′ single-strand overhangs on the same chain. (c) Both 5′ and 3′ overhangs invade the intact homologous duplex, displacing a D-loop. (d) Repair synthesis using both intact strands of the homologous duplex as template fills the gap, creating two Holliday junctions. One of the junctions of the double Holliday structure is resolved by cutting the outer strands (open up and down arrowheads). The other Holliday junction can then be resolved to give either (e) a crossover or (f) a noncrossover configuration.

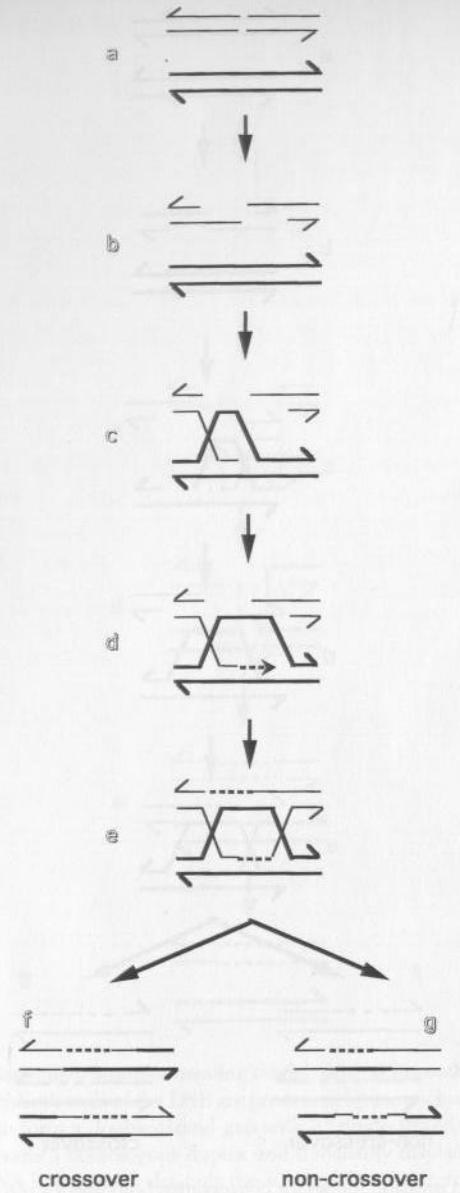


FIG. 4. Modified DSB repair model (*18*). (a) Recombination is initiated by a DSB in one duplex. (b) Extensive 3′ OH single-strand overhanging tails are exposed on either side of the DSB by 5′ → 3′ exonucleolytic digestion. (c) One of the two 3′ ends invades the intact homologous duplex, displacing a D-loop. (d) The D-loop is enlarged by DNA repair synthesis primed from the invading 3′ end, using the intact strand of the invaded homologous duplex. It anneals to the second 3′ end single-stranded DNA. (e) Repair synthesis from the second 3′ end takes place, and two Holliday junctions are formed. The Holliday junctions can then be resolved to give either (f) a crossover or (g) a noncrossover configuration.

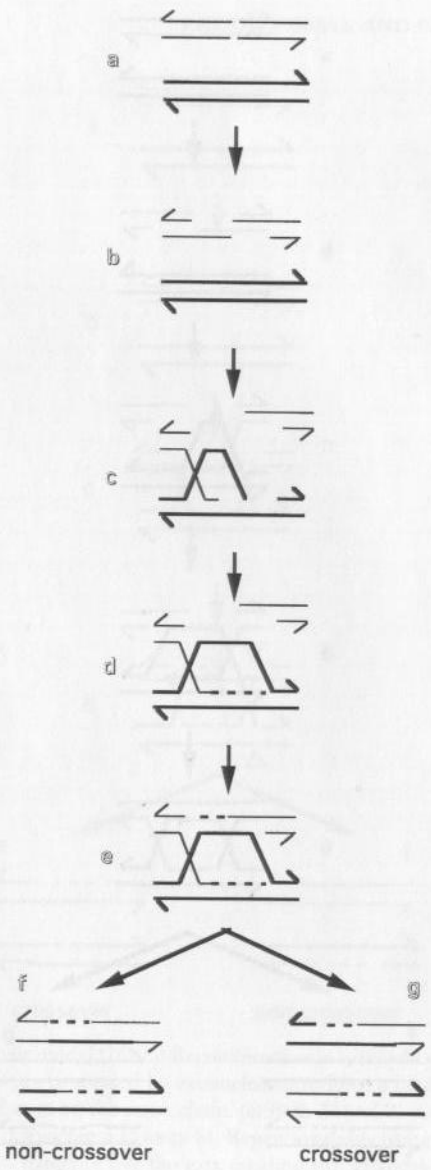


FIG. 5. One-sided invasion model for conservative homologous recombination (27). (a) Recombination is initiated by a DSB in one duplex. (b) 3′ OH single-strand overhanging tails are exposed on either side of the break by 5′ → 3′ exonucleolytic digestion. (c) One of the two 3′ ends invades the intact homologous duplex, displacing a D-loop. (d) DNA repair synthesis primed from the invading 3′ end, using the intact strand of the invaded homologous duplex, extends the D-loop. (e) Cutting at the front end of the D-loop with subsequent annealing with the noninvading end and DNA synthesis generates a Holliday junction. The other Holliday junction can then be resolved to give either (f) a noncrossover or (g) a crossover configuration.

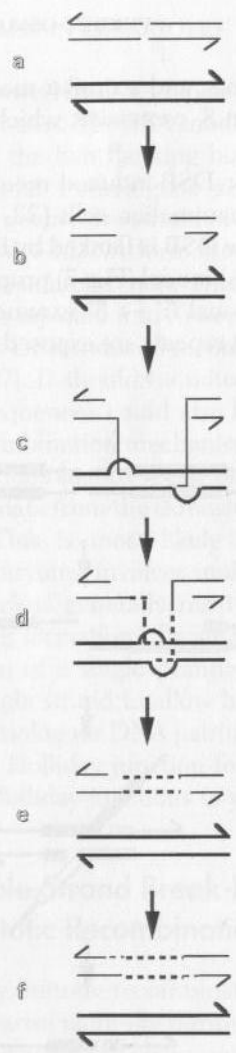


FIG. 6. Synapsis-dependent strand-annealing model (*30*). (a) Recombination is initiated by a DSB in one duplex. (b) Both ends of the DSB are processed by exonucleases that digest 5′ ends quicker than 3′ ends to form a double-strand gap with 3′ single-strand overhangs. (c) The 3′ ends independently invade a homologous duplex and transiently displace only a local loop or "bubble" of DNA. hDNA formation is also only transient. (d) Primed from the invading 3′ ends, new single strands are synthesized via a "bubble migration" mechanism using the intact strands of the invaded homologous duplex as template. The bubble migration mechanism proposes that the bubble is collapsed behind the DNA polymerase by rapid displacement of the newly synthesized single strands from the template. (e) Following synthesis, the new strands are completely displaced and anneal to one another. (f) The gap is then completed by extension of the ends of the annealed strands using each other as templates.

conversion-type recombinants, and a similar model (*32*) has been proposed for mating-type switching in *S. cerevisiae*, which also involves only conversion-type recombinants.

An alternative model for DSB-induced recombination, based on transformation experiments in mammalian cells (*33, 34*), has been proposed for DNA molecules in which the DSB is flanked by homologous sequences. The single-strand annealing (SSA) model (Fig. 7) proposes that the ends of a DSB undergo extensive bidirectional 5′ → 3′ exonuclease digestion, until complementary regions in direct repeats are exposed and can reanneal. Removal

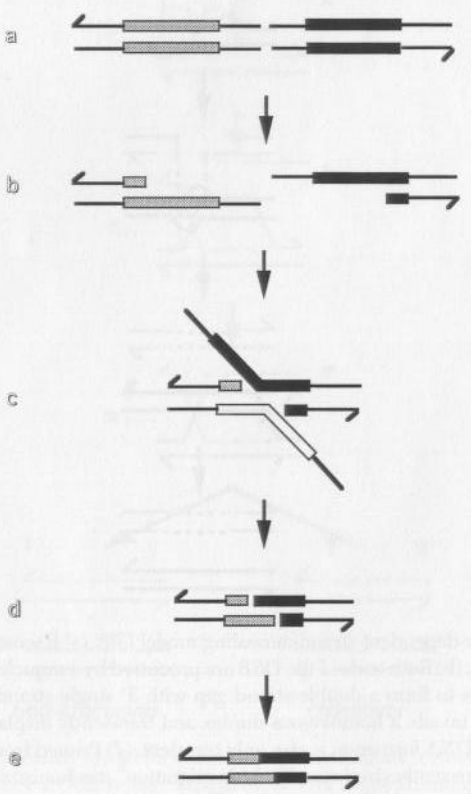


FIG. 7. Single-strand annealing model (*33, 34*). (a) Recombination is initiated by a DSB between flanking directly repeated homologous sequences (shaded rectangles). (b) Both ends of the DSB are subjected to extensive single-strand 5′ → 3′ exonuclease digestion until flanking homologous single-strand regions are exposed. (c) The complementary single strands anneal. (d) The nonhomologous tails are removed. (e) DNA repair synthesis and ligation yield a deletion product in which the intervening sequence is lost.

of nonhomologous tails, followed by DNA repair synthesis and ligation, completes the recombination process. The SSA model is inherently nonconservative, as the DNA between the two flanking homologous sequences is degraded and the two parental homologous sequences give rise to one recombinant duplex. It does not involve strand invasion or formation of a Holliday junction, but does involve hDNA formation at the annealed junctions (Fig. 7e) that is subject to mismatch repair. This model is supported by the analysis of products of DNA injected into *Xenopus* oocytes (*35, 36*), and by the analysis of the kinetics of DSB-induced recombination between direct repeats in *S. cerevisiae* (*19, 37*). It should be noted that these recombination events between repeated sequences could also be accounted for by a nonconservative one-sided recombination mechanism (*27*).

None of the models for homologous recombination in eukaryotes is consistent with all the available data from the extensive studies on recombination in a variety of systems. This is most likely because recombination in eukaryotes, like that in prokaryotes, involves multiple pathways utilizing different mechanisms. Nevertheless, generally, most models postulate at least six stages: (i) initiation involving formation of a single-strand nick, a DSB, or a DSG, followed by formation of a single-stranded DNA; (ii) presynapsis involving activation of the single strand to allow homology searching; (iii) the search for homology and homologous DNA pairing; (iv) strand exchange leading to hDNA formation; (v) Holliday junction formation and branch migration; and (vi) resolution of Holliday junctions to yield recombinant products.

II. Double-Strand Break-Induced Mitotic Recombination

As mentioned previously, mitotic recombination is important for the repair of DNA DSBs that can arise naturally during the life cycle of a cell or in response to a DNA-damaging agent. The consequences of unprocessed DSBs are blockage of DNA replication and loss of genome integrity leading to lethality. Moreover, DSB-induced chromosomal mitotic recombination is involved in a number of basic cellular processes seen in a wide variety of eukaryotic systems, including mating-type switching in *S. cerevisiae* (*38*) and *S. pombe* (*39*); transpositions of P-elements in *Drosophilia melanogaster* (*31*); and mammalian site-specific V(D)J gene rearrangements that give rise to immmunoglobin and T-cell receptor diversity (*40*).

There are several different fates of a mitotic DSB: homologous recombination, nonhomologous (illegitimate) recombination, or the addition of new telomeres at the break site. All of these types of mechanisms have been observed in eukaryotes as diverse as yeasts and mammals, although the relative

efficiencies of these events vary considerably in different eukaryotic cells. In *S. cerevisiae*, DNA DSBs are primarily processed by homologous recombination pathways (reviewed in *41*). In contrast, in mammalian cells evidence suggests that an illegitimate recombination mechanism, DSB end-joining, rather than homologous recombination is the prevailing mechanism (*42, 43*). Other eukaryotes appear to lie in between these two extremes with respect to relative efficiencies of homologous and illegitimate recombinational processing of DSBs. It is not clear whether these differences reflect additional capacity for nonhomologous DNA DSB joining or diminished pathways for homologous recombination in other eukaryotes compared to *S. cerevisiae*.

DNA-damaging agents that induce DNA DSBs, such as ionizing radiation, have been shown to stimulate mitotic chromosomal recombination in a wide variety of organisms. This could lead to conversions, crossovers, deletions, duplications, inversions, and translocations. Although such studies have been very informative, and were in part responsible for Resnick's model of DSB-induced recombination (*12*; Fig. 2), the damage-induced DSBs are difficult to study because they are randomly distributed and infrequent at biologically relevant doses. Generalized spontaneous mitotic recombination also occurs randomly at low frequency. The genetic and physical consequences of a mitotic DSB are best examined at a defined DSB. Three approaches to study the fate of a defined mitotic DSB have been employed in eukaryotes: (i) using extrachromosomal substrates with defined DSBs introduced *in vitro* prior to delivery into cells; (ii) examining recombination associated with the cellular processes involving a defined DSB mentioned earlier (i.e., mating-type switching in yeasts, P-element transposition in *Drosophila melanogaster* and V(D)J recombination in mammalian cells); and (iii) the use of components of some of these endogenous systems to induce site-specific DSBs elsewhere in the genome. This section focuses on current studies of defined DSB-induced mitotic homologous recombination in *S. cerevisiae*, but also includes studies on nonhomologous recombination and studies in other eukaryotes.

A. Recombination Events Associated with DSBs Induced *in Vitro*

Extrachromosomal circular DNA molecules linearized with DSBs and DSGs produced *in vitro* have been introduced into cells of many eukaryotic organisms. This has allowed the fates of the *in vitro*-produced DSBs and DSGs to be monitored *in vivo*. In these experiments, recombination efficiency is inferred from transformation efficiency. During transformation of *S. cerevisiae*, it was found that DSBs and DSGs stimulate transformation frequencies by as much as 3000-fold (*13, 14*). The integration of linearized nonreplicative plasmids bearing DSBs or DSGs within sequences homologous

to chromosomal sequences occurs by a homologous recombination mechanism (*13, 14*). Integration is accompanied by the repair of the termini through a gene conversion event copying the missing plasmid-borne information from homologous chromosomal sequences. Replicative plasmids with DSGs undergo similar events, with equal numbers of transformants containing integrated (gap repair with crossing over) and nonintegrated (gap repair without crossing over) plasmids. Recircularization during transformation of a replicative plasmid linearized by a DSB was found to occur at high efficiency by recombination using either a homologous chromosomal sequence (*13*) or a homologous sequence on a co-transformed plasmid (*44*). Reducing homology also reduced the frequency of recombination events associated with DSBs and DSGs (*45*). As mentioned previously, these observations were, in part, resposible for the DSB–gap repair model for homologous recombination (*15*; Fig. 3). Replicative plasmids linearized by a DSB and bearing homologous repeats could also recircularize to yield deletion-type recombinants associated with the loss of one repeat and the intervening sequences (*46, 47*). It was suggested (*47*) that these recombinants could arise via three pathways: gene conversion associated with reciprocal exchange, nonconservative one-sided invasion events, and SSA. These and numerous other such studies (reviewed in *6* and *48*) suggest that *in vitro*-produced, plasmid-borne DSBs and DSGs are primarily processed by homologous recombination mechanisms in *S. cerevisiae*. However, nonhomologous recombination events occurring at low frequency (at least 100-fold less efficient than homologous recombination events) have also been described. When *S. cerevisiae* cells are transformed with linearized replicative plasmids lacking homology to genomic DNA, recircularization can occur via direct rejoining of the ends (*46*), or via interaction of the ends of two linear molecules, resulting in the formation of head-to-head plasmid dimers (*49*). These illegitimate recombination events are associated with short deletions or insertions around the ends of the DSB. Nonhomologous integration of linearized plasmids utilizing little or no (4 bp or less) end-sequence homology can also occur at low frequency in *S. cerevisiae* and is also associated with small deletions–insertions at the join sites (*50, 51*). Illegimate recombination in *S. cerevisiae* may reflect the existence of a separate end-to-end joining mechanism, or, alternatively, it has also been suggested (*52, 53*) that it occurs via mechanisms similar to SSA or one-sided events involving small stretches of overlapping, locally homologous sequences (microhomology).

Studies with extrachromosomal substrates in several other eukaryotes have defined similar homologous and illegitimate recombination activities, although illegitimate events are more efficient in other eukaryotes compared to *S. cerevisiae*. For example, in *S. pombe*, although linearized self-replicating plasmids are efficiently recircularized by homologous recombination in

the presence of homology, 1 in 26 (compared to less than 1 in 100 for *S. cerevisiae*) are recircularized by nonhomologous end-joning (*54*). In the absence of homology, recircularization by nonhomologous end-joining pathways is efficient in *S. pombe* (*54*). As with *S. cerevisiae*, end-joining involved interaction of short patches (*1–4* bp) of sequence homologies and generated deletions at the ligation points. Similarly, integration of linearized plasmids by homologous recombination is efficient in *S. pombe*, but there is a higher frequency of nonhomologous integration compared to *S. cerevisiae* (*55, 56*).

In mammalian cells, illegitimate events are the primary mechanism for processing DSBs and DSGs in extrachromosomal substrates. Despite the great desire for efficient gene targeting in mammalian cells, less than 1 in 100 DSB-induced integrative transformation events involves homologous recombination. Nevertheless, DSBs and DSGs do stimulate homologous integration events in mammalian cells, and strategies have been developed to select for homologous integration against a large background of nonhomologous integrations (e.g., *57, 58*). In many respects these rare homologous recombination events strongly resemble the well-characterized events in *S. cerevisiae*. However, there are some notable differences, since homologous DSB-induced integrative transformation can be accomplished in mammalian cells by one-sided events when only one end of the transforming DNA integrates by homologous recombination (reviewed in *27*). DSBs and DSGs induce extrachromosomal homologous recombination between co-injected or co-transfected DNA molecules in mammalian cells (reviewed in *59, 60*), albeit at a reduced frequency compared to *S. cerevisiae*. Again, these homologous recombination events, involving gap filling, conversion, and reciprocal exchange, strongly resemble those in *S. cerevisiae*. As mentioned previously, it was also shown that SSA is a major homologous recombination pathway for linearized extrachromosomal molecules containing direct repeats transformed into mammalian cells (*33, 34*) or injected into *Xenopus* oocyte nuclei (*35, 36*). SSA has also been invoked to explain some types of recombination between linearized plasmids in the smut fungus *Ustilago maydis* (*61*) and plant cells (*62*).

Efficient illegitimate recombination events, end-joining, and end-to-end joining of linearized extrachromosomal DNA molecules has been observed in *Xenopus* (*63, 64*), mammalian cells (*42, 43*) and other eukaryotes.

In contrast to the similarities of recombination pathways for processing DSBs and DSGs among eukaryotes, there are also notable exceptions. For example, in a study in *U. maydis* (*65*) it was found that recombinational repair of a plasmid-borne DSG gap using chromosomal sequences was only very rarely accompanied by crossing over. Processing of the DNA ends flanking the gap was unequal, and a migrating D-loop model was proposed (*65*), similar to the synthesis-dependent strand annealing model shown in Fig. 6.

B. Recombination Events Associated with Artificial Site-Specific DSBs Induced *in Vivo*

1. Recombination Events in *S. cerevisiae*

HO endonuclease initiates mating-type switching in *S. cerevisiae* by producing a DSB at its target site, the *y/z* junction within the recipient *MAT* locus (*66, 67*). I-Sce-I is a mitochondrial intron-encoded, site-specific endonuclease that produces a DSB at its target site to initiate insertion of the intron into a new site (*68*). A modified version of I-Sce-I can be expressed in the nucleus. In *S. cerevisiae* both of these site-specific endonucleases, under the control of inducible promotors, have been used to study recombination initiated by a single, site-specific *in vivo* DSB by inserting their respective recognition sites at specific locations within defined DNA sequences (reviewed in *3*). In all cases DSB induction stimulated recombination. Only a few studies have utilized the I-Sce-I system (*69, 70*) compared to the numerous studies with HO. The studies with I-Sce-I used recombination substrates similar to those used in the studies with HO endonuclease. An important consideration is that the I-Sce-I-induced and the HO-initiated events, described later, are indistinguishable. This argues that HO and I-Sce-I endonucleases play no other role in the recombination events under study except DSB induction.

HO endonuclease has been used extensively to initiate DSB-induced intramolecular recombination events between repeated sequences in *S. cerevisiae*, both intrachromosomal and intraplasmid, by inserting the HO recognition site within artificially created duplications. Typically, the duplication consists of either two different alleles of the same gene or two overlapping segments of a gene. The repeated sequences can be in direct or inverted orientation and are typically separated by unique DNA, commonly with a marker gene also present within the intervening sequence. The HO recognition site has been placed either within duplicated DNA in one of the repeat elements (*19, 28, 37, 71–73*), or within unique DNA in the intervening sequence between the repeats (*20, 72, 74*).

The use of such substrates has allowed a genetic analysis of DSB-induced recombination by the recovery and analysis of recombinants arising from interaction between the repeated elements. Two main classes of recombinants were recovered: conversion-type recombinants, which still have two copies of the repeat element and have presumably arisen by nonreciprocal transfer of information from one element to the other without loss of the intervening sequences; and deletion-type recombinants, which have a single copy of the repeat element with accompanying loss of the intervening sequences.

The relative frequencies of conversion-type and deletion-type recombinants depended on whether the DSB was induced within the homologous sequences of one of the repeated sequences, or within the unique sequences

between the repeats. In studies in which the *in vivo* DSB was made in duplicated DNA, both conversion-type and deletion-type events were stimulated, for both intrachromosomal (*28, 71–73*) and intraplasmid (*19, 28, 37*) recombination substrates. The spectrum of DSB-induced recombination events depended on the particular substrate employed but remained the same as spontaneous events, indicating that the pathways involved in spontaneous and DSB-induced mitotic recombination may be the same.

For both direct and inverted repeats, for the induced conversion-type recombinants the cleaved repeat sequence acted almost exclusively as the recipient of genetic information, and these recombinants could be accounted for by a DSB–gap repair pathway. With the repeats in inverted orientation, it was shown that deletion-type recombination was also consistent with a conservative DSB–gap repair pathway, that is, a conversion event associated with reciprocal crossing over (*28*). For repeats in direct orientation, spontaneous and induced deletion-type recombinants could result by any of the following means: gene conversion associated with crossing over; an unequal sister chromatid exchange at G_2 (for chromosomal substrates); a nonconservative one-sided strand invasion pathway; or SSA. If gene conversion is accompanied by reciprocal crossing over, the segment of DNA that is internal to the two halves of the repeat will be excised as a circle. However, the majority of spontaneous and induced conversion-type events were not accompanied by crossing over, and for chromosomal substrates unequal sister chromatid exchange only occurred at very low levels (*19, 28, 37, 71–73, 75*), suggesting that deletion-type recombinants arose via the SSA pathway and/or via a nonconservative one-sided strand invasion pathway. At present it is not clear whether deletion-type recombinants arise via SSA or nonconservative one-sided events, or via both mechanisms, as suggested by Prado and Aguilera (*47*).

For intrachromosomal and extrachromosomal recombination substrates in which the DSB was induced within unique DNA between direct repeats, there was a predominance (>99%) of deletion-type mitotic recombinants (*20, 71, 72, 74*). Their production was also shown to be consistent with a SSA mechanism.

In addition to the genetic analysis, these DSB-induced recombination events could also be monitored physically. The use of inducible promotors to express HO endonuclease allows the *in vivo* DSBs to be produced synchronously. Subsequent steps in recombination, and the appearance of recombination intermediates and final products, could then to be followed over time by physically analyzing DNA extracted from cells. A physical analysis of the kinetics of both DSB-induced conversion-type and deletion-type product formation provided evidence that DSB–gap repair and SSA (and/or one-sided events) are two independent competing pathways of DSB-induced recombination with a common intermediate (*19, 28*). There was a distinct difference

in the time of appearance of gene conversion- and deletion-type products, and the appearance of deletion-type recombinants could be delayed by increasing the distance between repeats without affecting the appearance time of conversion-type recombinants (*19*). The likelihood of conversion-type recombination was increased with increasing distance between repeats. These studies identified 3′ single-stranded DNA on both sides of the DSB as recombination intermediates (*19, 20*). These are intermediates common to the modified DSB repair model (*18*) and the SSA model (*33, 34*). Although nonconservative, one-sided strand-invasion models envisage extensive single-strand tails on one side of the break, it is possible nevertheless that such pathways still contribute to recombination between repeated sequences to give deletions. Deletion-type recombination between regions flanking a DSB, presumably by SSA, was linearly dependent on the length of flanking homology, and appeared to have a minimum homology requirement of 65–90 bp (*20*). This requirement may reflect the minimum length needed for homology searching, or it may be the length needed to form a stable intermediate structure. SSA as an alternative recombination pathway appears to be as efficient as DSB–gap repair (*19*).

HO-induced DSBs introduced in the ribosomal DNA or in the *CUP1* tandem gene arrays also stimulated recombination events resulting in loss of one or more repeat units (*76*), consistent with the observations for the artificial duplications.

In vivo HO endonuclease-induced DSBs have also been used to investigate recombination in other types of substrate in *S. cerevisiae*. For example, recombination between plasmid and chromosomal homologous sequences initiated by a HO DSB was used to investigate conversion tract length and directionality (unidirectional or bidirectional) and the effects of nonhomology or homeology at the ends (*77, 78*).

Interchromosomal mitotic recombination was also stimulated by a HO-induced DSB in one of the participating chromosomes in diploids of *S. cerevisiae* (*79, 80*). A HO-induced DSB stimulated triparental recombination between *his3* heteroalleles on heterologous chromosomes (*79*). The DSB was made at a site 8.6 kb from one of the *his3* heteroalleles. The cleaved chromosome acted as recipient of genetic information, and recombination was accompanied by repair of the DSB. In most cases the DNA between the break site and the *his3* heteroallele was intact and did not show enhanced recombination, as would be expected from most recombination models, prompting the suggestion of a discontinuous hDNA model (*79*). In the other study, interchromosomal recombination in diploids was monitored between homologous sequences at allelic sites (*80*). In most recombinants the chromosome with the DSB acted as recipient of genetic information. In general most of the data were consistent with the DSB–gap repair, with the forma-

tion of DSGs from a few hundred to a few thousand bases in size that are repaired by information from the uncut chromosome. However, heteroduplexes flanking the DSB could also be generated, resulting in discontinuous conversion tracts, and in some cases the cut chromosome acted as the donor of genetic information.

An inducible HO endonuclease was also used to show that, in the absence of homologous recombination, *in vivo* DSBs at the *MAT* locus could be processed by nonhomologous end-joining, resulting in small deletions or insertions at the join sites (52). Evidence suggests that these deletions and insertions are formed by different nonhomologous end-joining pathways in *S. cerevisiae* (81).

Finally, HO endonuclease was used to investigate the formation of new telomeres at chromosome break sites in *S. cerevisiae* (82, 83).

2. Recombination Events in Other Eukaryotes

a. Intrachromosomal Recombination in S. pombe. It has been shown that DSBs at the mating-type locus can initiate mitotic recombination in *S. pombe* (39). These studies illustrated some of the general features of the DSB–gap repair model. No other studies had examined DSB-induced mitotic intrachromosomal recombination at loci other than the mating-type loci in *S. pombe*. In our laboratory we sought to determine whether the pathways of DSB-induced intrachromosomal recombination in *S. cerevisiae* were conserved in *S. pombe*. We showed that the *S. cerevisiae* HO endonuclease, expressed from an inducible *S. pombe* promotor, and its *MATa* target site could successfully be used to introduce site-specific DNA DSBs *in vivo* within intrachromosomal recombination substrates in *S. pombe* (84). The recombination substrates were similar to those used in *S. cerevisiae* and consisted of nontandem direct repeats of *ade6* heteroalleles. The *MATa* cutting site was located either within duplicated DNA in the left-hand *ade6* heteroallele or within unique DNA between the *ade6* repeats. Induction of DSBs resulted in a 2000-fold stimulation in the frequency of recombinants compared to spontaneous events. The DSB-induced recombination frequency was high enough so that it was not necessary to select for recombinants and all cells were analyzed, permitting an unbiased evaluation of all the different fates of the recombination substrate.

Analysis of the recombinants illustrated that DSB-induced intrachromosomal mitotic recombination in *S. pombe* was very similar to that in *S. cerevisiae*. When the DSB was located in duplicated DNA in one of the *ade6* heteroalleles, both conversion-type and deletion-type recombinants were induced, and in the same relative proportions as spontaneous recombinants. This suggested that the majority of spontaneous recombinants could also be arising due to spontaneous DSBs within duplicated DNA. For DSB-induced

conversion-type recombinants, the copy of *ade6* in which the DSB was made was the recipient of genetic information, which is a prediction of the DSB–gap repair model for recombination (15). Several different types of conversion-type recombinants were observed: those in which only the *MATa* site was lost; those that co-converted both the *MATa* site and the *ade6* mutation to wild type; and those that converted all the information of the recipient heteroallele to that of the donor heteroallele. When the DSB was situated within unique DNA between the *ade6* heteroalleles, over 99.8% of DSB-induced recombinants were deletion types. No *ade6* triplications, which are diagnostic of sister-chromatid reciprocal exchanges, were observed in our study, regardless of whether the DSB was made in duplicated or unique DNA.

DSB–gap repair and SSA (and/or nonconservative one-sided events) could account for the data. DSB-induced conversion-type recombinants, in which all the information of the recipient heteroallele is converted to that of the donor heteroallele, show that the DSG or hDNA postulated by the DSB–gap repair model (Fig. 3), or the 3′ single-strand tails postulated by the modified DSB repair model (Fig. 4), could be extensive, covering almost the entire *ade6* locus. Our results also suggested that, during SSA, 5′ → 3′ exonuclease digestion on both sides of the DSB exposed extensive, more than 1-kb, complementary homologous 3′ single-strand regions in the two *ade6* repeats. Annealing would result in extensive hDNA formation covering almost the entire *ade6* locus, with hybrid DNA at both the *ade6* heteroallelic mutation sites (located 1.3 kb apart on the *ade6* locus). The mutated bases of these two hybrid sites are in *trans* with respect to which strand of the duplex they are located on (i.e., +/− and −/+), and a careful analysis of Ade$^-$ deletion-type recombinants revealed the absence of final recombinants that retained both mutations. This suggested that either of the single strands of the hDNA covering the the entire *ade6* locus was subject to unidirectional mismatch repair. In addition, genetic intermediates in the form of half-sectored colonies were isolated, analyzed, and interpreted as evidence of hDNA formation during the SSA pathway.

b. Recombination Events in Higher Eukaryotes. Previously, induction of *in vivo* DSBs in higher eukaryotic cells relied on introducing bacterial restriction endonucleases into the cells by electroporation (85, 86). However, introduction of restriction endonucleases into higher eukaryotic cells causes wholesale genomic breakage that may induce cellular responses to global damage and obscure the effect of a single DSB. In recent years, *S. cerevisiae* I-Sce-I endonuclease has been introduced into higher eukaryotic cells to induce a single *in vivo* site-specific DSB into DNA containing the I-Sce-I recognition site. The I-Sce-I recognition site, which is 18 bp in length (87), is unlikely to occur randomly in the genomes of higher eukaryotes. I-Sce-I en-

donuclease has been introduced into the cells either by electroporation (*88, 89*) or by *in vivo* expression (*90–93*). *In vivo* I-Sce-I-induced DSBs have been used to study extrachromosomal, extrachromosomal–chromosomal and chromosomal recombination in higher eukaryotic cells.

I-Sce-I endonuclease-induced DSBs stimulated both intramolecular and intermolecular homologous recombination in extrachromosomal substrates in mammalian cells (*91*), plants cells (*93*), and *Xenopus* oocyte nuclei (*94*). DSB-induced recombination between direct repeats seemed to proceed via a SSA mechanism (*91, 93*). In contrast, DSBs in extrachromosomally replicating plasmids, generated *in vivo* by electroporation of restriction enzymes, were processed primarily by nonhomologous end-joining in mammalian cells (*85*).

Given the desire to effect efficient, precise gene targeting in mammalian cells, and the fact that most integrative transformation events occur randomly (even if the extrachromosomal molecule has a DSB), attention has been focused on recombination between sequences on extrachromosomal DNA molecules and homologous chromosomal sequences. In previous targeting experiments, the exogenous vector DNA had a DSB, but the chromosomal target did not. It was of great interest to determine whether a site-specific *in vivo* chromosomal DSB could stimulate homologous recombination with a homologous extrachromosomal sequence. Two reports (*90, 92*) have described the expression of the yeast endonuclease I-Sce-I in mouse cell lines to create site-specific *in vivo* chromosomal DSBs. The DSBs stimulated, by two to three orders of magnitude, homologous recombination between the two chromosomal sequences flanking the break and two homologous regions on a transfecting circular targeting vector, resulting in targeted integration of the vector. A DSB repair pathway, a SSA mechanism, or one-sided homologous recombination could account for the events. Nonhomologous end-joining between the cleaved chromosomal ends was also stimulated, either by direct ligation or associated with small deletions resulting from joining through short sequence homologies. One report (*88*) described the direct electroporation of I-Sce-I enzyme together with a targeting vector into mouse cells. Although cleavage and repair of the chromosomal target took place, no homologous recombination between the targeting vector and the chromosomal target was detected, in contrast to the other studies.

Recombination in *Xenopus* oocyte nuclei was monitored between homologous sequences on two extrachromosomal molecules, a linear DNA molecule and a circular one containing a I-Sce-I recognition site in a system designed to mimic a gene-targeting experiment based on a SSA mechanism (*94*). The linear DNA molecule contained two regions each homologous to the sequences flanking the DSB in the circular DNA molecule. *In vivo* DSB cleavage of the circular DNA stimulated homologous recombination with the linear DNA, resulting in a joint molecule. In plant cells, I-Sce-I-induced

chromosomal DSBs stimulated homologous integration of extrachromosomal targeting vectors by two different pathways, a DSB–gap repair mechanism involving both homologous ends and one-sided events involving one homologous end (*95*).

In mammalian cells, using intrachromosomal recombination substrates consisting of nontandem direct repeats, it was shown that *in vivo* I-Sce-I- or restriction enzyme-induced DSBs located within the duplicated homologous regions stimulated predominantly homologous recombination events (10-fold increase), with only a minority involving nonhomologous end-joining (*89*). Restriction enzyme-induced DSBs outside of the repeated regions, or between them, produced no change in recombination frequency. Godwin et al. (*96*) examined the effect on interchromosomal recombination between homologous sequences of *in vivo* DSBs induced by electroporation of a restriction enzyme. In their experiments they detected only nonhomologous end-joining events. Similarly, chromosomal deletions associated with nonhomologous end-joining have been shown to result from electroporation of mammalian cells with restriction enzymes (*86*).

Thus *in vivo* DSBs have been shown to stimulate predominantly homologous, nonhomologous, or both types of recombination depending on the systems used. The differences between these various results may have to do with variations in recombination substrates, the endonucleases chosen, or the methods used to introduce endonucleases into the cell.

C. Biological Systems Utilizing Naturally Occurring Site-Specific DSBs Induced *in Vivo*

As mentioned, several endogenous recombination systems in mitotic cells are associated with site-specific DSBs. An investigation of these systems has provided additional insights into the molecular mechanisms of DSB-induced recombination. It is beyond the scope of this review to describe studies with these systems in detail, but readers are directed to the reviews cited.

Homothallic switching of the mating-type genes in *S. cerevisiae* occurs by a highly regulated site-specific homologous recombination event (for reviews see *32, 38*). HO endonuclease makes a DSB in the *MAT* locus at its recognition site near the *MAT–y/z* border. This stimulates gene conversion to replace DNA at the *MAT* locus with sequences copied from one of two unexpressed donor loci, *HML* or *HMR*, located on the same chromosome. *MAT* conversion is not accompanied by reciprocal crossing over. The expression of HO endonuclease is normally confined to the G_1 phase of the cell cycle and only in cells that have previously divided. However, the use of an inducible HO gene made it possible to produce a DSB in the *MAT* locus at any time and follow the process kinetically. An early intermediate was a single

long 3′ single-strand tail beginning at one end of the induced DSB, followed by its invasion of the donor site and elongation by copying of the donor sequences (*21*). Strand invasion generated hDNA that was rapidly mismatch repaired, nearly always in favor of the donor sequences (*97*). Mating-type switching in S. *pombe* is similar to that in S. *cerevisiae* in that it involves initiation by a DSB at the *mat1* locus that stimulates gene conversion to replace DNA at the *mat1* locus with sequences copied from one of two unexpressed donor loci, *mat2-P* or *mat3-M*, located on the same chromosome (reviewed in *39*).

Detailed analysis of the P-element transposition in *Drosophila* (reviewed in 31) also involves a site-specific DNA DSB resulting in conversion without an associated crossover. It was shown that DSB-induced recombination events described in S. *cerevisiae* also occurred in *Drosophila*. These studies complement those from lower eukaryotes and reveal new aspects of recombination giving rise to the synthesis-dependent strand-annealing model of homologous recombination (Fig. 6).

In vertebrates, V(D)J recombination occurs during B and T lymphocyte development and is responsible for the tremendous diversity in antibody and T-cell receptor specificities (reviewed in *40*). During V(D)J recombination, three gene segments, the variable (V), joining (J), and diversity (D) elements, occurring at distinct locations in germ cells, become rearranged into a contiguous exon. V(D)J recombination is initiated by site-specific DSBs, acts between specific signal sequences, and does not require extensive sequence homology, although short sequence homologies have been implicated in coding joint formation. Attention has been focused on the mechanism of V(D)J recombination, since it was shown that there is significant overlap between V(D)J recombination and the ubiquitous nonhomologous end-joining mechanism for processing of DSBs in all other cell types (reviewed in *98* and *99*).

III. Double-Strand Break-Induced Meiotic Recombination

Homologous recombination is a major feature of meiosis in sexually reproducing plants and animals. Meiosis is the central vehicle for the exchange of genetic information in eukaryotes, and it is in meiosis that recombination in eukaryotes achieves its highest frequency. As well as being responsible for the reassortment of the genetic material, genetic studies in many organisms have shown that recombination between homologous chromosomes is necessary for proper disjunction during meiosis I in organisms in which recombination usually occurs (reviewed in *100*).

In the classical view of meiosis, the process that brings about homologous alignment of chromosomes in close apposition (chromosome synapsis) during meiotic prophase I, culminating in the formation of the synaptonemal complex, occurs prior to, and is required for, meiotic recombination. However, despite inconsistencies and exceptions, alternative ideas have developed in which DSB formation in early meiosis I prophase initiates genome-wide searches for homology and DNA–DNA exchanges, which precede and mediate homologous chromosome pairing and synaptonemal complex formation (reviewed in *101–103*). One interpretation is that mechanisms for the repair of DSBs have been recruited from somatic cells to function in meiosis as a homology seeking mechanism (*104*). Most of what we know about the molecular biology and genetics of meiosis comes from work in lower eukaryotes, and in the last few years many of the details come from work in S. *cerevisiae*. This section focuses on current compelling evidence that DSBs initiate meiotic recombination in S. *cerevisiae*.

Meiotic recombination events, either crossovers or gene conversions, occur at high frequencies in certain regions (hot spots) of the S. *cerevisiae* genome (reviewed in *105*). These hot spots are associated with elevated levels of meiosis-specific DSBs. Hot spots associated with DSBs have been localized near the *ARG4*, *HIS4*, *HIS2*, and *CYS3* loci, a centromere-linked region of chromosome III, and a *Tn3*-derived transposable element (see *105* and references therein). A prominent DSB has also been associated with a high level of recombination observed in an artificial hotspot, *HIS4-LEU2*, created by the insertion of a *LEU2*-containing fragment distal to the *HIS4* gene on chomosome III (see *105* and references therein). As well as occurring at these hot spots, meiosis-specific DSBs have also been detected at a number of preferred sites on every chromosome assayed in S. *cerevisiae* (*104, 106, 107*). Several lines of evidence suggest that these DSBs are responsible for the initiation of meiotic reccombination. First, these DSBs appear at the time of commitment to recombination. Second, mutations that alter recombination frequencies at hot spots also alter the frequency of nearby meiosis-specific DSBs in a directly correlated way. Third, the position of the DSB correlates with gene conversion polarity.

Meiotic DSBs are not DNA sequence specific, but occur preferentially in intergenic regions that contain transcription promotors and are hypersensitive to nuclease digestion in chromatin isolated from both meiotic and vegetative cells (*107–109*), indicating that chromatin structure plays a major role in determining the sites of meiotic DSBs and transcriptional regulation. Meiotic DSB cleavage at these sites must be catalyzed either by a meiotically induced endonuclease or by a constitutive endonuclease that is somehow activated or recruited by meiosis-specific gene products. Evidence suggests an interaction between the meiosis-specific endonuclease and transcription factors (*109*).

DSBs at hot spots are processed by 5′ → 3′ DNA exonuclease activity to

generate DNA molecules with 3′ overhangs several hundred base pairs in length (*18, 110*), similar to those observed during mitotic recombination. These tails are presumably used to form strand-exchange products. Double Holliday junction intermediates in *S. cerevisiae* meiotic recombination have been described (*29*).

The *M26* point mutation of the *ade6* gene is the best studied meiotic hot spot in *S. pombe* (*111*). *In vitro* mutational analysis showed that *M26* creates a specific 7-bp sequence that is crucial for hot spot activity (*112*), although this sequence is not sufficient to create a hot spot when inserted into other chromosomal locations (*112, 113*). Proteins binding to this heptanucleotide sequence have been purified (*114*). Meiotic and mitotic recombination in *ade6* can also be increased by fusing the gene to a strong *ADH1* promotor (*115*). However, to date, DSBs have not been shown physically to be associated with meiotic recombination in *S. pombe*, as they have in *S. cerevisiae*. DSBs have also not been shown to be associated with the *M26* hot spot physically, and genetic evidence is consistent with *M26* creating an initiation (or termination) site for gene conversion by the introduction of a single-strand break in its vicinity (*116*). In contrast to most other eukaryotes, meiotic recombination in *S. pombe* occurs in the absence of detectable synaptonemal complexes and crossover interference (reviewed in *117*). Given this fundamental difference, we were interested in whether DSBs are the initiators of meiotic recombination in *S. pombe*.

It had previously been shown that mitotically induced DSBs at the *mat1* mating-type locus of *S. pombe* persisted during mating-stimulated homologous meiotic recombination (*118*). However, DSBs as stimulators of meiotic recombination at other loci in *S. pombe* had not been investigated. In *S. cerevisiae*, HO-induced, site-specific *in vivo* DSBs were shown to stimulate meiotic intrachromosomal recombination between nontandem direct repeats of *ade4* heteroalleles (*73*). In our experiments, crosses involved two *S. pombe* strains of opposite mating type. One strain contained direct repeats of *ade6* heteroalleles, with the HO recognition site either in unique or duplicated DNA, but with no *HO* gene. The other strain contained the *HO* gene downstream of an inducible *S. pombe nmt1* promotor, but no HO recognition site. Thus the experiments were designed so that the HO endonuclease and HO recognition site would only come together during meiotic nuclear fusion, thus ensuring the DSBs were induced during meiosis. However, no stimulation of meiotic recombination was observed (F. Osman and S. Subramani, unpublished results). This either implies that DSBs did not induce detectable meiotic recombination events, or that functional HO endonuclease was not expressed from the *nmt1* promotor during meiosis. This is a potentially interesting result that requires additional experiments, perhaps involving fusion of the *HO* gene to a meiosis-specific *S. pombe* promotor.

IV. The Genetic Control of Double-Strand Break-Induced Recombination

An investigation of the genetic control of the pathways of recombination in eukaryotes depends on the isolation of recombination-defective mutants. The analysis of such mutants can identify genes whose products are required for recombination and can define the components of the different recombination pathways. The subsequent cloning of these genes, and the determination of the biochemical activities of the encoded gene products, are crucial steps in elucidating the molecular mechanisms of recombination. The genetic control of recombination has been most extensively analyzed in *S. cerevisiae*. Numerous mutants affecting meiotic and mitotic homologous recombination in *S. cerevisiae* have been isolated and characterized, and several excellent, detailed reviews have been published (*6, 41, 119, 120*). Similarly, reviews have dealt in depth with the genetic control of nonhomologous recombination (end-joining) in mammalian cells (*121–123*). Homologs of mammalian nonhomologous recombination genes have been described in *S. cerevisiae* (*124, 125*). In the presence of homologous recombination, they play only a minor role in processing DSBs in *S. cerevisiae*. On the other hand, despite initial doubt that homologous recombination pathways in *S. cerevisiae* were even conserved in mammalian cells, mammalian homologs of *S. cerevisiae* homologous recombination genes have now been identified, and an understanding of the significance of homologous recombination for processing DSBs in mammalian cells is developing rapidly (reviewed in *126*).

We are interested in the genetic control of DSB-induced mitotic intrachromosomal recombination between direct repeats in *S. pombe* compared to *S. cerevisiae*, and this is the main focus of this section.

A. The Genetic Control of DSB-Induced Mitotic Recombination in *S. cerevisiae*

As discussed earlier (Section III,B,1), genetic and physical analyses of DSB-induced mitotic intramolecular recombination between direct repeats in *S. cerevisiae* suggested that there are at least two independent competing pathways with a common intermediate (*19, 28*). The two proposed pathways are based on a DSB–gap repair mechanism and the SSA mechanism. Additional evidence for at least two distinct pathways of homologous recombination between nontandem intramolecular repeats in *S. cerevisiae* comes from an examination of its genetic control.

The best-studied mutants affecting recombination in *S. cerevisiae* are those in the *RAD52* epistatic group, isolated mainly on the basis of their sensitivity to x-rays (reviewed in *6, 41, 119, 120*). The *RAD52* epistatic group in-

cludes at least eight genes: *RAD50*, *-51*, *-52*, *-54*, *-55*, and *-57*, *MRE11*, and *XRS2*. The *rad52* mutant has been extensively studied and manifests a pleiotropic phenotype with regard to deficiency in meiotic and mitotic DSB-induced homologous recombination involving a variety of substrates (*6, 41, 119, 120*). Several studies have shown that, for DSB-induced recombination between direct repeats, DSB–gap repair to give conversion-type products is *RAD52* dependent (*19–21, 72, 74, 76, 127*). The effect of *rad52* mutations on the formation of deletion-type recombinants during DSB-induced recombination between direct repeats is more complex: it occurs at a reduced efficiency in *rad52* cells but is *RAD52*-independent (*19–21, 72, 74, 76*). Experiments in which HO-induced DSBs were introduced into the ribosomal DNA cluster or an 18-fold repeated *CUP1* gene locus demonstrated that deletion formation, resulting in loss of one or more repeat units, can occur by a *RAD52*-independent mechanism (*76*). However, when the number of *CUP1* repeats was reduced from 18 to 3, the events were *RAD52* dependent. Consistent with the physical analyses, this suggested an additional pathway(s) for deletion formation. Surprisingly, although other members of the *RAD52* epistatic group play crucial roles in both meiotic and mitotic recombination, they are not required for both conversion-type and deletion-type product formation during DSB-induced recombination between direct repeats (*3, 127*). This was despite the observation that single-stranded DNA formation was slower in *rad50* cells than wild type (*20, 127*).

RAD1 and *RAD10* belong to the *RAD3* epistatic group and are required for nucleotide excision repair of ultraviolet (uv)-damaged DNA in *S. cerevisiae* (for a review see *128*). They have also been shown to have a role in mitotic recombination. Mutations in *RAD1* and *RAD10* decreased the efficiency of integration of circular and linearized plasmids (*129, 130*). They also reduced the frequency of recombinants, primarily deletion types, during spontaneous intrachromosomal recombination between direct repeats (*47, 129–132*). Analysis of double mutants showed that *RAD1* and *RAD10* functioned in the same recombination pathway. For *rad52–rad1* and *rad52–rad10* double mutants, the reduction in mitotic intrachromosomal recombination between direct repeats was greater than with the single mutants, suggesting that *RAD52* and *RAD1–RAD10* functioned in separate recombination pathways (*47, 129–132*). Together with the kinetic data on DSB-induced recombination between repeats (*19*), this suggested that *RAD1–RAD10* functioned in the SSA pathway. It was subsequently shown that *rad1* and *rad10* strains were deficient in DSB-induced repeat recombination in which the DSB was introduced by HO cleavage at HO recognition sequences within duplicated DNA. This was due to the inability to remove small regions of nonhomologous DNA (the HO recognition sequences) at the site of the induced DSB (*37, 133*). This suggested that *RAD1* and *RAD10* coded for an endonuclease

activity that was required to remove nonhomologous DNA from the 3′ ends of recombining DNA, a process required for SSA (Fig. 7) and analogous to excision of photodimers during repair of uv-damaged DNA. Subsequently, it was shown that Rad1 and Rad10 proteins form a complex that functions as a single-strand DNA-specific endonuclease that can cleave at a junction between duplex DNA and 3′ single-stranded DNA tails (*134*), which explains the role of these proteins in excision repair and for the removal of nonhomologous DNA during SSA. This function would be required for recombination between direct repeats if the initiating DSB was made in unique DNA between the repeats or in nonhomologous regions within duplicated sequences. The Rad10 protein has also been shown to promote renaturation of complementary DNA strands (*135*), so it may also function in other steps of SSA. Other NER genes (*RAD2*, *-3*, *-4*, *-14*, *-16* and *-25*) were not required for spontaneous intrachromosomal recombination (*129, 130*) or for removal of nonhomologous DNA during DSB-induced repeat recombination (*133*). However, it has been shown that two mismatch repair genes, *MSH2* and *MSH3*, are also required in the *RAD1–RAD10* pathway for spontaneous intrachromosomal recombination between direct repeats and for homologous integration of linearized plasmids (*136*). Mutations in two other mismatch repair genes, *PMS1* and *MLH1*, had no effect.

It is not clear how many pathways there are for DSB-induced intramolecular mitotic homologous recombination between direct repeats in *S. cerevisiae*, but studies indicate that there may be at least three pathways: a *RAD52*-dependent DSB–gap repair pathway, a *RAD1–RAD10*-dependent SSA pathway, and a third alternative pathway for the generation of deletion-type recombinants.

The *rad1–rad52* and *rad10–rad52* strains still exhibit a residual capacity for intrachromosomal mitotic recombination between direct repeats (*47, 129–132*). One possible explanation is that additional alternative recombination pathways exist. Studies on the genetic control of direct-repeat and inverted-repeat recombination in *S. cerevisiae* (*47, 137, 138*) suggest that, as well as a *RAD52*-dependent DSB–gap repair pathway for the generation of conversion-type recombinants and a *RAD1–RAD10*-dependent SSA pathway for the generation of deletion-type recombinants, there exists a third pathway based on nonconservative one-sided recombination events that requires *RAD52*, *RAD1*, and *RAD10* and generates deletion-type recombinants.

Using an approach similar to that taken to identify multiple recombination pathways in *Escherichia coli*, a mutation in the *RFA1* gene, a gene encoding a single-stranded DNA-binding protein, was isolated in a screen for mutations that increased intrachromosomal recombination between direct-repeats in a *rad52–rad1* background (*139*). The *rfa1* mutation on its own

caused an increase in recombination that was, unlike most other hyper-recombination mutants, independent of *RAD52* function. Additionally, the *rfa1* mutant strain was uv sensitive and exhibited decreased levels of inter-chromosomal mitotic recombination in diploids. These results indicate that *RFA1* may function in an alternative recombination pathway for direct-repeat recombination. Interestingly, a novel allele of *RFA1* was also isolated in a screen for mutants that decreased homologous recombination between plasmid and chromosomal sequences stimulated by an *in vivo* HO-induced DSB within the plasmid sequences (*140*).

Finally, a series of *S. cerevisiae* mutants has been isolated directly on the basis of a deficiency in intrachromosomal mitotic recombination between repeated *ura3*$^-$ heteroalleles stimulated by an *in vivo* HO-induced DSB within duplicated sequences (*141*). To date only one of these mutations has been described and was found to be an allele of the essential *CDC1* gene (*141*). The mutation completely eliminated DSB-induced recombination to yield Ura$^+$ recombinants, and this was shown not to be due to stimulation of sister-chromatid exchanges. The mutation also caused moderate sensitivity to methylmethane sulfonate and ionizing radiation, but did not affect spontaneous recombination or cell viability. Although the precise effect of the *cdc1* mutation on DSB-induced recombination is not known, its effect could be due to the blocking of the normal pathways of recombination to allow an alternative pathway (*141*).

B. The Genetic Control of DSB-Induced Mitotic Recombination in *S. pombe*

Intrachromosomal direct-repeat recombination substrates have been used to investigate the genetic control of DSB-induced mitotic recombination in *S. pombe*. Earlier studies had shown that these substrates undergo gene conversion and deletion events by the DSB–gap repair and SSA pathways of recombination, respectively (*84*). While recombinants generated by the DSB–gap repair pathway in *S. cerevisiae* are *RAD52* dependent, the production of similar recombinants in *S. pombe* is independent of *rad22* (homolog of *S. cerevisiae RAD52*) (*142*). Similarly, while the production of deletion-type recombinants in *S. cerevisiae* is dependent on the *RAD1–RAD10* genes, the generation of analogous recombinants in *S. pombe* is independent of the *S. pombe rad10* (homolog of *S. cerevisiae RAD1*). Neither the deletion nor the gene conversion events were affected by mutations in the *S. pombe rad5* (homolog of the *S. cerevisiae RAD3* gene), *rad21*, *rad1*, or *rad3* genes (*142*). These results suggest that, although the pathways of DSB-induced recombination may be similar in *S. cerevisiae* and *S. pombe*, their genetic control is likely to be different. In this context, it is worth noting that the gene for a uv endonuclease has been cloned from *S. pombe* (*143*). This endonuclease, like the *S. cerevisiae* Rad1–Rad10 proteins, cleaves 5′ to the uv damage, and might function in intrachromosomal recombination in an *S. pombe rad10* mutant.

We have isolated a number of mutants deficient in intrachromosomal, DSB-induced mitotic recombination in *S. pombe*. The analysis of the phenotypes of these mutants and the genes that complement them should yield additional insights regarding the DSB–gap repair and SSA models of recombination.

V. Concluding Remarks

It should be evident from the preceding account that considerable progress has been made in the last decade in elucidating the general mechanisms by which mitotic recombination proceeds in lower and higher eukaryotes. There is evidence for multiple pathways that appear to be common among all eukaryotes, but their prevalence and genetic control might vary from organism to organism. In the near future, most of the progress will come in the identification and characterization of the genes and proteins involved in these processes. The yeast systems are likely to be at the forefront because they are amenable to genetic screens for recombination-deficient mutants, and these mutant phenotypes can be complemented easily with DNA libraries. A complete understanding of the enzymatic activities of the proteins involved, and of the possible redundancies, in these pathways will keep investigators in this field busy for some time to come.

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