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A Life Investigating Pathways That Repair Broken Chromosomes

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Abstract

Double-strand breaks (DSBs) pose a severe challenge to genome integrity; consequently, cells have developed efficient mechanisms to repair DSBs through several pathways of homologous recombination and other nonhomologous end-joining processes. Much of our understanding of these pathways has come from the analysis of site-specific DSBs created by the HO endonuclease in the budding yeast *Saccharomyces cerevisiae*. I was fortunate to get in on the ground floor of analyzing the fate of synchronously induced DSBs through the study of what I coined "in vivo biochemistry." I have had the remarkable good fortune to profit from the development of new techniques that have permitted an ever more detailed dissection of these repair mechanisms, which are described here.

INTRODUCTION

Double-strand breaks (DSBs) of eukaryotic chromosomes pose a severe threat to cell viability. DSBs can be repaired in several ways, including adding new telomeres to a broken end, ligating the broken ends by one of several nonhomologous end-joining (NHEJ) pathways, or using homologous sequences located elsewhere in the genome to patch up the lesion. In eukaryotes, the foundation for studying DNA repair of DSBs came from radiobiology, through exposing cells to X- or γ-rays and identifying mutants, both in simple model organisms such as the budding yeast *Saccharomyces cerevisiae* and in mammalian cells, that rendered cells sensitive to these assaults. Subsequently, radiomimetic chemotherapeutic agents and clastogens such as bleomycin, phleomycin, zeocin, and methyl methane-sulfonate were added to the arsenal. Additional important insights came from studies of repair in bacteria, notably *Escherichia coli* and its bacteriophages, where again it was possible to identify mutations that caused both radiosensitivity and deficiencies in recombination, as well as defects in phage propagation.

Biochemical analysis of some key recombination proteins began with bacterial proteins, the recombinase RecA and its cofactors. Mutations in RecA were first identified in 1969 (152) and the basic RecA strand exchange activity was first described in 1979 (93, 101, 119). Mutations in RecA's eukaryotic homolog, Rad51, were found in budding yeast in 1974 (29), but it was not until 1992 that yeast Rad51 was definitively identified as the eukaryotic homolog of RecA and not until 1994 that its biochemical activity was established (1, 134).

Research into DSB repair was guided by insightful molecular models of DNA repair that reflected the evolving understanding of recombination and repair in several model organisms, including bacteria and various fungi. The key concepts of recombination were laid out by Robin Holliday, who envisioned the exchange of single strands of DNA to form heteroduplex DNA (43). Holliday also envisioned the necessary resolution of the four-armed branched DNA recombination intermediate structures we now call Holliday junctions.

Holliday's model and the refinements suggested by Meselson & Radding (95) imagined that the initiating lesions for recombination were single-strand nicks, but it soon became clear that most recombination was initiated by DSBs. These ideas were first laid out by Michael Resnick (110) on the basis of the repair of damage from ionizing radiation, but such mechanisms became widely appreciated with the description of a DSB model of recombination articulated by Szostak et al. (135). Their model was motivated by experiments using linearized plasmid DNA that was transformed into budding yeast, where it would recombine and integrate selectively at a site homologous to the plasmid's DSB ends, but it was also influenced by the discovery that a programmed genomic rearrangement in budding yeast, *MAT* gene switching, also involved a DSB (125).

My involvement with DSBs began with budding yeast *MAT* switching after learning about it from Osamu Takano, who was visiting Brandeis in Harlyn Halvorson's laboratory in 1972. Takano and his remarkable mentor, Yasuji Oshima, had already published two insightful papers in 1970 and 1971, respectively, describing how *MAT***a** cells carrying the *HO* gene could switch to *MAT*α and back to *MAT***a** as often as once every generation (103, 136). These events required two donor loci that we now call *HML*α and *HMR***a**. Takano and Oshima invoked Barbara McClintock's notion (92) of controlling elements that would transpose from *HML*α or *HMR***a** and cause a change of mating type. The idea that *MAT* switching involved "jumping genes" was stunning, and I decided to investigate this fascinating subject when I set up my laboratory in 1972.

As I have recounted elsewhere in more detail, because I had almost no training in genetics, I stumbled my way into this field (32). Looking for mutants defective in *MAT* switching, I isolated a single allele of *swi1*, a gene controlling the expression of the HO endonuclease, and one allele of *chl1*, the first chromosome loss gene (34, 77). Had I been properly schooled, it is likely that

either I would have isolated many alleles of *swi1*, *swi2*, *swi3*, *swi4*, and so on and ended up studying chromatin remodeling and gene regulation, or I would have recovered alleles of the more than 20 other *CHL* (also known as *CTF* or *CIN*) genes and directed my studies toward chromosome segregation. At the same time, the course I was teaching compelled me to understand meiotic as well as mitotic recombination, and it became clear to me that *MAT* switching was some sort of gene conversion mechanism. *MAT* switching depends on the *RAD52* gene, which is required for most homologous recombination (HR) and for survival after X-irradiation. We had identified several inconvertible (*inc*) or stuck (*stk*) mutations in *MAT* that prevented or impaired switching and showed that these mutations prevented *HO rad52* cells from dying. We isolated survivors of *HO rad52* that all proved to have mutations—deletions—in *MAT*. In collaboration with one of the pioneers of DNA sequencing, Michael Smith, we identified single base pair mutations in *MAT* that proved to lie within the HO endonuclease cleavage site (147), but we did not sequence the deletions (sequencing was far from easy in 1983). It was not until 10 years later that my laboratory used similar deletions to define several NHEJ pathways (66, 99).

The big breakthrough in showing that *MAT* switching was indeed an example of DSB repair came from Jeff Strathern, Amar Klar, and Jim Hicks, who had cloned *MAT* and shown that *MAT* switching involved the formation of a DSB (125). Rich Kostriken and Fred Heffron then demonstrated that the HO protein was indeed a site-specific endonuclease (65). Soon thereafter, Kim Nasmyth showed that *SWI1* controlled the expression of HO (100). Finally, Rob Jensen & Ira Herskowitz created a galactose-inducible version of HO that provided the key to virtually all the work my laboratory and others carried out for the next 30 years (59).

Site-specific endonucleases have proved to be invaluable tools in the elucidation of DNA repair mechanisms, not only in budding yeast but in many model organisms and recently in mammalian cells. Here I focus on what we have learned from HO and extend these lessons to results with other site-specific endonucleases. I have often characterized our work as in vivo biochemistry, but at almost every step, our progress depended on the contributions made by real biochemists who do not always receive the credit they deserve in this review.

HO ENDONUCLEASE AND *MAT* SWITCHING IN BUDDING YEAST

The molecular and genetic analysis of the switching of *MAT* alleles has provided much of the foundation of what we know about DSB-induced gene conversion in budding yeast and, by extension, in other eukaryotes. This subject has been extensively reviewed (33, 38, 67); here, I summarize the main conceptual advances without crediting every step along the way. The *MAT* locus can express two distinct pairs of alleles that are encoded by entirely different DNA sequences. *MAT***a** encodes the Mat**a**1 corepressor protein and a second, poorly characterized open reading frame, Mat**a**2. *MAT*α encodes the Matα1 transcriptional activator of α-specific genes and Matα2, a repressor of **a**-specific genes that also acts with Mat**a**1 as a corepressor of haploid-specific genes (**Figure 1**). In homothallic (*HO*) strains, *MAT***a** can be replaced by *MAT*α, or vice versa, as often as once every cell division, in a pattern that ensures that adjacent **a**- and α-mating cells will conjugate to form diploids, after which switching is repressed by the Mat**a**1-Matα2 corepressor. The replacement of 650 bp of *MAT***a** sequences with 750 bp of *MAT*α occurs by DSB-mediated gene conversion, initiated by HO endonuclease. HO recognizes and cleaves a degenerate 24-bp site, cutting either *MAT***a** or *MAT*α close to the junction of the mating-type-specific (Y**a** or Yα) sequences and the Z region shared by *MAT* and its donors. Repair of the break involves HR with one of two donors, *HML*α or *HMR***a**. These donor regions are maintained as heterochromatic and transcriptionally silent sequences that share different extents of homology with *MAT*. The use of a galactose-inducible *HO* gene makes it possible to create a DSB rapidly in all cells in the

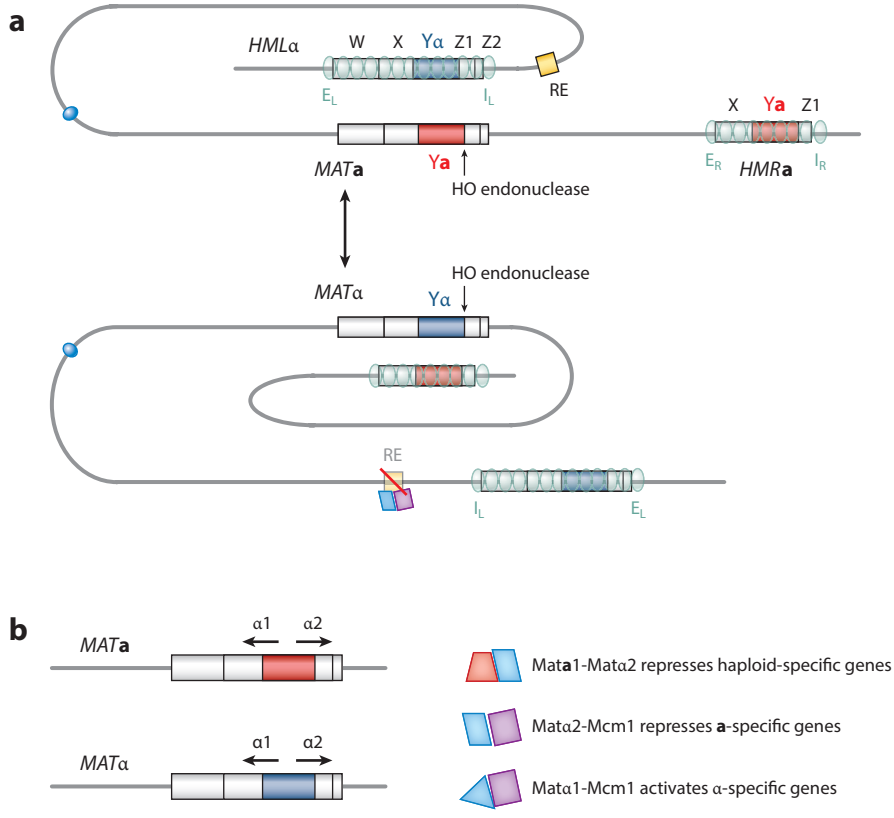

Figure 1

Mating type in *Saccharomyces cerevisiae*. (*a*) Mating type is determined by the Ya (*red*) and Yα (*blue*) regions of the *MAT* locus in the middle of chromosome 3. Two heterochromatic donor loci, *HML*α and *HMR*a, are located near the left and right telomeres, respectively; their silent state is maintained by the Sir2 histone deacetylase complex between the E and I regions surrounding the donors. Switching between *MAT*a and *MAT*α is catalyzed by the site-specific endonuclease, leading to double-strand break (DSB) repair by gene conversion. Donor preference is determined by a *cis*-acting recombination enhancer (RE; *yellow*) close to *HML* that positions *HML*α near the DSB. RE is inactivated in *MAT*α cells. (*b*) *MAT*a encodes two open reading frames that are distinct from the two transcripts in *MAT*α. Mating type–dependent regulation of a-, α-, and haploid-specific genes is enforced by combinations of the Mata1 and Matα2 corepressors with the Mcm1 protein or Matα1 transcriptional activator, again with Mcm1.

population and then to use Southern blot, polymerase chain reaction (PCR), or other assays to monitor the kinetics of repair and identify the key intermediates in DSB repair. *MAT* switching is surprisingly slow, taking an hour or more for the new *MAT* allele to be detected. The process consists of a number of slow steps (**Figure 2**).

5′ to 3′ Resection of the Double-Strand Break Ends

Essentially all HR takes advantage of several types of nucleases that preferentially remove one strand of the DSB in a 5′ to 3′ direction. White & Fox (151) had interpreted phage λ recombination in terms of a 3′-ended single-strand tail, but White & Haber (150) first detected single-stranded DNA (ssDNA) as a recombination intermediate by using Southern blots after denaturing gel

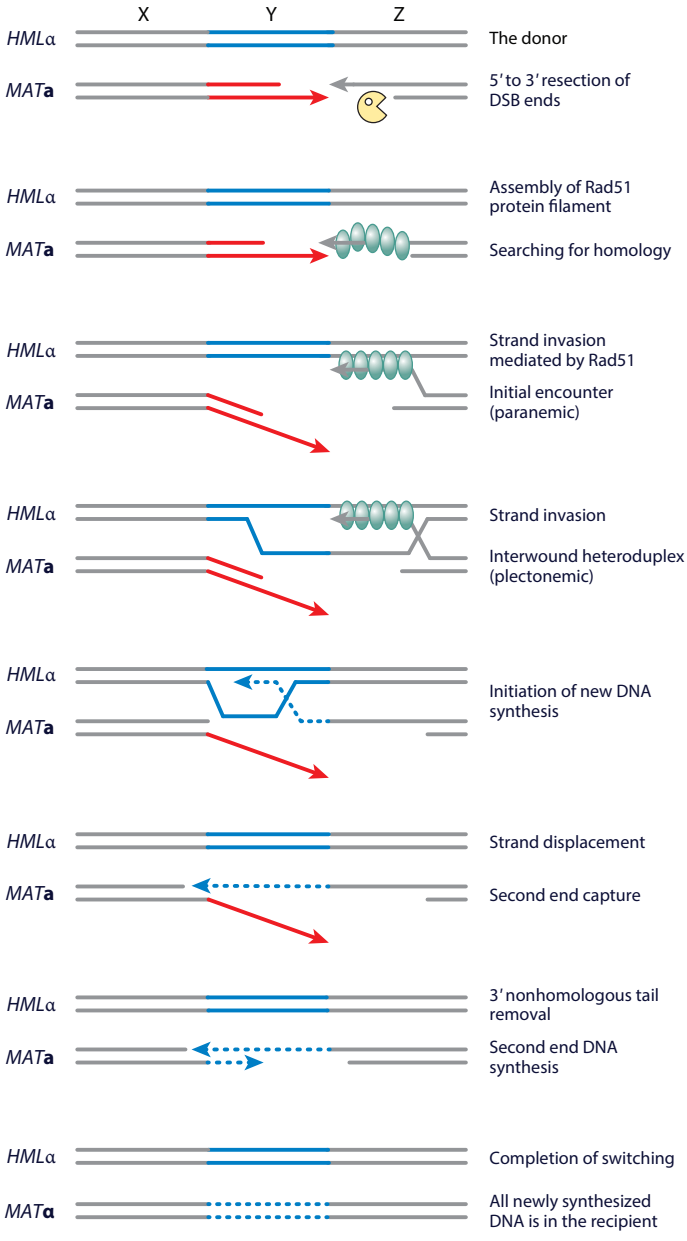

Figure 2

Steps in the switching of *MAT***a** to *MAT*α using *HML*α as the donor. Abbreviation: DSB, double-strand break.

electrophoresis. Because most restriction enzymes cannot cleave ssDNA, novel restriction fragments appear, representing instances in which first one and then another site is rendered single-stranded. Resection in budding yeast is slow at approximately 1 nt/s but also inexorable (27). In the absence of repair of a DSB (e.g., when *HML* and *HMR* are deleted or when *RAD52* is deleted),

resection can be detected even with native Southern blots by the progressive loss of restriction fragments as far as 50 kb from the DSB (161).

Several other assays have more recently been developed that provide a more quantitative estimate of resection rates by the progressive loss of sequences (128). The most quantitative assay was developed by Zierhut & Diffley (162), who used PCR primers that span a convenient restriction site (**Figure 3**). Soon after HO endonuclease induction, DNA is fully cleaved and there is no PCR product. However, when resection removes one strand, the DNA is no longer cleaved and a PCR

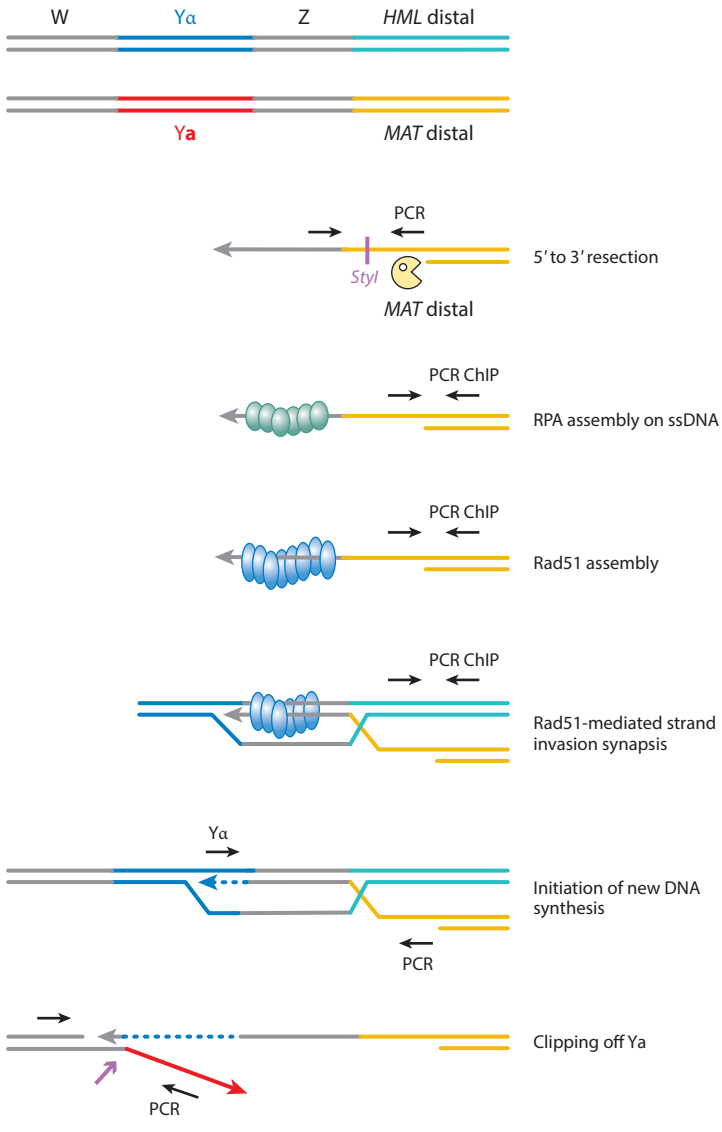

Figure 3

Assays for intermediates arising during *MAT* switching. Abbreviations: ChIP, chromatin immunoprecipitation; PCR, polymerase chain reaction; RPA, replication protein A; ssDNA, single-stranded DNA.

product appears, amplifying the remaining ssDNA and providing a positive signal for resection rather than one measuring loss. This assay has also proved valuable in evaluating resection in mammalian cells (160).

We now know that the DSB ends are processed by three different complexes: (*a*) the exo/endonuclease Mre11-Rad50-Xrs2 (MRX), associated with Sae2, whose precise role continues to be debated; (*b*) the exonuclease Exo1; and (*c*) the helicase/endonuclease Sgs1-Rmi1-Top3-Dna2 (30, 52, 96, 161). In vitro, Mre11 exhibits 3′ to 5′ and endonuclease activity, and it has been argued that the MRX complex initially creates a 3′-ended ssDNA tail by resecting 5′ to 3′ from a nick toward the DSB end; this is similar to Mre11's role in processing meiotic DSBs, where the Spo11 protein is covalently attached to the 5′ end of a DSB. Deletion of any of the MRX genes or Sae2 slows down resection, although the effect is seen principally near the DSB. In logarithmically growing cells, the absence of one of the MRX proteins reduces resection a few kilobases from a DSB by approximately 50%, but in G2/M-arrested cells, the absence of these proteins blocks all resection (20). Under this specific condition, then, MRX is required for any further resection, but this does not seem to be generally the case. Nevertheless, MRX is frequently represented at the apex of a resection process. The absence of Sae2 does not prevent resection in G2/M-blocked cells.

In G1-arrested cells, where the B-type cyclins that in combination with the Cdk1 kinase regulate progression through the cell cycle are turned off, there is also no resection (5, 51). Indeed, it seems that several key proteins must be phosphorylated to have proper activity. One such protein is Sae2, in which a phosphomimetic mutant allows at least some resection in Cdk1-inhibited cells (46). Sae2's homolog in mammals, CtIP, plays a central role in resection (47). Dna2 is also phosphorylated by Cdk1 (10). However, these modifications are not essential for resection, because deleting one of yeast's Ku proteins provides access for Exo1 to promote resection sufficient to allow efficient *MAT* switching in G1-arrested cells (97).

One surprising aspect of the resection studies is that the 3′-ended ssDNA tail is remarkably stable; apparently, there are no 3′ to 5′ ssDNA exonucleases in the nucleus. However, secondary structures can form that can result in degradation, but this is prevented by the single-strand binding protein complex replication protein A (RPA) (9). The apparent useful lifetime of ssDNA, for example, the period of time it can participate in ectopic recombination, can be extended by overexpressing RPA (70).

Formation and Function of the Rad51 Filament

Once 3′-ended ssDNA is generated, it can be incorporated into a Rad51 nucleoprotein filament that is the machine that searches for homologous sequences elsewhere in the genome and facilitates strand invasion and the initiation of repair. The loading of Rad51 onto the DSB end can be monitored by chromatin immunoprecipitation (ChIP), illustrated in **Figure 3** (131, 145, 155). Surprisingly, there is an approximately 10-min delay in robust recruitment of Rad51 onto the DSB end after the DSB itself is apparent. During this time, it appears that the ssDNA binding protein complex, RPA, first binds and is then displaced by Rad51 in a process that, based on in vitro biochemical studies, requires the mediator Rad52 and the Rad51 paralog heterodimer Rad55-Rad57 (39, 78, 132). Recent work from Wolf-Dietrich Heyer's laboratory has suggested that Rad55-Rad57 heterodimer is itself incorporated into the predominantly Rad51 filament. HO-induced recombination fails without Rad55 or Rad57 (78), although curiously, repair (presumably by sister chromatid recombination) of X-rays is competent at 30°C without Rad55-Rad57. At 18°C, X-rays can be repaired only if Rad55 and Rad57 are present, Rad51 is overexpressed, or Rad51 carries mutations that improve its activity (80, 86).

Much of what we know about the way Rad51 facilitates strand invasion has come by analogy with the revelatory X-ray crystallographic analysis of the bacterial RecA protein carried out by Nikola Pavletich's laboratory (11). RecA and, by inference, Rad51 contain a principal ssDNA binding site and a secondary site that binds double-stranded DNA (dsDNA). A monomer binds 3 nt and stretches both ssDNA and dsDNA to 150% of B-form DNA length. Surprisingly, the three bases within a monomer have an almost B-form configuration and most of the stretch occurs between the triplets. Mutation of three residues at site II allows strong binding of Rad51 to ssDNA but prevents the nucleoprotein filament from carrying out strand exchange (14).

Recently, the laboratories of Patrick Sung and Eric Greene have collaborated to examine RecA and Rad51 homology recognition by single molecule techniques (69). They find that these proteins require eight consecutive base-pairings to achieve minimal stable homology recognition. Base-pairings proceed in triplets. A similar conclusion has been reached by Mara Prentiss and her colleagues for RecA, although the two approaches have reached different conclusions in terms of how single mismatches impair progression of strand exchange (158).

Homology Searching and Synapsis with a Donor

Once the Rad51 filament has formed, it can search for homology with a sister chromatid, with a homologous chromosome, or at an ectopic site, as it does in *MAT* switching. *MAT* shares only approximately 230-bp homology on one side of the DSB with *HMR*, but repair is nearly 100% efficient. In fact, intrachromosomal repair can occur with as little as 50- to 70-bp homology (49). Interchromosomal ectopic recombination is significantly slower and less efficient than intrachromosomal events (70); improvements in the efficiency of repair can be observed when the amount of homology on either side of the DSB is increased, up to approximately 3 kb.

Using an antibody against Rad51, ChIP again makes it possible to detect when Rad51 makes contact with a homologous donor sequence (**Figures 2** and **3**), presumably at the point when strand exchange is initiated (131, 145, 155). The kinetics of homology searching are slow, taking 15 min even in the case of *HML* pairing with *MAT*. This encounter between *MAT* and *HML* is exceptional because it is facilitated by a recombination enhancer (RE; discussed in more detail below). When RE is deleted, the time for the Rad51 filament to synapse with HMLα donor sequences, as observed by ChIP, increases to 30 min, and the time it takes for Rad51 to synapse with an ectopic donor is even longer.

Stefan Jentsch's laboratory used ChIP to demonstrate that Rad51 preferentially explores intrachromosomal sites, most likely by a series of collisions dictated by the persistence length of the Rad51 filament and the adjacent chromatin rather than a progressive sliding along the DNA (109). These data support many studies showing that the kinetics of intrachromosomal repair are significantly faster than those of interchromosomal repair. Interestingly, the timing of repair is closely monitored by the DNA damage checkpoint. Whereas *MAT* switching itself does not trigger Mec1 (ATR)-dependent phosphorylation of Rad53 (Chk2), interchromosomal repair using the same sequences is notably slower and provokes Rad53 phosphorylation (63).

A number of chromatin remodelers are needed during strand invasion and repair, but currently the list is incomplete. There is a suggestion that the Swi2/Snf5 chromatin remodeler is required for strand invasion, but because deleting these proteins prevents galactose-induced HO expression as well as normal HO expression, further investigation is needed. Similarly, the Swi/Snf homolog Rad54 is required to complete *MAT* switching, even when the donor is not heterochromatic (7, 41). Micrococcal nuclease has been used to analyze changes in the nucleosome positioning during strand invasion of *HML*. When the subsequent steps of new DNA synthesis are delayed, it is possible to see that Rad54 is required for the chromatin rearrangements associated with

strand invasion. Surprisingly, Rad54 is not needed for strand invasion itself, as seen by ChIP experiments, although the nature of the pairing between the invading strand and the donor might be a side-by-side (paranemic) rather than interwound (plectonemic) joint (41) (**Figure 2**). The loss of nucleosome positioning seems to reflect the changes needed to initiate new DNA synthesis, which is absent in a *rad54*Δ mutant.

An area of continuing interest is how homology searching is coordinated between the two ends of the DSB and whether the ends become more mobile after induction of a DSB. If the two sides of a DSB are fluorescently labeled by placing LacO and TetO arrays on either side and expressing LacI- and TerR-fluorescent proteins, the two ends do not behave independently. Their apparent tethering is only partially reduced by eliminating the end-tethering MRX complex or other recombination proteins (61, 79). Our recent genetic results support the notion that normally the two ends of a DSB behave as if they were tethered to each other during the search process, as determined by comparing the kinetics of HO-induced DSB repair when the two ends, LE and U2, are derived from a single site (in *cis*) or are created at two different locations (in *trans*), in each case using a third chromosome location as the *LEU2* template for repair (54). The slower kinetics of the *trans* case depend on the fact that the opposite ends of the two DSBs are themselves searching for homology (**Figure 4**).

When a fluorescently tagged region near a DSB is followed microscopically, its mobility (measured by mean squared displacement) and its plateau (the radius of confinement) are much greater than before DSB induction. This has led to the suggestion that the DSB makes the ends more mobile (21, 98). There is a much smaller effect on unbroken chromosomes. However, two recent studies have suggested that this increase in mobility does not improve the ability of a distant donor locus to repair a DSB, for example, in competition with an intrachromosomal donor (70, 126). In fact, much of the increase in mobility can be attributed to DNA damage checkpoint kinase phosphorylation of a key kinetochore protein, Cep3, and the untethering of telomeres from the

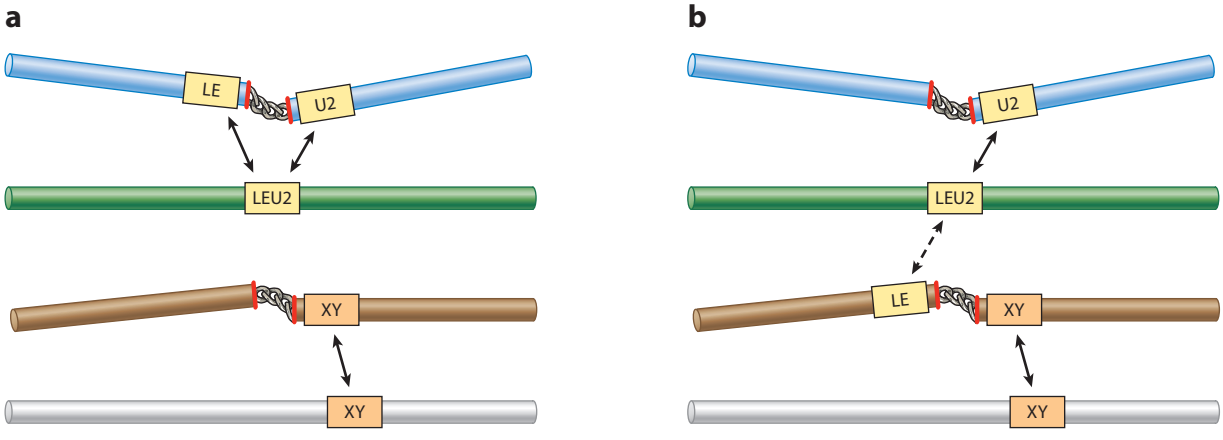

Figure 4

The ends of a double-strand break are coupled. When the chromosomes of budding yeast suffer two independent, site-specific double-strand breaks (DSBs; *red ends*), the breaks can be repaired by homologous recombination, using an ectopic template. (*a*) When the two ends of a single DSB (LE and U2) are homologous to a LEU2 template on another chromosome (ends in *cis*), repair occurs by gene conversion to patch up the break. (*b*) A second break has no influence on the outcome. However, if the same repair event (LE and U2 locating and repairing with a LEU2 template) occurs when the homologous ends come from two different breaks (ends in *trans*), the efficiency and kinetics of the repair can be greatly affected by the competing repair events involving the other ends of the two breaks, illustrated here by recombination between XY sequences. Thus, the ends of a single DSB appear to be linked and to act coordinately.

nuclear periphery (126). However, there is much to be explored here; the centromere/telomere modifications do not explain why mutations in a number of recombination proteins (Rad51, Rad54, Sae2, or chromatin factors such as Htz1 or Ino80) should reduce this increase in mobility. When DSB ends remain unrepaired, they become localized to the nuclear periphery, which might facilitate their end-joining, as Ku proteins and perhaps other NHEJ factors are normally found at telomeres (45).

Initiation of New DNA Synthesis

Strand invasion is followed by the initiation of new DNA synthesis (**Figures 2**, **3**). It is possible to detect this step by using a pair of PCR primers, one in the Yα segment of *HMLα* and one distal to *MAT***a**. Initially, there is no PCR product, but once 50 bp of new DNA is synthesized, there is an intermediate of repair that can be recognized by both primers (**Figure 2**). Hence, the beginning of new DNA synthesis can be detected (150). This intermediate arises only approximately 15–20 min after strand invasion is detected (by Rad5 ChIP) and points to a complex series of steps that prepare the 3′ end to act as a primer for new DNA synthesis. The alterations to the Rad51 filament or the recruitment of a DNA polymerase, most likely DNA Polδ, has not yet been carefully analyzed. This primer-extension assay revealed a remarkable delay of several hours in the initiation of break-induced replication (BIR) compared to gene conversion (55, 88). We believe that this delay reflects the surveillance of a recombination execution checkpoint (REC), which determines whether the two ends of a DSB are synapsed to the same template, within a distance of less than 2 kb and in opposite orientation (55). Recent studies by Suvi Jain, Neal Sugawara, and colleagues in my laboratory have implicated the Sgs1 and Mph1 helicases in enforcing this REC checkpoint. When both helicases are deleted, the kinetics of new DNA synthesis are as fast for BIR as for gene conversion (56). Interestingly, the REC is not activated if the two ends of a gene conversion event come from two different DSBs (54).

The use of temperature-sensitive (ts) mutations has made it possible to ask which parts of the normal replication machinery are required for mitotic gene conversion. As noted above, for cells arrested prior to the "start" point of the cell cycle, for example with a ts mutation of *CDC28*, *MAT* switching is blocked because Cdc28 is needed to initiate 5′ to 3′ resection. *MAT* switching does not require that cells be in S phase; recombination can be induced in G2/M-arrested cells or in cells blocked prior to S phase by inactivating Cdc7, when the cells have progressed beyond "start." Gene conversion does not require the Cdc45-MCM-GINS replicative helicase or most of the proteins needed for the initiation of normal DNA replication (83). However, some proteins used in establishing a replication fork are required, including Dpb11, Sld2, and Sld3 (41). The analysis of ts mutants also established that the lagging strand Polα-primase complex was not required (44, 146) (but see Was Allyson Holmes Right? below). Either DNA Polδ or Polε can apparently carry out the DNA synthesis steps, but several lines of evidence point to Polδ and the primary repair polymerase (90). Neither the bypass DNA polymerase Polζ nor Polη is required for *MAT* switching, but there is strong evidence that the error-prone Polζ is important (but not required) for the filling-in of single-strand regions associated with resection beyond the regions of shared homology between *MAT* and its donor, as there is a highly elevated rate of mutation in such regions in the presence of Rev3 (Polζ) (42).

The proliferating cell nuclear antigen (PCNA) clamp is required for *MAT* switching, but the clamp does not seem to be very efficient in the absence of the normal arrangement of proteins in the replication fork. There is a 1,000-fold increase in mutations associated with *MAT* switching (albeit the rate is still in the range of 10^{-5}), and many of these events appear to result from polymerase losing its place (40). The elevated rate of single base pair substitutions is independent

of the mismatch repair protein Msh2. This may indicate that the mismatch repair machinery, which is tightly coupled to a complete replication fork, is absent in DSB repair. Approximately 40% of the repair-induced mutations reflect some sort of template switching in which there must be a dissociation of the replicating polymerase from its template. The most striking of these events are dissociations of the partially copied strands, resulting in interchromosomal template switches between highly diverged sequences (140). There must be two jumps, into the distant sequence and then back to the original *HMR* template, to complete switching. If the second ectopic template is 100% identical to the sequences within *HMR*, 1 in 300 events includes this pair of template jumps! How often the polymerase jumps once and fails to return can only be surmised. Whether these jumps require Rad51 is not known, but the kinetics of these two-jump template switches occur with approximately the same kinetics as a simple gene conversion, so there is not the long delay seen in the initial Rad51-mediated search for a partner. Although the chromatin remodeler Rdh54 (Tid1) has no effect on a simple *MAT* switch, its deletion profoundly reduces interchromosomal template jumps (140). At which step Rdh54 is acting is not yet evident, but Rdh54 is also important for template jumps occurring in BIR (4).

Was Allyson Holmes Right?

As noted above, we initially concluded that DNA Polα-primase was required for *MAT* switching but later decided that this conclusion was in error. Our first experiments, carried out by Allyson Holmes (44), involved arresting growing cells at 25°C by raising ts mutants of Polα or primase to their restrictive temperature of 37°C and then inducing HO expression. *MAT* switching was blocked. However, later, Xuan Wang and colleagues (146) repeated this experiment by first arresting cells in nocodazole and then raising the cells to 37°C. Here, when cells had already completed normal replication, *MAT* switching was efficient in the absence of Polα and primase. We concluded that when DNA polymerase or primase was inactivated and cells were arrested at the beginning—or in the midst—of replication, some other replication factor necessary for *MAT* switching must have been sequestered. When these proteins were inactivated in nocodazole-arrested cells, however, this same factor must have been available. What that factor might be has not been identified.

But was Allyson right? If Polα-primase were needed, it would suggest that our assumptions that a 3′ end of an invading strand can serve directly as a primer might not be right. Perhaps new DNA synthesis is a form of replication restart, in which Polα-primase is needed to initiate new synthesis, as has been suggested for bacterial replication (159). Ligation of the Polα-initiated DNA to the invading 3′ end might be a slow step that would explain why the initiation of new synthesis appears to take approximately 20 min from the time strand invasion can be detected. Perhaps there was no mysterious other component trapped in S phase. Instead, perhaps the inactivation of Polα and primase ts mutants in nocodazole-arrested cells is, for some reason, less efficient than their denaturation and/or degradation in cycling cells? It seems important to revisit this question. One approach that we are taking uses auxin-inducible degrons that could be added to the ts alleles in Polα or primase to more completely deplete a ts-inactivated protein.

Rapid and Directional Mismatch Repair of Heteroduplex DNA

An *stk* mutation in the Z_{11} position within the HO cleavage site (8 nt from the HO-cut end) severely reduces HO cleavage of *MAT***a** but abolishes cleavage in *MAT*α. Induction of HO cleavage of *MAT***a**-stk resulted mostly in colonies with *MAT*α, erasing the *stk* mutation; however, in a *pms1* mutation, eliminating mismatch repair, 75% of the switches became sectored; that is, half the

offspring from a single switched cell were *MAT*α and the other half *MAT*α-inc (107). Regardless of whether the *stk* mutation was corrected to the wild-type sequence in *HML*α or remained as it was, the copying of the displaced first strand (which is the current model) to generate the second strand of the switched product should have yielded an unsectored outcome. Sectors imply that the second strand synthesis might have copied the donor locus rather than the newly copied first strand, leaving a mismatch at Z_{11}. Twenty years later, this result still confuses. One reasonable explanation, I think, is that some gene conversion events proceed via the double-Holliday junction (dHJ) pathway (50, 135). In this case, the two strands that anneal by dissolving the dHJ (156) will have independently copied the donor template and one of them could retain the *stk* mutation. But there is still an issue: For this to be the explanation, a minimum of 75% of the events in *pms1* would now have to be derived from this pathway. So, I am still confused. The *pms1* strain might also slow down repair so that what was presumed to be a single DSB repair event might have taken place after replication.

But in any case, Pms1 is needed to resolve a mismatch, presumably when the invading (Stk-containing) single strand creates a D-loop and a heteroduplex DNA segment with the donor. By sequencing the primer extension PCR product described above, we showed that mismatch repair was very rapid, occurring in the interval between strand invasion and the appearance of the PCR-amplifiable intermediate (35). These data also showed that heteroduplex DNA was repaired in a highly directional fashion, so that the base on the invading strand was corrected to the complementary base of the unbroken resident strand at *HML*.

Our recent studies have suggested that most mismatch correction is actually carried out by the 3′ to 5′ resection activity of the proofreading domain Polδ, which chews away the mismatch and then copies the donor sequence (R. Anand, unpublished observations).

Recently, Olga Tsaponina (140) examined mismatch repair in the interchromosomal template switching between 72% identical *URA3* sequences. In her experiments, the *HMR* donor contains a 32-bp deletion within the *Kluyveromyces lactis URA3* gene that is the only donor to be copied into *MAT*. The only way to create a Ura^+ cell is via a pair of template jumps to copy the region that includes the missing 32-bp region from the *S. cerevisiae ura3-52* locus on another chromosome. Deleting mismatch repair genes had little effect on the overall rate of such events but revealed an unexpected outcome. In the absence of Msh6, some Ura^+ events proved to have corrected the 32-bp deletion at *HMR*. These are the first events we have ever seen where the donor template is converted; they suggest to us that Msh6 plays a role in specifying whether the donor or recipient strand should be mismatch corrected. All of the conversion events depended on Msh3, which is needed for gene conversions involving large heterologies such as the 32-bp region. It should be profitable to explore how Msh6 is specifying invading and resident strands, as this is reminiscent of the question of how eukaryotes specify old and newly synthesized strands; for example, would an *msh6* mutation allow the *stk* mutation to end up in the donor template?

Second End Capture and Clipping of the Nonhomologous Y Region

Before *MAT* switching can be completed, the elongating strand must anneal with the opposite end of the DSB, but, in addition, the nonhomologous Y**a** tail must be removed before the second strand of new DNA synthesis can occur. We showed that removal of this nonhomologous tail requires the Rad1-Rad10 endonuclease (26). Soon thereafter, Rad1-Rad10, which was known to cleave one side of a mismatch in nucleotide excision repair, was shown to be a flap endonuclease, as we had predicted (139). Tail removal most likely requires the same factors (Saw1, Slx4, and to a lesser extent, Rad59 and Msh2-Msh3) that are needed to remove nonhomologous tails during single-strand annealing (SSA), but these have not been examined in *MAT* switching. In

the absence of Rad1, some *MAT* switching still occurs by a ligation between the newly copied strand and the resecting end of the DSB, but the 3′-ended strand of the second end still retains the nonhomologous sequences and normal repair cannot be completed. Thus, after the next round of DNA replication, one daughter cell is viable and has switched, whereas the second daughter cell is inviable (84).

Excision of the Y**a** strand can also be monitored by PCR. The level of PCR product drops to 50% when the first strand is resected, but further loss is delayed until the time of tail removal (41). This step only occurs at the time of apparent second-end capture.

Completion of Repair

Completion of repair, after removal of the nonhomologous tail, apparently occurs by primer extension of the second 3′ end. This step occurs quite soon after removal of the nonhomologous tail that blocks primer extension, but the details of these last steps are lacking. One curiosity is that the final ligation steps, joining the extended strands to the resecting ends of the DSB, have not been defined. Yeast supposedly has only two DNA ligases, ligase 1, which is active in normal DNA replication to join Okazaki fragments, and ligase 4, which is used in NHEJ. However, in a strain deleted for ligase 4 (Dnl4) and a ts mutation of ligase 1 (*cdc9-1*) that has been reported to block Okazaki fragment ligation, *MAT* switching yields apparently intact and ligated products, as seen in Southern blots of denaturing gel electrophoresis (146). There is possibly sufficient remaining residual, active Cdc9 to carry out this step, analyzed in G2/M-arrested cells, or perhaps there is another ligase.

A *MAT*-Specific Recombination Enhancer

MAT switching in general appears to be quite similar to DSB repair events induced in different sequences by HO endonuclease or by the I-SceI endonuclease, as discussed below. However, in one respect, *MAT* switching is distinct: The efficient use of *HMLα* to replace *MAT***a** is strongly dependent on a 250-bp RE located approximately 17 kb proximal to *HML* (157) (**Figure 1**). Without RE, *MAT***a** cells use the normally excluded *HMR* locus as the donor more than 90% of the time. RE contains multiple binding sites for the Fkh1 transcription factor, but it is only the phosphothreonine binding domain of Fkh1 that is required. When RE is replaced by four LexA binding sites, a LexA-Fkh1 fusion protein mimics the normal RE function and a LexA-FHA^{Fkh1} construct has full activity (76, 133). ChIP experiments show that RE binds close to DSB ends and GFP-FHA^{Fkh1} fusion protein localizes to multiple HO-induced DSBs (C.S. Lee, unpublished observations). Thus, RE brings *HML* close to *MAT* and accounts for the increased donor use of this distant locus. A mutation that is predicted to abolish FHA^{Fkh1} binding to phosphothreonine residues has no RE activity, but the target of the FHA domain has not been identified. Removal of DSB-induced γ-H2AX or the phosphorylation of histone H4-S1 has no effect, nor does the damage-associated phosphorylation of a number of recombination proteins. RE is portable and will enhance ectopic gene conversion between an HO-cleaved sequence and its donor (55, 70).

USE OF HO IN DIFFERENT RECOMBINATION CONTEXTS

HO endonuclease has been used in other contexts besides *MAT* switching by inserting a 24-bp cleavage site in other locations.

Gene Conversion Tract Length

The size of the gene conversion tract on either side of a DSB has been monitored by creating a DSB in the *URA3* gene where there is a mutated *URA3* template carrying mismatches across the region (148, 149). With mismatches 100 bp from the HO cleavage site, approximately 20% of the events converted only the HO cleavage site itself, but the average tract length is approximately 250 bp. Interestingly, strong transcription across the donor locus both reduced the frequency of events in which only the HO cut site was replaced and increased the frequency of bidirectional repair tracts (i.e., altering sites on both sides of the cleavage).

Analysis of Crossovers Associated with Mitotic Gene Conversion

In contrast to meiosis, where ≥30% of gene conversion events are associated with crossovers, reciprocal exchanges accompanying mitotic DSB repair are rare. This is especially true when the extent of homology surrounding the DSB is limited, as in ectopic recombination in a haploid cell. The paucity of crossovers can be attributed to at least two factors. First, the great majority of DSB repair events appears to proceed through a synthesis-dependent strand annealing (SDSA), mechanism (illustrated in **Figure 2**) that does not produce a stable Holliday junction (or a dHJ). Second, those DSB repair events that lead to formation of a dHJ are efficiently resolved as noncrossovers by the action of the Sgs1-Top3-Rmi1 helicase-strand passage complex that dissolves dHJs (8, 156). Deletion of Sgs1 or Top3 leads to a threefold increase in crossing over, as monitored on Southern blots (50). Deletion of another 3′ to 5′ helicase, Mph1, leads to a similar increase in exchanges, but this occurs independently of Sgs1, as the double mutant has an additive increase in crossovers, approaching the levels seen in meiosis (106). Mph1 apparently acts as a gatekeeper to channel mitotic DSBs into the SDSA pathway so that in its absence many more repair events must enter the dHJ process. A third 3′ to 5′ helicase, Srs2, also appears to increase crossovers, but in fact the physically measured level of crossovers is not elevated; instead, many noncrossover outcomes fail to be completed (50). This places Srs2 farther down the SDSA pathway, perhaps reflecting its role in limiting the extent of the Rad51 filament formed on ssDNA or some other as yet undefined role.

HO-Induced Crossovers in Meiosis

There are many differences between DSB-mediated repair in mitosis and repair in meiosis, where DSBs are created in a complex manner by the Spo11 topoisomerase (144). Crossovers are frequent in meiotic cells. When HO endonuclease was expressed from a meiosis-specific promoter, the frequency of crossovers was dramatically increased, suggesting that the differences between mitotic and meiotic recombination were not attributable to the manner in which the DSBs were formed but to the many meiosis-specific crossover control mechanisms that are overlaid on top of the basic DSB repair process (87). Hence, a deletion of the Msh4 protein that promotes crossovers via the ZMM pathway reduced crossovers for an HO-induced meiotic event as well. Fortuitously, HO expression in meiosis was not robust; thus, many tetrads gave evidence that only one of two sister chromatids was cleaved. When both sister chromatids were cleaved (as evident by the absence of the HO cleavage site on either sister chromatid), the level of exchange was significantly diminished compared to those tetrads where only one sister was cleaved. These data suggest that an important aspect of crossover control resides in the integrity of the axis established between the two sister chromatids.

Chromosome Architecture and Interchromosomal Ectopic Repair Efficiency

Chromosome conformation capture experiments have provided a three-dimensional picture of the arrangement of yeast chromosomes in mitotic cells based on the frequency of formaldehyde cross-linking of different chromosomal regions (contact frequency). Chromosomes are arranged in a Rabl orientation, with the 16 centromeres clustered at the spindle pole body and the telomeres associated with the nuclear envelope. DSB repair is constrained by this arrangement, such that a DSB induced near a telomere is three times more efficiently repaired by a donor at another telomeric location and a DSB in a sequence near a centromere is more efficiently repaired by a donor near another centromere (2). Repair is also highly variable for a DSB in interstitial locations. When HO creates a DSB in a *leu2* gene inserted at a chosen site and a *LEU2* donor is inserted at one of 20 different locations on different chromosomes, there is a very strong correlation between the efficiency of repair (which can vary from approximately 1% to more than 50%) and the contact probability between the two loci (70). Thus, chromosomal location matters. When the site of the HO-cleaved *leu2* recipient was placed in another location, some donors that had been very inefficiently used became very efficient donors, supporting the notion that the limitation on using a given donor did not reflect any intrinsic constraint on the donor region but was in fact defined by the contact probability between a given recipient and the donor location. Importantly, the kinetics of repair of a poorly used interchromosomal donor were identical to those of a well-used donor (2, 70). This observation implies that the limitation on repair is the likelihood of contact between donor and recipient; once there is productive contact, the kinetics are the same.

At a certain point, approximately 8 h after HO cleavage, repair stops; thus, poor donors do not have the chance to simply catch up slowly. Recombination becomes impossible if the resected ssDNA is degraded (the recombining *leu2* sequences are only the first 1 kb of what could be a 30-kb ssDNA resected broken chromosome). RPA seems to be the key protector of the integrity of ssDNA (there is not sufficient Rad51 to coat this much DNA). Thus, poor donors become better donors if the level of RPA is increased (70).

Poor donors can be made better donors in several ways. First, there is an improvement if the homology surrounding the donor is increased from 1 to 3 kb on each side of the DSB. Second, the RE, placed near the poor donor, improved its usage, although only to a limited extent. RE does not get a chance to work if it does not come into contact with the DSB.

BREAK-INDUCED REPLICATION

At telomeres and presumably at stalled and broken replication forks, a broken DNA molecule has only one end that shares homology with a sister chromatid, a homologous chromosome, or an ectopic location. No model yet exists for studying repair of a stalled and broken replication fork, although it should be possible to follow in real time the repair of a G1-created single-strand nick that is converted to a DSB on one sister chromatid, by using a mutant I-SceI (60), a FLP site-specific recombinase (91), or newly developed Cas9 enzymes that only cleave one strand at a defined location. But much has been learned by studying BIR in settings in which only one end of a DSB shares homology with either a homologous chromosome or an ectopic site. Indeed, there are two BIR pathways, the efficient one being Rad51-dependent and a second Rad51-independent pathway (18, 88, 89, 121). Although Rad51-mediated strand invasion occurs with similar kinetics to those seen for gene conversion, there is a delay of several hours before the initiation of new DNA synthesis. As discussed above, this REC is also seen when the two ends of a DSB share homology with a donor in which the regions are separated by approximately 5 kb. The two ends of the DSB must apparently signal their contact to each other, perhaps by creating a pair

of D-loops that are oriented toward each other (two invasions pointed in the same orientation do not eliminate the delay). This delay seems to be enforced by Sgs1 and Mph1 helicases, but precisely how is not known. When both helicases are absent, the kinetics of BIR become almost as rapid as those for break-repair gene conversion.

The DNA replication requirements for BIR include essentially all the proteins needed for origin-dependent replication, except for origin-specific factors such as Cdc6 (81–83). However, unlike normal replication, the initial steps of BIR can proceed in the absence of DNA Polε, but this polymerase is required to complete the copying of 30 kb of new synthesis. Such a distinction has not been seen in origin-dependent replication. BIR requires proteins not essential for replication or for gene conversion. Most interesting is the nonessential subunit of DNA Polδ, Pol32. Pol32 is required for ectopic BIR and is also needed for either of two alternative lengthenings of telomeres pathways when telomerase is ablated (81). A recent experiment in human cells demonstrated that the POLD3 homolog of Pol32 is needed when cells are under replication stress (when presumably BIR might be needed to restart replication at stalled or broken forks) but not in the absence of such stress (17). In addition, a FF248,249AA mutation of yeast PCNA blocks BIR but, again, not normal replication or gene conversion (83).

Recent physical analysis of BIR intermediates has forced a reevaluation of how BIR proceeds. It had been assumed that BIR involved establishment of a coordinated replication fork linking leading and lagging strand synthesis, but this is not the case. Instead, the leading strand synthesis, in the form of a replication bubble, creates a long single-stranded region that is only later converted to dsDNA by lagging-strand synthesis (114). Curiously, inactivation of Polα-primase blocks this initial step (81), raising the possibility that Polα-primase is needed to start first-strand copying rather than simply using the 3′ end of the invading strand as a primer. The migration of the replication bubble requires the 5′ to 3′ helicase Pif1, which again has no major role in normal replication or in gene conversion (153).

The discoordination between leading and lagging strand synthesis in BIR helps to explain why, as in gene conversion, there is a >1,000-fold elevation in mutations associated with BIR (19, 115). In addition to "local" template switches leading to deletions in homonucleotide runs, BIR displays a high level of interchromosomal template jumps. These were first seen when a linear fragment of DNA was used to initiate BIR in a diploid with polymorphic sites on two homologous chromosomes (123); there were frequent jumps from one template to the other, largely confined to jumps within the first several kilobases of new synthesis (possibly correlated with the late requirement for Polε in BIR). However, there are also very frequent template jumps between distant intrachromosomal regions that share a few hundred base pairs of homology. For example, when a DSB occurs near a UR sequence on the left arm of a chromosome, it initiates BIR with an RA sequence on the opposite chromosome arm more than 5% of the time, and the DSB jumps again to a more distal A3 sequence, producing a URA3 product as part of a nonreciprocal translocation (3). These second template jumps are largely Rad51- and Pol32-dependent as well as Rdh54-dependent.

SINGLE-STRAND ANNEALING

HO cleavage has also been used to analyze SSA where the DSB is flanked by repeated sequences. SSA is Rad51-independent but requires the strand annealing activity of Rad52. There is also an important role for Rad59, a homolog of Rad52, but Rad59 cannot substitute for Rad52. SSA generates nonhomologous 3′-ended tails that must be excised before repair can be completed. This system has made it possible to identify all of the components of the tail-cutting machinery, including Rad1-Rad10, Msh2-Msh3, Saw1, and Slx4 (75, 129, 130, 138). Slx4 is one of the few

repair proteins whose posttranslational phosphorylation by the ATM/ATR (Tel1/Mec1) kinases is required for its activity. SSA is efficient with 200 bp of flanking homology, but 3% mismatches in such sequences markedly reduce repair (127). This mismatch-provoked inhibition is suppressed by deleting Sgs1, other components of the STR complex, or Msh6. Interestingly, the inhibition of homologous SSA involves the unwinding rather than nucleolytic destruction of the mismatched strands, as a competition experiment revealed that the mismatched strand could persist and engage in a subsequent annealing with a fully matched partner.

Rad52-dependent SSA (or SSA-like) events can be detected even with flanking homologies of fewer than 20 bp; however, below approximately 12 bp, repair occurs by some form of NHEJ (122).

NONHOMOLOGOUS END-JOINING

Although most of the classic NHEJ machinery was identified in human immune-deficient patients, NHEJ repair of HO breaks has contributed significantly to our understanding. HO cleavage produces 4-bp 3′-overhanging ends. In most experiments, the strains used are deleted for *HML* and *HMR*; thus, there is no repair by gene conversion. Simple rejoining of the ends recreates the cleavage site, but because HO turns over very rapidly, it is possible to show that even simple ligation requires the core components of NHEJ, including DNA ligase 4, the XRCC4-like Lif1, and the yeast Ku70 and Ku80 proteins (99). Yeast NHEJ also largely requires the MRX complex. NHEJ plays an important role even when HR is available. If HO is turned on and then off after 90 min, approximately 10% of HO-induced repair in a *MAT***a** *HML*α *HMR*α strain remain *MAT***a**; these rejoining events are lost in the absence of yKu70 (141). Another NHEJ protein, Nej1, was identified independently by five laboratories, and in four different ways, after the discovery that there was a mating type–regulated component (28, 62, 102, 141, 154). Nej1 has little homology, but possibly has related functions, with mammalian XLF (37).

If HO is continually expressed, then simple rejoining of the DSB ends is futile, as they will be cleaved again. Approximately 0.2% of cells survive by alterations of the cleavage site, mostly by deletion or filling in of the overhanging ends. Larger deletions also arise. Interestingly, one can identify several subpathways of NHEJ by their dependence on different NHEJ components. In the absence of MRX proteins, the frequent 2- and 3-bp fill-ins are absent but a 3-bp deletion created by a different misalignment of the overhanging ends is still present (99). The fill-in events require the Pol4 DNA polymerase. Deleting yKu70 or yKu80 eliminates nearly all events, but in the special case where the overhanging ends share no homology (e.g., when HO cuts at *MAT*α and in an inverted nearby insert containing the *MAT***a** cleavage site), there are robust Ku-independent events (85) in which the junctions show substantial microhomology. Microhomology-mediated end-joining (MMEJ) events also require Rad1-Rad10, apparently to remove nonhomologous tails that are produced when the microhomologies lie at some distance from the cleavage site. MMEJ events are now recognized as a majority alternative pathway in mammalian cells, where they are both Ku-independent and XRCC- or DNL4-independent (94). Because yeast apparently only has DNA ligases 1 and 4, loss of Dnl4 removes the MMEJ events as well as classic end-joining, but in mammals, DNA ligase 3 appears to have a prominent role. Poly ADP-ribosyl polymerase, another important MMEJ factor in mammalian cells, is also absent from budding yeast.

ANALYSIS OF THE DNA DAMAGE RESPONSE

A single unrepaired DSB—for example, when *MAT* is cleaved in the absence of *HML* and *HMR*—provokes a robust DNA damage response (36, 104). Cells arrest prior to anaphase. This arrest

depends on the checkpoint kinase cascade, including the Mec1^{ATR} and Rad53^{Chk2} kinases, as well as the activators of the checkpoint response, the TopBP1 homolog Rad9 and the 9-1-1 checkpoint clamp complex. Tel1^{ATM} and Chk1 play less important roles (13, 116, 164).

Adaptation and Recovery

In the absence of repair, checkpoint-arrested cells eventually adapt and resume cell cycle progression, even though the lesion is unrepaired and 5′ to 3′ resection continues (117). Adaptation-defective mutants have been identified that reveal the complexity of the DNA damage response (71, 137). These include deletions of proteins associated with repair, such as Rad51 (but not Rad52), yKu70-yKu80, Srs2, Sae2 (but not Mre11 or Rad50), and Sgs1 (12, 24, 71, 73, 142). Mutants lacking Rdh54 (Tid1) protein, a homolog of Rad54, are also adaptation-defective, but *rad54* mutants are not (72). Deletion of another repair-associated protein, the chromatin remodeler Fun30, which regulates 5′ to 3′ resection, is also adaptation-defective (24). A simple idea that increased resection, or the products of resection, would cause permanent arrest was initially supported by the finding that a cell with two unrepaired DSBs was unable to adapt, as was the *yku70*Δ strain in which resection of the DSB ends was increased, but this idea has been rejected with the finding that deletions of proteins (Sgs1, Sae2, or Fun30) that reduce the rate of resection are also adaptation-defective. A single amino acid change in the Cdc5 kinase also blocks adaptation (137), whereas overexpression of Cdc5 (143) extinguishes cell cycle arrest.

The prolonged arrest of many of these adaptation-defective mutations depends on some sort of handoff to another checkpoint, the spindle assembly checkpoint (SAC) involving the Mad2 protein. A deletion of Mad2 provides an escape mechanism for many of the adaptation-defective mutations and suggests that permanent arrest depends on the activity of the SAC (23). The way in which Mad2 is involved remains under investigation, but it appears that its role requires an alteration of the centromeric chromatin on the chromosome with the DSB. Elimination of γ-H2AX phosphorylation, which spreads across the centromere region, also suppresses permanent arrest and is epistatic with *mad2*Δ. Indeed, deletion of the centromere of the DSB-damaged chromosome also shortens the arrest caused by an unrepaired DSB in otherwise wild-type cells (23).

Even if a DSB can be repaired, the DNA damage checkpoint must be actively turned off. The PP2C phosphatase Ptc2 is phosphorylated by casein kinase II (CKII) after DNA damage, and the modified Ptc2 is then bound by the FHA domain of Rad53, thus tethering the phosphatase to dephosphorylate Rad53 and to turn much of the checkpoint off. In addition to Ptc2, a homolog, Ptc3, also plays a role in regulating the checkpoint but has not been tested to see if it also interacts with Rad53 directly. Homologous phosphatase, Ptc3, CKII mutants and the *ptc2*Δ *ptc3*Δ double mutant are also adaptation-defective (31, 74). The *ptc2*Δ *ptc3*Δ double mutant also prevents cells that have been arrested by a 6-h-delayed SSA event from recovering and resuming growth (74).

Recently, another set of mutations proved to be adaptation defective and recovery defective. These mutations, which are found in the Golgi complex–associated retrograde protein complex, increase the level of autophagy in response to a single DSB and cause the partial degradation of a key mitotic regulator, securing (Pds1) (22). Simply increasing autophagy by expressing a dominant-active form of the Atg13 protein promotes relocalization of Pds1-GFP from the nucleus to the cytoplasm.

Studies of adaptation and recovery continue to reveal the ways in which DNA damage activates cell cycle arrest and how this signaling is reversed when cells finally repair the lesion. We have recently described a Mec1- and Tel1-dependent pathway of DNA damage–induced autophagy that is distinct from any other -phagy pathway, such as mitophagy or ribophagy (V.V. Eapen,

D.P. Waterman, A. Benard, N. Schiffman, E. Sayas, R. Kamber, B. Lemos, G. Memisoglu, J. Ang, A. Mazella, S.G. Chuartzman, R. Loewith, M. Schuldiner, V. Denic, D.J. Klionsky, J.E. Haber, submitted). In addition, we have found that turning off Mec1 seems to involve two autophosphorylated sites that are only phosphorylated at the time the checkpoint is turned off (G. Memisoglu & K. Lee, unpublished observations). How this is regulated is under investigation.

Formation of γ-H2AX (and γ-H2B)

The creation of single unrepaired DSBs has made it possible to examine in detail the phosphorylation of yeast histone H2A (which is in fact the ortholog of mammalian H2AX). Yeast γ-H2AX spreads robustly approximately 50 kb on either side of a DSB, within 30 min, and spreading can be accomplished by either $Mec1^{ATR}$ or $Tel1^{ATM}$ (120). But a closer examination revealed that there was little modification over transcribed genes (68). When transcription of galactose-regulated genes was shut off, γ-H2AX modification appeared within a few minutes, and when transcription was again activated, γ-H2AX disappeared almost as rapidly. The loss of γ-H2AX is much more profound than the reduction of histone proteins in the transcribed regions and suggests that the modification occurs more slowly than the replacement of histones. The reformation of γ-H2AX, 6 h after the DSB was induced, was carried out primarily by Mec1, consistent with cytological evidence that Tel1 is by then displaced from the end of the DSB.

Although γ-H2AX spreads rapidly over 50 kb within 30 min, it continues to spread to much greater distances in a slower process that only Mec1 can accomplish (64). Given that Mec1 associates with ssDNA through its interaction with the ATRIP homolog, Ddc2, this spreading appears to parallel the rate of continuing resection, still spreading approximately 50 kb ahead of the ssDNA.

γ-H2AX can also spread to sites that are not on the broken DNA molecule but instead are on other segments of DNA that are brought into close proximity with the DSB (68, 108). Modification of the region on the opposite chromosome arm occurs around the RE in *MAT***a** cells when RE is active, but not in *MAT*α cells when RE is repressed and does not bind near the DSB. Similarly, a DSB near the centromere of one chromosome can cause modification around the centromere regions of all of the other chromosomes that are clustered at the spindle pole body. Again, this is Mec1-dependent and Tel1-independent.

One yeast-specific discovery was the existence of γ-H2B, the phosphorylation of T129 on histone H2, in a TQ site modified by either Mec1 or Tel1 (68). This sequence is absent from fission yeast, flies, worms, mice, and humans. γ-H2B spreads over essentially the same domain as γ-H2AX, but more slowly, except it is nearly absent near telomeres. The kinetics of γ-H2B modification are accelerated in a strain where H2A-S129 is mutated to alanine or in a *rad9*Δ strain, apparently because Rad9 binds to γ-H2AX nucleosomes and somehow prevents access to the site on H2B.

I-SCEI AND OTHERS

HO is not special; any endonuclease should be able to create comparable results. One of the great advantages of HO is its intrinsic instability, so that when turned off, it is soon gone. But when one is only interested in the final outcome, when the nuclease is continuously expressed, then other site-specific nucleases should be able to produce comparable results. We first showed this was the case when Bernard Dujon's laboratory (16) created a synthetic version of the *S. cerevisiae* mitochondrial I-SceI homing endonuclease that could be expressed in the nucleus and successfully translated in the cytoplasm. I-SceI had been shown by the laboratories of both Ron Butow (163)

and Dujon (53) to catalyze a site-specific DSB-mediated gene drive in which the homing intron was copied from ω^+ to ω^- mitochondria. Anne Plessis, Arnaud Perrin, Dujon, and I (105) then showed that I-SceI would stimulate the same gene conversion events that Norah Rudin, Elaine Sugarman, and I (112) had first shown for HO (not *MAT* switching but gene conversion involving inverted *E. coli lacZ* sequences on a plasmid).

I-SceI has been exploited in budding yeast for a number of experiments comparable to those using HO but has been particularly interesting in the hands of Francesca Storici and her collaborators (118, 124), who have demonstrated that repair of a DSB can be mediated by ssDNA—and even RNA!—templates. She has also demonstrated that one can improve gene targeting by using an aptamer to tether the template close to the DSB site (113). Storici's laboratory (60) has also created a nicking version of I-SceI that should allow the analysis of single-strand nicks in repair. Another approach to this question has been carried out by Gregory Ira and colleagues (91) using the FLP-nick system.

But, until recently, I-SceI has also been the workhorse for studying DSB repair in flies and mammals. Maria Jasin has been especially important in this endeavor, beginning with her demonstration with colleagues in 1994 that I-SceI could be used to promote both gene editing and gene ablation in mouse cells (111). Many other laboratories have used Jasin's I-SceI-induced gene conversion assays in studying mammalian recombination and repair (58).

Another very powerful approach has been taken by Gaelle Legube and colleagues (48, 160), who have used an estrogen receptor–regulated AsiSI enzyme to create a defined number of DSBs in mammalian cells, allowing them to measure γ-H2AX formation. Recent work in her laboratory has distinguished two types of lesions, those that recruit XRCC4 and presumably the rest of the NHEJ proteins and those that recruit Rad51 and presumably attempt HR. Interestingly, transcriptionally active regions of the chromatin recruit the HR machinery (6).

THE FUTURE: CAS9 AND ITS COUSINS

The revelation of the past few years that virtually any sequence can be cleaved by using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 and an appropriate guide RNA has made it possible to efficiently knock out genes with small indels or, with less efficiency, to edit them by gene conversion (57). There are many exciting avenues to investigate beyond the question of how to make mammals more like yeast in gene-targeting efficiency (The best answer to this question is to reduce the genome complexity of a mammal!). I suspect that NHEJ is not much more avid in mammals than in yeast. The problem is that Rad51 must search a 300× larger genome, and increasing the size of the targeting sequence 300-fold is not the solution.

Yeast is still going to play an important role in sorting out how to make gene modification more efficient. We have been exploring several interesting questions. First, is Cas9's access to chromatin similar to HO? HO cannot cut its recognition site in *HML* or *HMR* when they are silenced, but there is no problem when silencing is eliminated. We have found that Cas9 cannot cut very close to the same site in silenced *HML* or *HMR*, but other sites in this heterochromatic region are in fact accessible.

Second, what are the consequences of cleaving DNA such that the ends of the DSB are not homologous to the donor sequence and need to be trimmed off? We had previously shown that such tails impair HR in budding yeast. Tails longer than approximately 20 nt have to be clipped off by the Rad1-Rad10 system described earlier, whereas shorter nonhomologies can be removed by the 3′ to 5′ exonucleases of DNA Polδ or Polε (15). Now, using Cas9, it is possible to take the same substrate and create shorter and shorter tails. Ranjith Anand in my lab has now shown that even a 3-bp tail poses a meaningful impediment to efficient repair.

OLD AND NEW QUESTIONS

I have been worrying about DSB repair for roughly 40 years and hope to be still worrying about it for a few more. With a remarkable set of colleagues, younger and younger (relative to me anyway) as time passes, we have solved a lot of questions. But some very important questions still remain. Here are some (that might end up in a grant proposal):

1. What, precisely, are the homology requirements for Rad51 to function in vivo? If, as it appears, the rules are not identical in vivo and in vitro, what are the key roles of Rad55 and Rad57, Rad54, and other associated proteins?
2. How can there be sectored colonies arising from a single gene conversion event with a mismatch between recipient and donor? I outlined some possibilities above, but it would be interesting to know whether blocking the dHJ dissolving activity would change this outcome.
3. Can we find PCNA mutations that would prevent DNA polymerase from being so error-prone? We have already shown that proofreading-defective DNA polymerase is more processive and makes far fewer slips and jumps.
4. How are the mismatch repair proteins engaged in discouraging recombination between mismatched sequences, and how is the directionality of mismatch repair exclusively in favor of the invading strand accomplished?
5. How are template jumps occurring? Do they require Rad51, and if so, why are they not delayed by the time required to initiate a homology search? And if not so, how is DNA polymerase δ doing this?
6. When template switches happen between highly mismatched sequences, is the only important consideration the microhomology at the junction? How long does the mismatched sequence have to be to get a jump to take place?

That should keep us busy for a while.

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