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Analysis of repair mechanism choice during homologous recombination

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ABSTRACT

Double-strand breaks (DSBs) occur frequently during cell growth. Due to the presence of repeated sequences in the genome, repair of a single DSB can result in gene conversion, translocation, deletion or tandem duplication depending on the mechanism and the sequence chosen as partner for the recombinational repair. Here, we study how yeast cells repair a single, inducible DSB when there are several potential donors to choose from, in the same chromosome and elsewhere in the genome. We systematically investigate the parameters that affect the choice of mechanism, as well as its genetic regulation. Our results indicate that intra-chromosomal homologous sequences are always preferred as donors for repair. We demonstrate the occurrence of a novel tri-partite repair product that combines ectopic gene conversion and deletion. In addition, we show that increasing the distance between two repeated sequences enhances the dependence on Rad51 for colony formation after DSB repair. This is due to a role of Rad51 in the recovery from the checkpoint signal induced by the DSB. We suggest a model for the competition between the different homologous recombination pathways. Our model explains how different repair mechanisms are able to compensate for each other during DSB repair.

INTRODUCTION

The natural occurrence of DNA lesions is a constant threat to genome stability. DNA double-strand breaks (DSBs) arise due to intracellular events or to environmental insults. Failure to repair DSBs can lead to cell death and apoptosis, while inaccurate repair can cause genomic instability and cancer. In order to overcome the cytotoxic and mutagenic properties of DSBs, eukaryotes have two sets of competing mechanisms: a relatively error prone mechanism termed non-homologous end-joining (NHEJ) and a more accurate one termed homologous recombination (HR) (1). Although classified as relatively error free, HR can also be mutagenic and lead to chromosomal rearrangements and to loss of heterozygosity. Despite the high conservation of these mechanisms of repair in evolution, different organisms use HR and NHEJ repair pathways to different extents (1).

The term 'homologous recombination' describes a set of mechanisms, all of which use homologous sequences to repair DNA. Most current models of HR are initiated by a DSB; the most common models are: the double-strand break repair (DSBR) (2), the synthesis dependent strand annealing (SDSA) (3), the single-strand annealing (SSA) (4) and the break induced replication (BIR) models (5,6) (Figure 1). These HR mechanisms have several common features: all HR reactions are catalyzed by a number of proteins that belong to the *RAD52* epistasis group (7), although some enzymes are more important for specific pathways. For example, Rad51, the eukaryotic ortholog of bacterial RecA, is essential for DSBR and SDSA, but not for SSA (8). All HR mechanisms begin by resecting the DSB, leaving a 3′ single-stranded tail at both ends (9,10). This creation of single-stranded DNA (ssDNA) is believed to serve as the molecular signal for the DNA damage response, in addition to its requirement in the repair process itself (Figure 1).

A very important difference between HR mechanisms is the end products left following the repair. Repair of even a single DSB can result in gene conversion (GC), deletion or tandem duplication depending on the mechanism and the sequence chosen as partner for the recombinational repair. Thus, to make the repair as efficient and risk-free as possible, cells must balance between these potentially competing DSB repair mechanisms. Despite the fact that the way the repair mechanism is chosen is still largely an enigma, some of the factors influencing it are known. These include the stage in the cell cycle at which the break has occurred and the chemical nature of the ends (11).

Although DSB repair has been extensively explored, the choice of competing repair mechanisms was systematically studied only in a handful of studies (12–14). Here we use yeast strains in which a single, defined DSB can be

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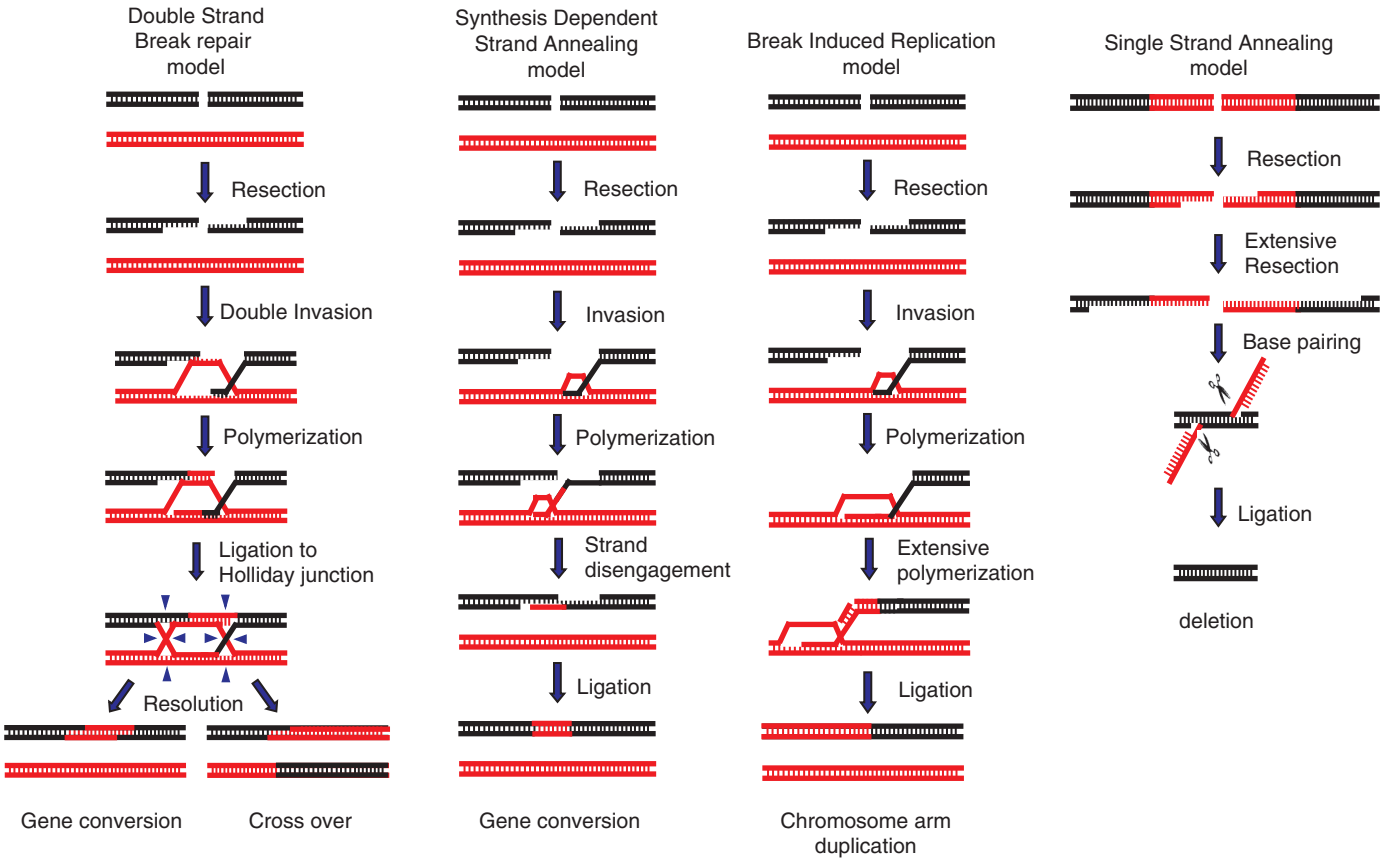

Figure 1. Schematic representation of the different DSB repair models. In the DSBR model (2), a double Holliday junction (HJ) can be resolved by endonucleolytic cleavage (indicated by triangles) to generate crossover or gene conversion (non-crossover) products. In the SDSA model (3), D-loop extention and invading strand displacement produce a GC product. In the BIR model (5,6), the invading strand continues DNA synthesis to the end of the DNA molecule, producing a duplication of the chromosome arm. In the SSA model (4), a DSB made between direct repeats results in deletion of one of the repeats and the intervening DNA.

inflicted at will, to explore the parameters affecting the choice between competing repair mechanisms.

MATERIALS AND METHODS

Yeast strains

All the yeast strains used in this study are isogenic with strain MK203 (*MAT***a**-*inc ura3-HOcs lys2::ura3-HOcs-inc ade3::GALHO ade2-1 leu2-3,112 his3-11,15 trp1-1 can1-100*) (15), a derivative of W303. Intrachromosomal repeats were created by integration of plasmids pM53 to MK203 to create NA3 (6.5 kb). Repeats at shorter distances were created by using PCR products carrying a *KanMX* cassette with homologies to the plasmid sequence transformed to NA3 replacing variable fragments of the plasmid sequence in NA3 leaving the appropriate distances, creating strains: NA14 (3 kb), NA30 (4.5 kb) and NA29 (5.5 kb). Homologous *URA3* fragments of different lengths were inserted at an *Hpa*I site within *LYS2* sequences as described (16) to create NA14 (1.2 kb), NA41 (5.6 kb) and NA42 (12.8 kb).

NA15 was created by integration of a modified pM53 with *KanMX::HOcs* cloned in its *SspI* site. Homologous pM53 fragment was created by insertion of a *ScaI* PCR fragment from pM53 into *LYS2*.

Deletion of the *RAD51* gene was carried out by one-step transplacement using plasmid pAM28 (17). The *mec1 sml1* alleles were introduced by crossing NA3 to U953, provided by Rothstein. Genes were deleted by transformation with a PCR product produced on the appropriate strain from the *Saccharomyces* Genome Deletion Project array. All chromosomal configurations were verified by Southern blot and/or PCR analysis after transformation.

Media and growth conditions

Saccharomyces cerevisiae strains were grown at 30°C, unless specified otherwise. Standard YEP medium (1% yeast extract, 2% Bacto Peptone) supplemented with 3% glycerol (YEP–Gly), 2% galactose (YEP–Gal) or 2% dextrose (YEPD) was used for non-selective growth. We added 1.8% Bacto Agar for solid media.

Repair efficiency measurement

Each strain was streaked onto YEP–Gly plates. Individual colonies were resuspended in water, appropriately diluted, and plated on YEPD and YEP–Gal plates. Colonies were counted after 3–5 days of incubation at 30°C.

Repair choice measurement

Single colonies were resuspended in rich YEP–Gly medium, grown to logarithmic phase, centrifuged, and resuspended in YEP–Gal. After 10 h cells were plated on YEPD and counted following 2 days incubation in 30°C. To measure repair choice colonies were analyzed both by replica plating to selective media as well as by PCR analysis of the repaired sequence.

Induction experiments

Single colonies were resuspended in rich YEP–Gly medium, grown to logarithmic phase, centrifuged, and resuspended in YEP–Gal. At timely intervals, samples were plated on YEPD plates to score viability and commitment to repair, and DNA and protein was extracted and subjected to the different assays. Commitment to deletion was examined by replica plating to YEPD media containing G418 (Sigma).

Western blot analysis

Rad53 was detected using anti-Rad53 polyclonal antibodies kindly provided by J. Diffley (Clare Hall, London, UK). Secondary antibodies were purchased from Jeckson ImmunoResearch Laboratories and proteins were visualized by an enhanced chemiluminescence system according to the manufacturer.

RESULTS

Experimental system

Previous studies from several laboratories have shown that following the creation of a DSB, a genome-wide search for homology allows the broken chromosome to interact with similar sequences, irrespectively of their genomic position; this homology search can be extremely efficient, allowing repair of almost all cells (18). We have created in the past strains in which a single DSB can be induced at will (15,16,19) and its repair kinetics can be monitored using different assays.

Spontaneously arising DSBs may be repaired by using various substrates and mechanisms. In order to mimic a situation in which multiple donors for repair exist, we constructed a yeast haploid strain (NA14) with three *URA3* gene sequences: two copies on chromosome *V* and one on chromosome *II* (Figure 2A). One of the *URA3* copies carries a recognition site for HO (*HOcs*) inserted at an *Nco*I site. The second sequence is the wt copy of the *URA3* gene, which has a single *Nco*I site. In this construct, the two homologous sequences are direct repeats sharing 1.2 kb of homology and separated by a 3 kb interval (Figure 2A). A third 1.2-kb fragment of the *URA3* gene is present at the *LYS2* gene on chromosome *II*. It carries the HO recognition site with a single-base pair mutation that prevents cleavage by the endonuclease (*ura3-HOcs-inc*) and two RFLPs, located downstream (*Bam*HI) and upstream (*Eco*RI) of the *HOcs-inc* insertion (15). This strain also bears the *HO* gene under the transcriptional control of the *GAL1* promoter. Upon transfer of cells in the mid-log phase of growth to galactose-containing medium, the DSB thus created can be repaired using, as donors, two alternative homologous sequences: one located on the same chromosome (intrachromosomal donor), and one present in a different chromosome (ectopic donor). The RFLPs (presence/absence of *Nco*I and *Bam*HI) can be used to identify the sequence that served as donor in the GC event (Figure 2).

The efficiency of DSB repair can be estimated by comparing the ability of the cells to form colonies on galactose-containing medium (HO induction) to that seen on glucose (no HO induction) (15). Strain NA14 showed 87.1% efficiency of repair when plated on galactose (Table 1). This value is similar to those obtained with other strains undergoing a single DSB (15,16).

In order to assess DSB repair choice, cells grown on YP–Gal were tested for Uracil prototrophy and for their ability to grow on medium containing G418. We could thus distinguish between the different repair products [Figure 2A: intrachromosomal gene conversion (IGC): Ura^+ $G418^R$; deletion: Ura^+ $G418^S$; ectopic gene conversion (EGC): Ura^+ $G418^R$)]. IGC and EGC events can be distinguished by indicative colony-PCR and restriction enzyme digestion of the repaired sequence. The presence of *Nco*I sites in the repaired chromosome points to an intrachromosomal origin, whereas a *Bam*HI site can only be acquired through EGC. Surprisingly, a fourth category was also detected. These cells were Uracil auxotrophs and sensitive to G418. Analysis of these cells revealed that a single *URA3* gene replaces the repeats on chromosome *V*, and it carries the RFLPs present on chromosome *II*. We call this class tri-partite deletion (T-deletion), as it requires interactions between both *URA3* copies on chromosome *V* and the one on chromosome *II*. It is important to note that we did not encounter any products of imprecise or precise NHEJ (the latter are expected to be continually re-cleaved in our experimental conditions).

An analysis of these results reveals several interesting observations. First, in concurrence with previous findings (4,20), most (60%) of the repair events led to a deletion product (Table 1). Second, there was a clear preference for using the intrachromosomal homologous sequence for the repair; about 97% of the events were intrachromosomal. It is important to stress that in an isogenic strain lacking the intrachromosomal donor 100% of the repair is carried out by EGC (15). Thus, addition in chromosome *V* of a second copy bearing homology to the broken ends (1.2 kb) completely changes the donor preferences towards the intrachromosomal sequences. Third, we were able to detect the formation of an unexpected product, in which the three *URA3* copies participate, and which accounts for 1.5% of the repair events (Figure 2A and Table 1).

HR mechanisms can compensate for each other

To better understand the repair mechanisms involved in the formation of the repair products detected, we examined the repair in cells deleted for two main repair enzymes: Rad51 and Rad1. Rad51 forms a nucleofilament that participates in strand invasion (19) and was found to play a central role in GC but not in SSA. Rad1 forms,

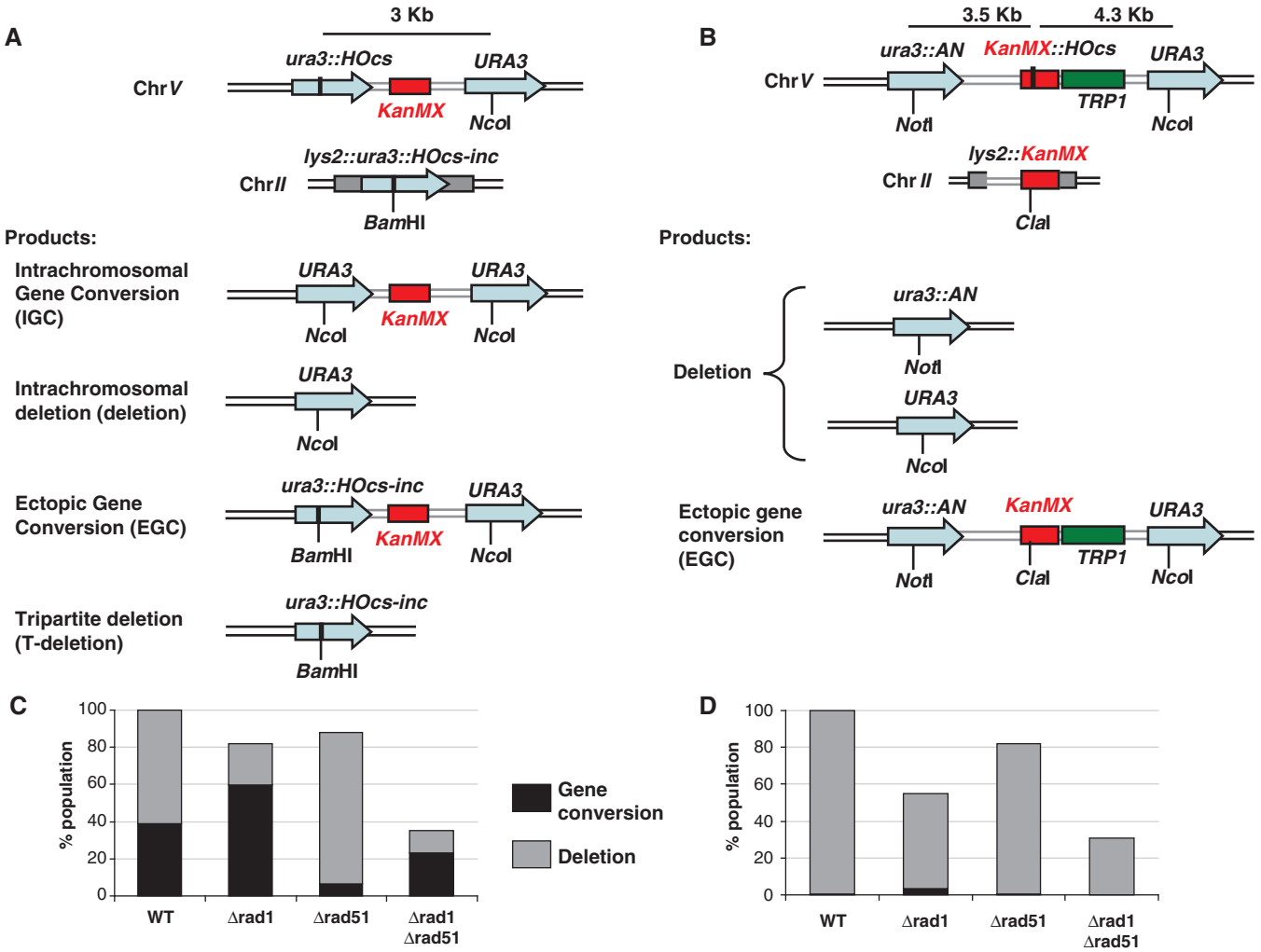

Figure 2. Repair choice in a strain which undergoes a DSB. (**A**) Schematic representation of the yeast strain NA14 (DSB *within* one of intrachromosomal repeats) and its repair products following induction in YEP–Gal medium. (**B**) Schematic representation of strain NA15 (DSB *between* intrachromosomal repeats) and its repair products. (**C**) Graphic representation of the distribution of repair products in NA14 wt, *Δrad1*, *Δrad51* and *Δrad1Δrad51* strains. (**D**) Graphic representation of the distribution of repair products in NA15 wt, *Δrad1*, *Δrad51* and *Δrad1Δrad51* strains. Column height represents the viability on YEP–Gal medium compared to YEPD medium. Each column shows the percentage of the deletion events (gray) and GC events (black).

Table 1. Repair choice in our experimental system

Strain	Location of the break	*n*	Viability[a]	ICG[b]	Deletion[b]	EGC[b]	T-deletion[b]
NA14 (3kb[c])	Inside repeat	6390	87.1 (1)	37.6	59.5	1.4	1.5
NA14 *Δrad1*		1189	71.5 (0.82)	67.6	25.2	5.1	2.1
NA14 *Δrad51*		1111	77.1 (0.88)	7.4	92.6	0	0
NA14 *Δrad1 Δrad51*		748	31.1 (0.35)	65.8	34.2	0	0
NA15	Between repeats	2918	93.2 (1)	0	99.5	0.5	0
NA15 *Δrad1*		505	51.5 (0.55)	0	94.3	5.7	0
NA15 *Δrad51*		601	76.6 (0.82)	0	99.5	0.5	0
NA15 *Δrad1 Δrad51*		1182	29.4 (0.31)	0	100	0	0
NA3 (6.5 kb[c])	Inside repeat	5495	83.9 (1)	49.9	44.3	3.8	2
NA3 *Δrad51*		1141	38.8 (0.46)	7.4	92.6	0	0
NA3 *Δrad59*		407	28.8 (0.34)	28.7	67	4	0.3
NA3 *Δrad51 Δrad59*		1191	11.25 (0.13)	16.8	83.2	0	0
NA3 *Δpol32*		2004	60.7 (0.72)	45.4	53.2	0	1.4
NA3 *Δmre11*		352	31.4 (0.37)	37.7	51.1	6.6	6.3
NA3 *Δlig4*		1633	73.2 (0.87)	40.1	54.8	2.6	2.5
NA3 *Δku70*		1352	69.6 (0.82)	52.4	40.1	3.5	4

[a]Viability on YEP-Gal plates compared to YEPD. The numbers in brackets represent the relative repair efficiency.
[b]IGC: Intrachromosomal gene conversion; Deletion: Intrachromosomal deletion; EGC: Ectopic gene conversion; T-deletion: Tripartite deletion.
[c]Distance between intrachromosomal repeats.

together with Rad10, an endonuclease able to cut at flap junctions and is believed to be essential for SSA but less important for GC (7).

We found that, as previously seen (8), *Δrad51* cells showed an evident decrease in GC events and an increase in deletion events. Similarly, *Δrad1* cells showed a decrease in deletion events, which was accompanied by an increase in GC events (Figure 2C and Table 1). It is important to note that in both mutants, the dramatic changes in the repair products were accompanied by only a mild decrease in the ability of the cells to repair (Figure 2C and Table 1). Taken together, these results clearly show that the different HR repair mechanisms are able to compensate for each other. As expected from such a conclusion, *Δrad1 Δrad51* double mutant showed a dramatic decrease in repair efficiency (Figure 2C). An examination of the surviving colonies yielded, surprisingly, a high level (65%) of GC products (Table 1). This result shows that in the absence of both Rad1 and Rad51 additional, less efficient repair mechanisms are active (see 'Discussion' section).

The relative usage of SSA is dependent on break location

In the yeast strain analyzed, NA14, the DSB occurs *within* one of the repeats. To examine the effect that break location might have on repair choice, we constructed an isogenic strain (NA15) in which the single HO cut site is in the sequence 'between' the two *URA3* intrachromosomal sequences. As NA14, this strain carries an ectopic insert homologous to the sequences surrounding the break (Figure 2B). Importantly, in both strains the distance between the break and the homologous sequences used for repair is similar (~3 kb). The repair efficiency of NA15 was 93.2%, comparable to that of NA14 (Figure 2D and Table 1). However, in NA15 repair results almost exclusively (99.5% of the cases monitored) in deletion products (presumably by SSA), despite the presence of a 1.2 kb potential ectopic donor (Figure 2D and Table 1). Importantly, in a strain identical to NA15 but lacking one of the intrachromosomal repeats, survival on galactose is high and 100% of the repair is carried out by EGC (data not shown), again indicating that in the absence of an intrachromosomal repeat repair is efficiently carried out using this ectopic donor as partner. Examination of the genetic requirements of NA15 revealed that while Rad1 is important for the repair in this strain (reducing the survival to 50%), deletion of *RAD51* had little effect, and *rad51* strains show results similar to those seen in the strain with the break located inside one of the repeats (compare Figure 2D to C). Notably, very little compensation by EGC was observed in the *rad1* strain (see 'Discussion' section). A NA15 *Δrad1 Δrad51* double mutant, however, shows a reduction in repair efficiency, compared to the single *Δrad1* mutant, indicating that a Rad51-dependent pathway is contributing to survival (Figure 2D). All the repair events in this case were deletions and no compensation by GC (possible in this chromosomal configuration) is observed. Thus, the location of the DSB (whether in the context of homology or at an adjacent, non-homologous region) has a strong effect on the mechanism by which the break is repaired.

The relative usage of the ectopic donor is size dependent

Ectopic sequences are used with high efficiency when they are the only homologous sequences present. However, in all the cases in which homologous sequences are also present in the chromosome undergoing the DSB, these are preferentially used, both in GC events, or to create deletions. To better understand the choice between intra- and inter-chromosomal sequences, we sought to improve the competitiveness of the ectopic donor by increasing its size. We thus created a series of yeast strains similar to NA14 (DSB within one of the *URA3* sequences) that carry, on chromosome *II*, ectopic donors of increasing size (1.2, 5.6 and 12.8 kb). In addition, we constructed a diploid strain, which has a full homologous chromosome *V*, providing 577 kb of homology.

We examined the repair efficiency, as well as the relative use of each donor, as described before. The results show that as the size of the ectopic donor increases, its relative use for repair also increases, from 2.9% for a 1.2-kb ectopic donor to 23.2% for a 12.8 kb ectopic donor (Figure 3A and Table S1, strains NA14, NA41 and NA42). Importantly, despite a 10-fold increase in ectopic donor size, the intrachromosomal sequences still acted as the preferred donor for GC. Note that even in the presence of a full-length homologous chromosome (577 kb of homology), the 1.2 kb sequence located on the same chromosome serves as donor for IGC in 15.9% of the cases and participates in intrachromosomal deletion events in 18.3% of the cells (Figure 3A and Table S1 NA14 diploid). Thus, these results indicate on one hand that the size of the ectopic donor has an effect on its ability to compete with the intrachromosomal sequences as donors for repair; on the other hand these results underscore the strong preference for intrachromosomal sequences as donors of information.

To investigate the T-deletion product observed, we examined repair choice in the NA14 diploid strain. This strain is constructed in such a way that in addition to the configuration of NA14 (Figure 2A), it carries a homologous chromosome *V* with different *URA3* RFLPs (*Pvu*II and *Xba*I). Note that since diploid cells show higher levels of recombination, we refer only to the choice of repair observed in this strain. Examination of the galactose-grown colonies revealed that, among the ~68% of deletion products, the majority of events are also tri-partite products (a deletion carrying the *Pvu*II and *Xba*I markers of the allelic chromosome). We conclude that tri-partite events occur at high frequencies during DSB repair.

The relative usage of the ectopic donor depends on the distance between repeats

Another characteristic that might play a role in repair choice is the distance between the repeats. The effect of varying the distance between repeats has been previously investigated, using strains in which a break was introduced 'between' repeats (21,22). We have seen that when the DSB occurs within one of the direct repeats, both GC and deletion events take place. To study the effect of donor distance in this case, we constructed a series of isogenic strains in which we systematically varied the

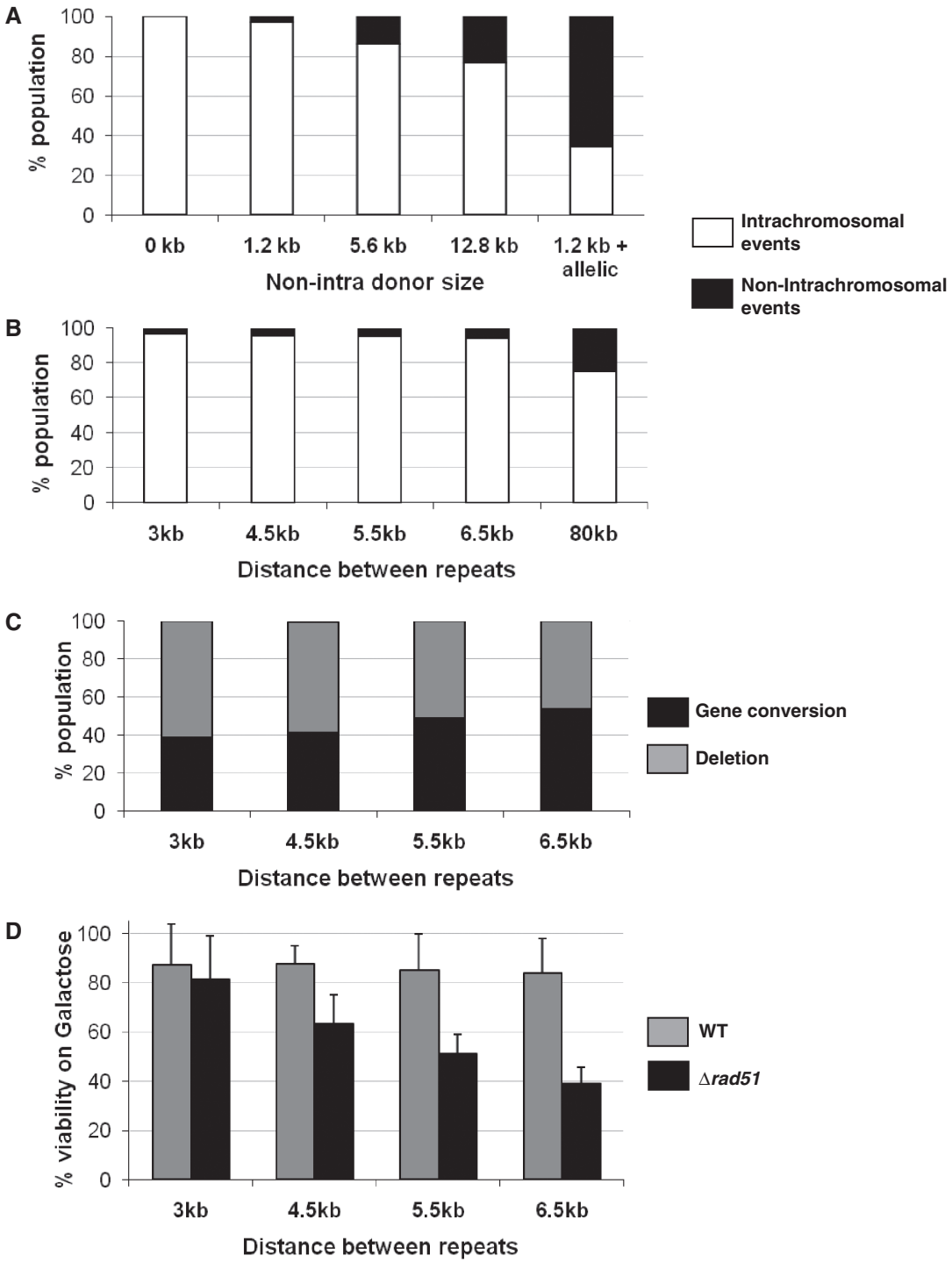

Figure 3. Graphic representation of the distribution of repair products in different strains. (**A**) Distribution of intra- (white) and inter-chromosomal events (black) in strains harboring an ectopic/allelic donor of increasing length in wt background. (**B**) Distribution of intra- (white) and inter-chromosomal events (black) in strains harboring an increasing distance between the intrachromosomal repeats in wt background. (**C**) Distribution of GC events (black) and deletion events (gray) in strains harboring an increasing distance between the intrachromosomal repeats in wt background. (**D**) Graphic representation of the repair efficiency of wt and *Δrad51* strains with an increasing distance between intrachromosomal repeats.

distance between the two *URA3* repeats (4.5, 5.5, 6.5 and 80 kb), while leaving the HO-cs 'within' one of the repeats.

We found that with increasing distance there is an increase in the use of the ectopic donor for repair, ranging from 2.9% when the repeats are 3 kb apart to 5.8% for a distance of 6.5 kb (Figure 3B and Table S1, strains NA14, NA30, NA29 and NA3). This result indicates that increasing the distance between the repeats enhances the ability of the ectopic donor to compete for the repair. However, even when the repeats are 80 kb apart there is still a

preference for using the intrachromosomal sequence (75.5% of the cells; Figure 3B and Table S1). With increasing distance between the repeats there is a slight increase in GC accompanied by an equal reduction in deletion events, without affecting the general repair efficiency (Figure 3C and Table S1). This result is in agreement with previous findings in a yeast extrachromosomal system (8) as well as in human cells (20).

The dependence on Rad51 increases with distance between repeats

We also examined the effect that a deletion of *RAD51* has on the repair of strains with increased distances between repeats. Surprisingly, [as Rad51 is not supposed to be required for SSA (8)], we found that the repair efficiency in *Δrad51* strains was *reduced* with increasing distance between repeats, reaching about 40% efficiency when the repeats are 6.5 kb apart (Figure 3D). The choice of repair mechanism in these strains was almost similar at all distances, with about 92% of the events resulting in deletion and 8% in GC (Table S1).

To explain the decrease in repair efficiency seen in *Δrad51* strains with increased distance, we first assumed the possibility that a Rad51-dependent mechanism (different from SSA) may participate in deletion formation; the use of this alternative mechanism would be distance-dependent. Four Rad51-dependent mechanisms could result in a deletion product: intrachromatid crossing over (pop-out), unequal sister chromatid recombination (23), gap repair with the intrachromosomal repeat (24) or BIR involving the intrachromosomal repeat (6). We constructed a yeast strain in which the two intrachromosomal homologous sequences are inverted with respect to each other and are located 6.5 kb apart. In this construct, crossing-over (CO) leads to an inversion of the sequences between the repeats, a product that can be easily identified by PCR analysis. Examination of the repair product revealed that CO occurs in only ~2% of events (1 out of 50 colonies), a frequency too low to explain the striking reduction in repair in Δ*rad51* strains with repeats located far from each other. Similarly, we found that unequal sister chromatid exchange (which should generate a triplication of the *URA3* repeats in a similar ratio as deletion events) is extremely low (0/50 colonies analyzed) and could not account for the distance effect in *Δrad51* strains. In order to examine the possibility that BIR is responsible for the deletion products, we deleted the *POL32* gene. The Polymerase δ subunit Pol32 has been shown to play an important role in BIR (6,12). Examination of repair efficiency in *NA3Δpol32* showed only a mild reduction compared to wt cells (60% compared to 80% in wt cells; Table 1). In addition, when examining repair choice in this strain, deletion products were less affected by *POL32* deletion than GC events (Table 1). These results together indicate that BIR does not play a major role in deletion formation in our system.

An alternative possibility is that the low cell survival observed on galactose plates reflects a Rad51 function that is different from actual repair. For example, repair could be completed in all types of cells, but successful recovery and cell cycle resumption may depend on Rad51 (22). To discriminate between these possibilities, we compared the repair kinetics of two *Δrad51* strains: one with repeats 3 kb apart (NA14*Δrad51*) and another one with repeats 6.5 kb apart (NA3*Δrad51*). Cells were grown to mid-logarithmic phase in medium containing glycerol, and a DSB was induced by transferring the cells to galactose-containing medium. Cells were harvested for examination at timely intervals and subjected to FACS and to PCR analysis.

First, we used PCR analysis to monitor the creation of the deletion following repair (Figure 4A). Formation of the deletion product in the strain harboring repeats 3 kb apart (NA14*Δrad51*) starts about 1.5 h after transfer to galactose, while in NA3*Δrad51* (6.5 kb apart) the deletion product appears half an hour later. A similar difference is seen in the isogenic Rad+ NA14 and NA15 strains (data not shown), in accordance with the need for more extensive resection when repeats are further apart and with the resection rate of ~35 nt/min as previously calculated (4,15). Other than this delay, both strains show very similar kinetics of repair (Figure 4A). Quantization of the deletion and the control products shows that in both strains about 80% of the cells complete repair by creating a deletion (Figure 4A). Nonetheless, one strain (NA14*Δrad51*) produces >80% viable colonies, whereas the other (NA3*Δrad51*) leads to about 40% survival on galactose-containing medium. We thus conclude that actual repair is completed, in the absence of Rad51, in both strains, likely by SSA, but that survival is lower in the strain with the repeat farther away. NA3*Δrad51* cells are unable to recover, despite having completed repair of the break (as observed by PCR). Consistent with this idea, FACS analysis (Figure 4B) and microscopic observation of the cells (Figure 4B) show that NA3*Δrad51* cells, harboring repeats 6.5 kb apart, arrest with normal timing, but remain arrested even 8 h after DSB induction.

One of the commonly used markers for G2/M arrest is the Mec1-dependent phosphorylation of the effector kinase Rad53 (25). This phosphorylation was shown to occur in response to Mec1 recognition of the ssDNA formed by resection of the DSB (26). We compared the Rad53 phosphorylation kinetics in NA3*Δrad51* to NA14*Δrad51*. In NA14*Δrad51*, there is a very low level of phosphorylation following 2 h of DSB induction, a level that remains constant at all time points (Figure 4C). In contrast, NA3*Δrad51* shows a higher level of Rad53 phosphorylation 2 h following DSB induction, and there is an increase at the 6 and 8 h time points (Figure 4C). This result indicates that there is an apparent distance-dependent difference in the level of Rad53 phosphorylation, i.e. G2/M arrest signal, in the absence of Rad51. This high level of phosphorylated Rad53 prevents the cells from recovering and reinitiating growth.

The inability to recover from the G2/M arrest is reflected in the rate of cell growth on galactose plates: whereas both NA14 and NA14*Δrad51* cells, (with repeats 3 kb apart) and NA3 (with repeats 6.5 kb apart) formed medium sized colonies after 3 days, NA3*Δrad51* cells needed an additional 48 h to reach the same colony size (Figure S1A). These colonies exhibited death sectors

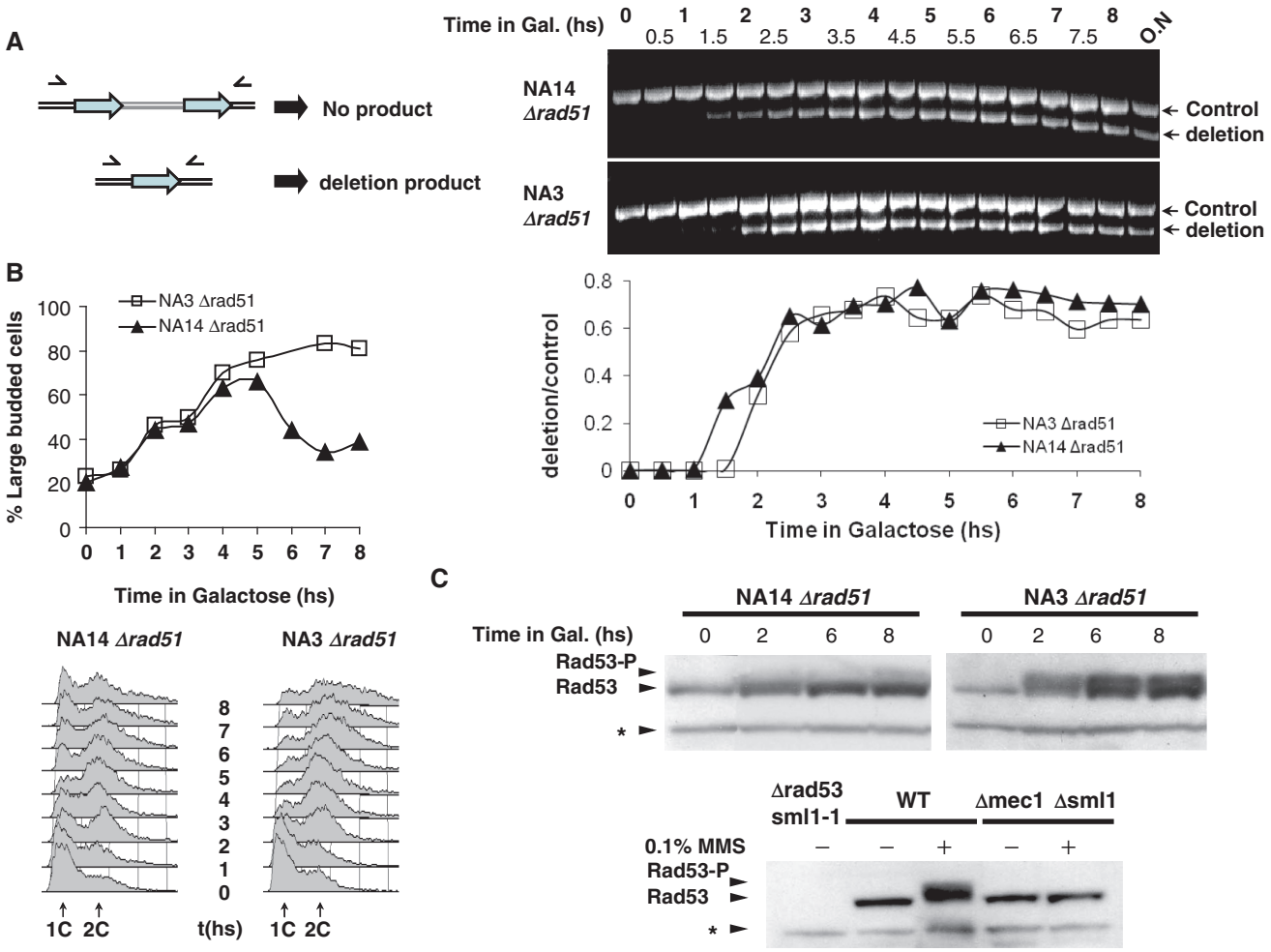

Figure 4. Analysis of repair kinetics in *Δrad51* strains. Repair kinetics was analyzed in yeast strains carrying intrachromosomal repeats 3 kb apart (NA14*Δrad51*) or 6.5 kb apart (NA3*Δrad51*). **(A)** Quantative PCR measuring the relative amount of deletion product at timely intervals following induction of DSB. The picture on the left depicts the assay conditions. The graph below shows the quantification of the deletion product normalized by the control (*STE4*) band. **(B)** Graphic representation of the fraction of large budded cells (up) and FACS analysis (bottom) of cells following induction of DSB. (**C**) Upper panels show a western blot analysis of the activation kinetics of the Rad53 kinase in NA14*Δrad51* and NA3*Δrad51*. The lower panel shows controls: No protein in a *Δrad53sml1-1* strain, MMS- treated/untreated wild-type and *Δmec1Δsml1* strains. The phosphorylated form of Rad53 (Rad53-P) migrates more slowly than the un-phosphorylated form (Rad53). Asterisk marks the location of a non-specific band.

(Figure S1B), indicating a high frequency of cell death during the first generations after recovery in *rad51* cells with far-apart repeats. This effect is short-termed, as no difference in growth rate was observed when cells from 5-day-old colonies on galactose were re-plated on glucose or galactose, (Figure S1B). Since the G2/M arrest is Mec1-dependent (25), we examined the colony formation in a *Δmec1* mutant and in a *Δmec1Δrad51* double deletion strain [these strains are also deleted for the *SML1* gene, necessary for the viability of *mec1* strains, (27)]. When examining the colony formation kinetics in the *Δmec1Δrad51* double mutant the 48 h delay was abolished (Figure S1A), indicating that the defects in recovery depend on the Mec1-controlled G2/M arrest. In contrast to this result, deletion of *RAD9* had no effect on colony formation in either wild type or *Δrad51* backgrounds (data not shown). We thus conclude that repair of a DSB in a repeated sequence in the absence of Rad51 leads to a checkpoint-dependent cell death that delays colony formation. This Rad51-dependence is elevated as the distance between the repeated sequences increases.

Genetic requirements of repair choice

We next investigated the relative importance of various genes that play a role in DSB repair. *RAD59* was first identified in a screen for mutants that abolish Rad51-independent spontaneous mitotic recombination (28). It was found to play a major role in SSA but to have only a mild effect on GC (29). Surprisingly, we found that deletion of *RAD59* causes a dramatic reduction in repair efficiency (28.8% compared to 83% in wt cells; Table 1). Among survivors, deletion products were more abundant (67%) than GC products (33%). These results suggest that Rad59 plays a major role in repair choice in our system. We further deleted *RAD51*, as in the absence of Rad51 the best candidate mechanism for deletion formation is SSA (8). As shown above, NA3*rad51* (6.5 kb between repeats) has a reduced repair efficiency

compared to wt and more than 90% of the repair products are deletions (Table 1). The *rad51 rad59* double mutant exhibited an additional reduction in repair efficiency and showed products similar to those observed in *rad59* cells. Thus, as observed before, *rad51* and *rad59* show additive relations with respect to repair efficiency, but in this case *rad59* is epistatic to *rad51* for the mechanism of repair choice.

The MRX complex, composed of the Mre11, Rad50 and Xrs2 proteins, plays important roles in end processing and checkpoint signaling (1). Deletion of *MRE11* has a significant effect on repair efficiency in our system (31% compared to 83% in wt cells; Table 1) but only a mild difference in the total amount of deletion compared to GC products (Table 1). Interestingly, however, there is a significant *increase* in ectopic events compared to wt cells ($P < 0.005$). This might be due to the known effect Mre11 has on resection rate following the formation of the break (see 'Discussion' section).

We also examined the effect on the choice of repair mechanism when NHEJ was inactivated. Mutations in *DNL4* or in *YKU70* (30) did not show a significant effect on either repair choice or efficiency (Table 1). These results show the specificity of our system for HR mechanisms.

DISCUSSION

Although many laboratories have carried out detailed analyses of the various mechanisms of DSB repair by HR, only few studies have concentrated on the nature of the interactions between them (12–14). We present here for the first time a detailed and systematic analysis of repair choice using a series of isogenic yeast strains, all of which carry several alternative donors for the repair of a single DSB.

Intrachromosomal donors are preferred

In the basic strains presented (NA14, NA15), the cells can choose to repair the DSB using information from either an intrachromsomal or an ectopic donor, both of which are of similar size. We showed that, when faced with this decision, the cells choose to repair the break by interacting with the intrachromosomal sequence in more than 90% of the cases. This result is similar to previous studies carried out at the mating type locus of yeast (14) or at the Rr3 construct in flies (13). The preferential use of intrachromosomal sequences for repair is affected by the size of the ectopic donor (Figure 3A), suggesting an active competition between intrachromosomal and ectopic sequences, probably at the stage of the homology search. Importantly, however, even when using an ectopic donor that is more than 10 times larger than the intrachromosomal one, the choice is still mainly intrachromosomal repair. We found that only in a diploid strain, in which there is an entire chromosome sharing homology with the broken chromosome, there is a preference to repair from the allelic donor over the intrachromosomal donor. However, one must remember that in this strain the intrachromosomal homologous sequence, which is only 1.2-kb long, is used as a donor of information for GC as frequently as the allelic donor, which shares 577 kb of homology with the broken chromosome. Notably, even when the repeats are 80 kb apart there is still ~75% repair using the intrachromosomal donor (Figure 3B).

A model for repair mechanism choice

Our results indicate that the broken chromosome is identified, to be distinguished from the rest of the genome, and that the whole chromosome, and not a restricted region around the DSB, is involved in homology search. This is in accordance to previous results indicating a preference for repair carried out by sequences located at a distance to the DSB (31,32).

The strong preference for repair using intrachromosomal donors may imply that the search is carried out in two consecutive stages, first intra-, then inter-chromosomal. Alternatively, the search encompasses both sequences in *cis* and in *trans*, but homologous sequences are found more readily when present on the same DNA molecule (even at extreme distances) or are utilized more efficiently. An implicit idea in most HR models is that the search for homologous sequences somehow involves the ssDNA filament created by the resection (18). We propose that the homology search is carried out concomitantly with the resection. A stationary helicase pumping DNA in from both broken arms (33), could in principle allow to recognize intra- and inter-chromosomal homology while carrying out extensive resection (Figure 5). If the resection rate is high enough to expose homologous repeats before homology is found and invaded by the searching ends, then the event results in a deletion. If intrachromosomal sequence identity is recognized first, it produces an intrachromosomal GC event. However, if interchromosomal homology is recognized first, depending on resection rate, it could either lead to interchromosomal GC or T-deletion due to gap repair.

Compensation mechanisms

We have shown that in strains in which the DSB occurs within the repeated sequence several repair mechanisms can act, and if one of them is compromised a different one can compensate, resulting in only a slight decrease in repair efficiency (Figure 2). In contrast, DSBs created outside the shared homology resulted almost exclusively in a deletion product, despite the presence of a possible ectopic donor (Figure 2). The strong dependency on Rad1 implies that this repair is by SSA; in the absence of Rad1, no compensation by GC is observed. This result is puzzling, as we know that isogenic strains lacking one of the two repeats are able to repair the DSB using the ectopic donor with almost 100% efficiency [data not shown (15)]. In fact, the reduced levels of repair observed in the double *rad1 rad51* compared to the single *rad1* mutant imply that Rad51 is in fact contributing to survival; interestingly, however, even in this case all the repair events were deletions. We must therefore conclude that the presence of direct repeats (even far apart from each other) determines the fate of the repair choice; when SSA

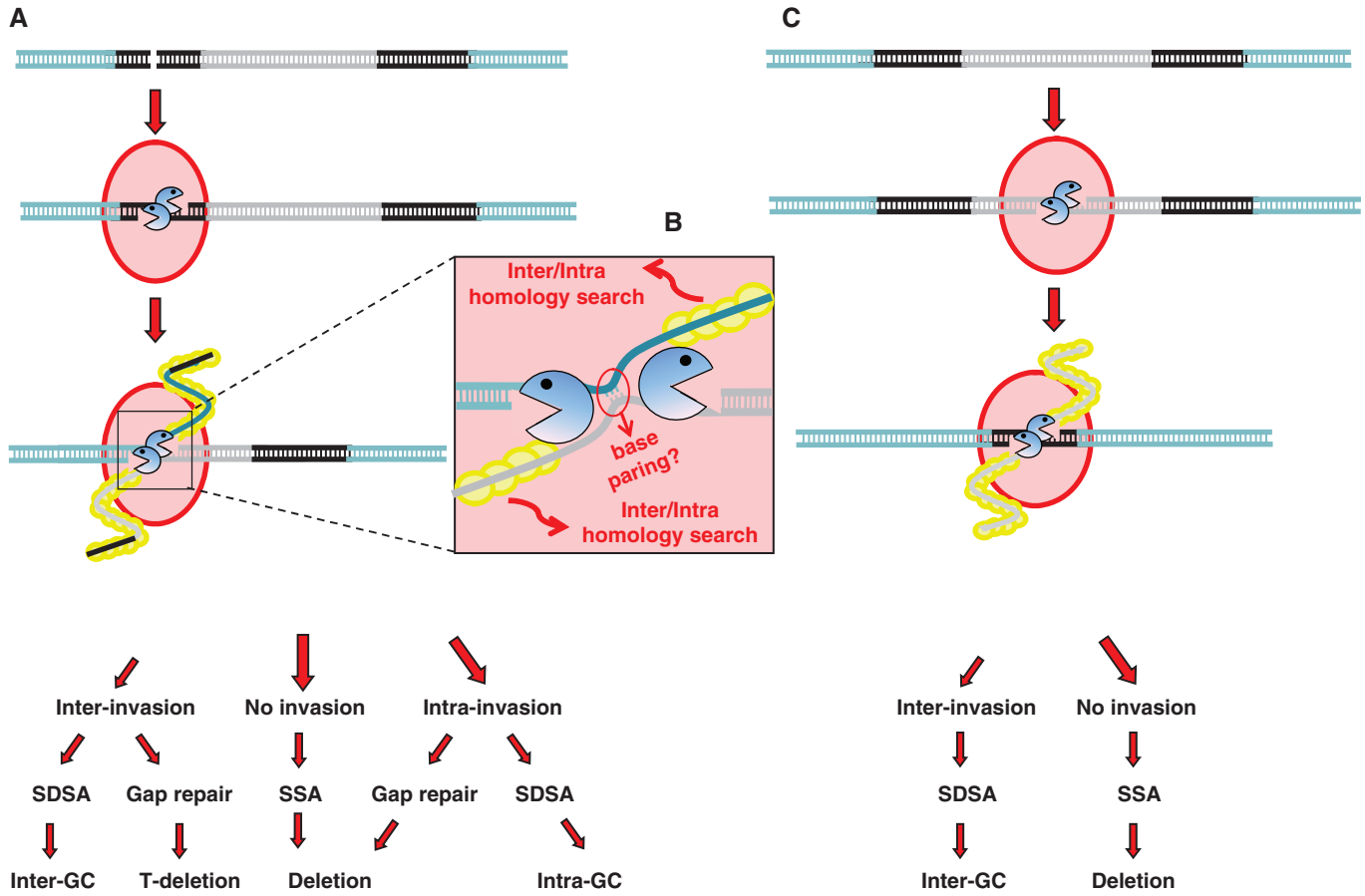

Figure 5. A model for repair pathway choice. (**A**) Repair choice in a strain where the break is located inside one of the intrachromsomal repeats (NA14). Following DSB formation resection is carried out at both sides of the break by a complex recruited to the break (depicted as a pink circle around the lesion). Among these proteins there is a helicase/nuclease that processes the ends and extrudes ssDNA out to allow homology search. The ssDNA exposed following resection is covered by several proteins such as RPA, Rad51 and checkpoint components (yellow circles). (**B**) The zoom-in image shows that while resection is carried out base pairing is examined in the repair complex to find homology between the resected strands (homology in *cis*), while inter- and intra-chromosomal homology is searched by the ends (homology in *trans*). (**C**) Repair choice in a strain where the break is located between the intrachromsomal repeats (NA15). Resection and homology search take place as in (A). The end products of the repair is determined by resection reaching intachromosomal homology first.

is possible other repair mechanisms available are either repressed or unable to act.

We propose that the presence of homologous sequences in the same chromosome can be readily identified during homology search (maybe by an ssDNA strand annealing mechanism) (Figure 5B); if such homology is detected, the homology search can be aborted. In wild type NA14 cells, resection will reach the homologous sequences (in *cis*), and the homology search mechanism will find and invade the homologous sequences (in *trans*), roughly at the same time, resulting in about 40% GC events and 60% deletions (Figure 2C). In contrast, in NA15 resection uncovers the repeats and allows annealing, preventing further homology search. In the absence of overhang removal (*rad1* cells) GC can compensate in the first case, but not in the second (Figure 2D). Interestingly, in NA14 *Δrad1 Δrad51* there is an increase in GC events compared the single *Δrad51* strain. This result indicates the presence of a rad51-independent repair mechanism that can produce GC events, possibly a mechanism which is dependent on resection reaching the intrachromosomal homologous sequence to compensate for the lack of invasion.

Tripartite events

As mentioned above, we reported the occurrence of a repair product consisting of a deletion of the sequences between the two intrachromosomal repeats, but carrying information donated by an ectopic repeat (Figure 2). Similar repair products were previously observed in various systems and were suggested to occur due to GC accompanied by gap repair (24). There are several possible models that could account for such a repair product: (i) A regular GC event could be followed by an independent SSA. (ii) During invasion by one of the broken arms, the second arm may undergo degradation of both DNA strands, leading to a gap that can be repaired during reannealing (Figure 1). (iii) While resection is carried out, both ends can independently invade two different donors (34); when resection reaches the intrachromosomal repeat it can anneal with either one of the ends.

Effect of distance between homologous sequences

With increased distance between repeats, a reduction in deletion events accompanied by an equal increase in GC

events is seen, without diminution in the general repair efficiency. Choosing between GC and deletion might again be dependent on timing. If resection reaches intrachromosomal homology before invasion occurs, homology search through the ends is aborted, and deletion will occur. The further apart the intrachromosomal repeats are from one another, the higher the chances that invasion will occur, leading to an increase in GC events at the expense of deletion events.

An unexpected result observed was that in a *Δrad51* background increasing the distance between the two intrachromosomal repeats reduced the survival (Figure 3D). We showed that this was not the result of a decreased ability to carry out the repair, but rather to recover from the DSB-induced G2 arrest (Figure 4). In *rad51* cells with far apart repeats Rad53 phosphorylation remains high (Figure 4) and colony formation is severely delayed. This delay could be abrogated by deleting the *MEC1* kinase (Figure S1A). In Rad51$^+$ cells, RPA-covered ssDNA appears to be the recruiting substrate of Mec1 (35), which then gets displaced by Rad51, effectively shutting off the checkpoint signal. In the absence of Rad51, D-loop formation is prevented, and when the repeats are separated by longer and longer stretches of heterologous sequences, the extent of resection increases accordingly. Rad51 is not present to displace Mec1 from the chromatin, resulting in Mec1 hyper-activation, which disrupts cell cycle resumption following the completion of DSB repair. Mec1 activity is maintained within the cells at such high levels that it affects cell survival in the successive cell divisions, creating a delay of ~48 h in colony formation. Our results are consistent with observations made by Dubrana *et al.* (36) showing that in the absence or Rad51 there is an elevation in Mec1 recruitment to a DSB site.

Genetic control of repair choice

We have analyzed the role played by various genes in the choice between repair mechanisms. Mutations in *YKU70* or *DNL4*, genes involved in NHEJ, had very little effect, as expected from the relatively minor role of NHEJ in our system (Table 1). Strains deleted for the *POL32* gene had only a slight decrease in DSB repair efficiency, but the reduction was preferentially in GC events: there was a relative increase in deletions, and no EGC was observed. Using a different system, *POL32* has been recently shown to affect BIR, but not GC (6). The fact that deletions are not reduced rules out the possibility that the deletions we observe are BIR-initiated. However, we did observe a small effect of *pol32* on GC events. A role for Pol32 in initiating DNA replication across gaps has been recently proposed (12). It is conceivable that some of the GC events detected in our strains (such as those created by invasion of the intrachromosomal repeat) are created by gap repair.

Rad59, a protein with similarity to Rad52 that was shown to promote DNA annealing *in vitro*, was shown to play a major role in deletion formation by SSA (29,37). We saw a dramatic effect for the *rad59* deletion both in repair efficiency and in repair choice. However, in contrast to previous findings, deletion events were less affected than GC events (Table 1). Thus, Rad59 seems to have additional roles to simply annealing of the homologous sequences during SSA. The epistatic relationship between *rad59* and *rad51* (in which the double mutant displayed the repair products characteristic of *rad59*, and not of *rad51*) suggest an early role for Rad59 in the repair process. The precise mechanism by which Rad59 exerts its effect remains to be elucidated.

We have also examined the role played by Mre11 in our system. As expected for a protein with such a central role in DNA processing and in checkpoint establishment, the efficiency of DSB repair was strongly affected by deletion of *MRE11*. Surprisingly, however, we observed a significant increase in the use of the ectopic donor for repair in *mre11* strains. Deletion of *mre11* was previously shown to decrease the rate of resection compared to wt cells (38). Thus, consistent with our model, slower resection provides more time for the homology search, resulting in GC events. It is important to note, however, that intrachromosomal events are still very strongly preferred over ectopic events (87% vs. 13%, intrachromsomal vs. ectopic events, respectively) even in *Δmre11* cells.

CONCLUSION

The presence of repeated sequences in all genomes sequenced to date represents a challenge for mechanisms of DNA repair based on homology, such as homologous recombination. HR can lead to cancer through loss of heterozygozity or chromosomal rearrangements mediated by HR between highly similar duplicated sequences (39). Moreover, it is becoming increasingly clear that many genomic disorders are due to genomic rearrangements rather than a single mutation (40). Thus, it is important to understand how cells differentiate between the various possible partners for HR. We have systematically explored the parameters affecting the choice of repair mechanism. We provide insights on the mechanisms that allow the cells to make the repair of DSBs as efficient and risk-free as possible, balancing between potentially competing DSB repair pathways.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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