

Gene conversion and deletion frequencies during double-strand break repair in human cells are controlled by the distance between direct repeats

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ABSTRACT

Homologous recombination (HR) repairs DNA double-strand breaks and maintains genome stability. HR between linked, direct repeats can occur by gene conversion without an associated crossover that maintains the gross repeat structure. Alternatively, direct repeat HR can occur by gene conversion with a crossover, or by single-strand annealing (SSA), both of which delete one repeat and the sequences between the repeats. Prior studies of different repeat structures in yeast and mammalian cells revealed disparate conversion:deletion ratios. Here, we show that a key factor controlling this ratio is the distance between the repeats, with conversion frequency increasing linearly with the distances from 850 to 3800 bp. Deletions are thought to arise primarily by SSA, which involves extensive end-processing to reveal complementary single-strands in each repeat. The results can be explained by a model in which strand-invasion leading to gene conversion competes more effectively with SSA as more extensive end-processing is required for SSA. We hypothesized that a transcription unit between repeats would inhibit end-processing and SSA, thereby increasing the fraction of conversions. However, conversion frequencies were identical for direct repeats separated by 3800 bp of transcriptionally silent or active DNA, indicating that end-processing and SSA are not affected by transcription.

INTRODUCTION

DNA double-strand breaks (DSBs) are potentially lethal events that can be repaired by homologous or

non-homologous repair pathways. If left unrepaired, DSBs can lead to chromosome loss or cell death. DSBs are induced by ionizing radiation, X-rays, free radicals, chemicals, nucleases, and they also arise at stalled replication forks (1). DSB repair can occur by non-homologous end-joining (NHEJ) or homologous recombination (HR). Although DSB repair by NHEJ or HR can be accurate, misrepair can have serious genetic consequences. Genomic rearrangements associated with the misrepair of DSBs may lead to carcinogenesis through the activation of proto-oncogenes or inactivation of tumor suppressor genes (1,2). The critical role for HR is underscored by the marked genome instability observed in cells with defects in HR proteins, including BRCA1, BRCA2 and the RAD51 paralogs XRCC2, XRCC3, RAD51B, RAD51C and RAD51D (3–8).

Unlike single-strand breaks and other single-strand damage for which a repair template is readily available, the repair of DSBs by HR requires a search for a homologous template. In genomes with large quantities of repeated sequences, there may be many possible homologous templates. Potential interaction partners include homologous chromosomes, sister chromatids and ectopic sequences linked to the damaged locus or at unlinked sites on homologous or heterologous chromosomes. HR can result in significant genomic changes, including localized or large-scale loss of heterozygosity (LOH), gene deletion and duplication, inversions and translocations. The particular outcome depends on the type of HR event and the arrangement of the interacting regions (2).

HR can occur by conservative and non-conservative mechanisms. Gene conversion is conservative, involving non-reciprocal transfer between donor and recipient loci; for DSB-induced events, the broken locus is almost always the recipient. Gene conversions without crossovers preserve the gross structure of the genome, leading only to localized LOH. However, conversions with associated crossovers in homologous chromosomes result in LOH of all genes from the point of the crossover to the telomere in 50% of subsequent

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mitotic divisions. Crossovers between linked direct repeats result in deletion of one repeat and sequences between repeats as a circular molecule that is usually mitotically unstable. Crossovers between sister chromatids (unequal sister chromatid exchange) yield the same deletion in one daughter cell and a triple-repeat structure in the other daughter cell. Single-strand annealing (SSA) in direct repeats is a non-conservative HR mechanism that also deletes one repeat and sequences between repeats, but in this case the deleted DNA is degraded. SSA between unlinked loci can result in translocations, but this is thought to require DSBs at both loci, as for NHEJ-mediated translocations (9). Because crossovers are suppressed in mammalian cells (9–11), most direct repeat deletions are thought to result from SSA. For simplicity we describe gene conversions without associated crossovers as ‘conversions’ and deletions by any mechanism as ‘deletions.’

Several factors may influence the direct repeat conversion: deletion ratio. For example, in yeast, conversions accounted for 20–50% of DSB-induced HR between 1.2 kb repeats (12–14), but 94% with 6.5 kb repeats (15). Similarly in mammalian cells, conversions accounted for 17–60% of events with two different repeats ~0.7 kb in length (16–18), but 97% with 1.4 kb repeats (19). These results suggest that conversion is favored with longer repeats. However, the yeast repeat systems also differed in that the longer repeats flanked an autonomously replicating sequence (15). To date, there have been no systematic studies of the effects of the composition of sequences between repeats on the conversion:deletion ratio. It should also be noted that increasing repeat size effectively increases the distance between homologous sequences within repeats. In a yeast plasmid-based system it was shown that the conversions increase with the increasing distance between repeats (20). However, plasmid-based systems may not mimic chromosomal systems, particularly when HR occurs immediately after plasmids are introduced into cells. For example, essentially all DSB-induced HR between extrachromosomal direct repeats in mammalian cells results in deletions (21,22), but chromosomal repeats yield conversions as a common or predominant outcome (16–19).

In the present study we tested the effect of varying the distance between otherwise identical 1.4 kb *neo* direct repeats in human cells. All HR substrates were targeted to a specific locus to eliminate potential position effects. We show that the conversion:deletion ratio increases with increasing distance between repeats. However, even when repeats were separated by 3.8 kb, the conversion frequency was only ~40%, whereas a related HR substrate in CHO cells with repeats separated by the same distance gave 97% conversions (19). One difference between the low and high conversion substrates was the presence of an active transcription unit between repeats in the latter substrate. We hypothesized that an active transcription unit between repeats inhibits end-processing and SSA, and thereby increases conversions. However, adding a transcription unit did not affect the conversion frequency. These results indicate that the relative frequencies of direct repeat conversion and deletion in human cells are influenced by the distance between repeats, and by factors other than the transcriptional status of the intervening sequences, such as chromosomal position or cell type.

MATERIALS AND METHODS

Plasmid DNAs

Plasmids were manipulated and prepared as described previously (23). Plasmids pcDNA5/FRT and pOG44 were purchased from Invitrogen (Carlsbad, CA). pcDNA5/FRT was modified such that the Flp-recombinase target site (FRT) and hygromycin-resistance gene (*hyg*) were flanked by MfeI sites, creating plasmid p2XMfeI. Plasmid pOG44 carries the Flp-recombinase gene regulated by the CMV promoter. Plasmids pDR1/FRT, pDR2/FRT and pDR3/FRT each contain 1.4 kb *neo* direct repeats. The distance between *neo* repeats differs in these plasmids; in pDR1/FRT and pDR2/FRT, the DNA between repeats is from pSV2neo, and in pDR3/FRT there is an additional 2.2 kb fragment carrying the yeast *LEU2* gene. Plasmid pDR4/FRT is identical to pDR3/FRT except that *LEU2* was replaced by an active transcription unit comprising an SV40 promoter driving the *Escherichia coli gpt* gene (*SVgpt*). Plasmid pDP/FRT is identical to pDR1/FRT except it carries a third copy of *neo*. The pDR and pDP plasmids all have the FRT site and *hyg* from p2XMfeI. One copy of *neo* (recipient) is regulated by the SV40 promoter but is inactivated by a 29 bp insertion containing the I-SceI cleavage site within the natural BanII site (19). The other copies of *neo* (donors) have wild-type coding capacity but are inactive because they lack promoters. Donor *neos* have either a wild-type BanII site or a silent mutation that converts the BanII site to a BsaI site; the silence of the BsaI mutation was confirmed in direct transfection experiments and in HR experiments in which both the donors were present (E. Schildkraut and J. A. Nickoloff, unpublished data). Because the wild-type BanII site and silent BsaI mutation occur opposite of the I-SceI insertion, these donors present identical homologous regions and are interchangeable. The I-SceI expression vector pCMV(3xNLS)I-SceI, and negative control vector pCMV(I-SceI⁻) with the I-SceI nuclease in reverse orientation, were described previously (24,25).

Targeting HR substrates to human 293 cells

Flp-In 293 (human embryonic kidney) cells (Invitrogen) were cultured in DMEM with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified incubator with 6% CO₂ at 37°C. Plasmids with *neo* direct repeats and *hyg* were targeted to Flp-In 293 cells by co-transfection with pOG44. Correct targeting activates the *hyg* gene and interrupts an endogenous zeocin-resistance gene (*zeo*). Stable transfectants were selected in medium with 100 µg/ml hygromycin and then tested for sensitivity to zeocin (Invitrogen). Correct targeting was confirmed by PCR using primers P1 (5'-GGCCTCTGAGCTATTCCAGA) and P2 (5'-GACCAA-TGCGGAGCATATAC) (Figure 1). Southern hybridization was used to ensure that HR substrates were intact and also to further verify targeting. Genomic DNA was digested with EcoRI, separated on 0.8% agarose gels, transferred to nylon membranes and hybridized with a ³²P-labeled *neo* fragment (Figure 2).

Recombination assays

DSB-induced HR was assayed as follows. For each experiment, 5 × 10⁵ cells were seeded into 3.5 cm (diameter) wells, incubated for 24 h and lipofected with 2 µg of either

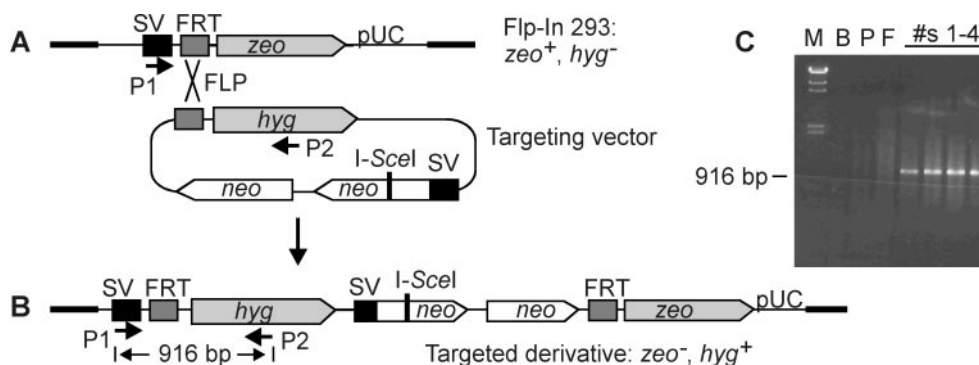


Figure 1. Targeting strategy. (A) The parent Flp-In 293 cells have a single integrated copy of a vector with an FRT target site between an SV40 promoter and *zeo*. Each targeting vector has an FRT site upstream of a promoterless *hyg* gene and *neo* repeats (not to scale). (B) Flp-mediated targeting links the SV40 promoter to *hyg*, creating *hyg*⁺, *zeo*⁻ derivatives. (C) Confirmation of targeting by PCR. Primers P1 and P2 amplify a 916 bp fragment in correctly targeted substrates. Lane M = λ /HindIII size marker; lane B = blank (no template) control; lane P = parent human 293 DNA; lane F = Flp-In 293 DNA; and #s 1–4 = four targeted derivatives.

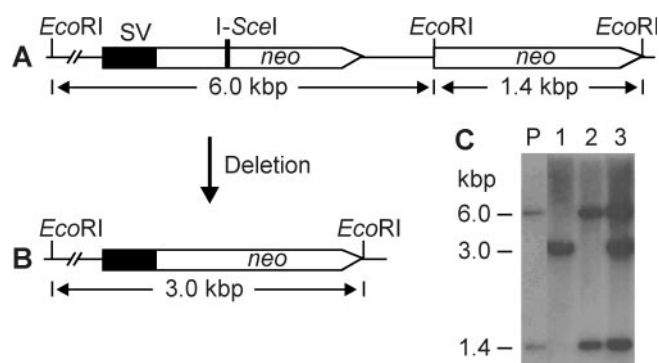


Figure 2. Southern blot strategy. (A) Map of *neo* direct repeats. In this example, *neo* genes are separated by 1.6 kb. Parent and gene conversion structures both yield 1.4 and 6.0 kb EcoRI fragments when probed with *neo*. (B) Deletion produces a single 3.0 kb EcoRI fragment. (C) Representative Southern blot patterns with the 1.6 kb spacer. Lane P = parental DNA. G418^r HR products are shown in lane 1 (deletion), lane 2 (gene conversion) and lane 3 (mixed deletion + gene conversion). Analogous patterns were obtained with 0.85 and 3.8 kb spacers (data not shown).

pCMV(3xNLS)I-SceI to induce DSBs or the negative control vector pCMV(I-SceI⁻). Twenty-four hours post-transfection, 10⁵ cells were seeded into each of the four 10 cm dishes. After an additional 24 h, G418 was added to a final concentration of 750 μ g/ml and the cells were incubated for 14 days. Cell viability was measured by seeding appropriate numbers of transfected cells into non-selective medium and scoring colony formation after 12–14 days. HR frequencies were calculated as the ratio of the total number of G418-resistant (G418^r) colonies to the number of viable cells plated in the selective medium. G418^r colonies were expanded in 10 cm dishes and genomic DNA was prepared using DNeasy tissue kits (Qiagen Inc., Valencia, CA). The recipient allele was amplified by PCR using primers specific for the SV40 promoter (5'-GCC-CAGTTCGCCCATCTC) and the 3' end of *neo* (5'-CGA-AATCTCGTGATGGCAGG), and HR was confirmed for each G418^r product by showing that the resulting PCR products were cleaved by BanII or BsaI, and resistant to cleavage by I-SceI. Southern hybridization of EcoRI digested genomic DNA, as described above, distinguishes gene conversions from deletions (Figure 2). For each cell line, G418^r products

were isolated from 1 to 3 independent experiments. In cases where products were isolated from more than one experiment, similar product spectra were observed and all data for a single cell line were pooled.

RESULTS

Experimental design

To investigate factors that regulate conversion:deletion ratios for DSB-induced HR events in human cells, we constructed cell lines with one of the five HR substrates shown in Figure 3. Each substrate had 1.4 kb *neo* repeats. One *neo* was regulated by the SV40 promoter and inactivated by insertion of an I-SceI recognition site; the other copies had wild-type coding potential but were inactive because they lacked promoters. In four of the HR substrates the sequences between the *neo* repeats included vector DNA or vector DNA plus a 2.2 kb fragment carrying the yeast *LEU2* gene; hence these intervening sequences were not transcriptionally active in human cells. In the fifth substrate, the intervening sequences included an active transcription unit (SVgpt). All HR substrates were targeted to an FRT site in human 293 kidney cells; targeting eliminates variation in HR outcome caused by chromosome position effects and allows direct comparisons among the HR substrates. HR was stimulated by transient expression of I-SceI nuclease and G418^r HR products were selected, and HR frequencies and outcomes were determined as described in Materials and Methods. HR frequencies with the five HR substrates are shown in Table 1. Variations in spontaneous and DSB-induced HR frequencies among HR substrates probably reflect differences in the number of pre-existing recombinants in each population, and day-to-day variation in transfection efficiency. Nonetheless, HR was increased by ~10-fold or more upon I-SceI expression with each HR substrate, thus ensuring that the resulting HR product spectra reflect primarily DSB-induced events.

Conversion:deletion ratios with double and triple repeat HR substrates

We previously reported that DSB-induced HR between 1.4 kb *neo* direct repeats in CHO cells resulted in 97%

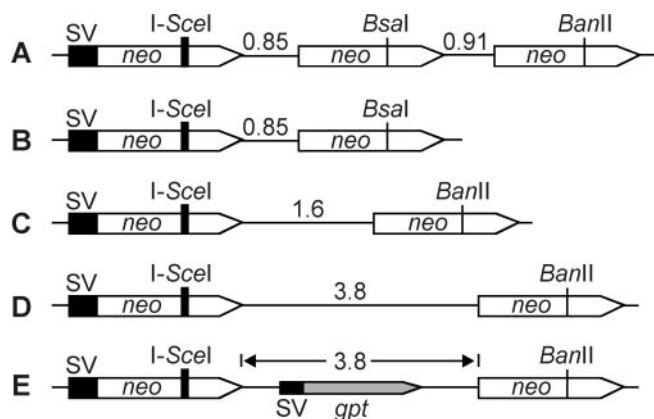


Figure 3. Structures of HR substrates. Each HR substrate contains a recipient *neo* regulated by the SV40 promoter and inactivated by an I-SceI site insertion. Donors lack a promoter and contain either the wild-type BanII site or a silent BsaI mutation in place of BanII. Distances (in kb) between *neo* repeats are indicated. Each *neo* repeat is 1.4 kb. (A) Triple repeat substrate. (B–D) Direct repeat substrates with increasing amounts of non-transcribed DNA between *neo* genes. (E) Direct repeat substrate with a transcriptionally active SVgpt gene between *neo* repeats.

Table 1. HR Frequencies in *neo* direct repeat strains

HR substrate ^a	HR frequency ($\times 10^3$) ^b	
	Spontaneous	DSB-induced
A	0.38	11.2
B	0.31	3.2
C	1.4	14.7
D	2.4	18.9
E	0.45	12.3

^aShown in Figure 1.

^bHR frequencies for cells transfected with control vector (spontaneous) or I-SceI expression vector (DSB-induced).

conversions (19). In that study, the repeats were separated by 3.8 kb. In a separate study of donor preference using HR substrates with three 1.4 kb *neo* repeats Flp-targeted to human 293 cells, including the pDP/FRT substrate with one recipient and two donors (Figure 3A), we found that only 2 of the 14 products (14%) arose by conversion. These markedly different conversion frequencies could be due to several factors including differences in the number of repeats, the distance or composition of the DNA between repeats, chromosomal position and cell type. To test if the third *neo* enhances deletions, we examined HR products from a related HR substrate with two *neo* genes targeted to the same locus in 293 cells (Figure 3B). Of 38 HR products from this strain, only 1 arose by conversion (2.6%). Thus, the low conversion frequency with the triple repeat HR substrate was not due to the third copy of *neo*.

The conversion:deletion ratio is proportional to the distance between direct repeats

The low frequency of conversion when *neo* repeats were separated by 855 bp (Figure 4) is consistent with a general trend in the literature indicating that conversion rates are proportional to distances between repeats in mammalian cells

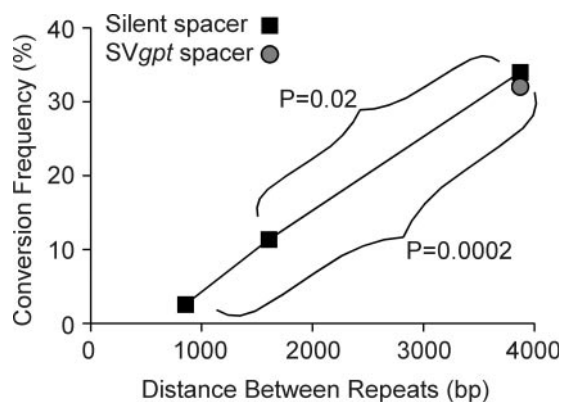


Figure 4. Conversion frequency increases with the increase in distance between the repeats. Significant differences are indicated (*P* values calculated with Fisher's exact tests).

Table 2. Reported DSB-induced HR outcomes in mammalian chromosomal direct repeats

Cell type	Repeat length (bp)	Distance between repeats (bp)	Conversion frequency (%)	Reference
CHO	6800	9600	82	(47)
CHO	1400	3800	97	(19)
Human HT1080	1400	3800	98	(32)
CHO	700	2000	33	(18)
Mouse ES	700	2000	17	(16)
Mouse ES	659	1300	43–61	(17)

(Table 2). However, the systems used in these studies differ in several other respects, such as repeat size, chromosomal position and cell type. We therefore tested whether the conversion frequency would increase with increasing distance between repeats by targeting to human 293 cells two HR substrates with *neo* repeats separated by 1.6 kb (Figure 3C) and 3.8 kb (Figure 3D). We analyzed 37 G418^r products from the 1.6 kb strain and found two arose by complex rearrangement, and four of the remaining 35 (11%) arose by conversion. We then analyzed 53 HR products from the 3.8 kb strain and found 18 (34%) arose by conversion. Thus, conversion increases as a linear function of distance between direct repeats (Figure 4).

The conversion:deletion ratio is not affected by a transcription unit between direct repeats

Although increasing the distance between repeats increased conversion rates, dissimilar conversion rates were obtained with 3.8 kb separating 1.4 kb *neo* repeats in CHO cells (97%) (19) and human 293 cells (34%), indicating that conversion frequency is influenced by additional factors. In the CHO HR substrate, the *neo* genes were separated by an active transcription unit (SVgpt) whereas in the substrate used here the *neo* genes were separated by SV40 DNA and yeast *LEU2*, which are not transcriptionally active in human cells. Because most or all deletions arise by SSA, and SSA requires extensive strand resection to expose complementary single strands in the *neo* repeats, we reasoned that a transcription unit between

repeats might slow resection, for example, if the transcription machinery interfered with the enzyme(s) responsible for end-resection. Reduced end-resection would inhibit SSA and increase the frequency of gene conversion. To test this concept, we replaced the 2.2 kb *LEU2* fragment with a 2.2 kb *SVgpt* fragment (Figure 3E) and targeted the resulting HR substrate to human 293 cells. We inserted the *SVgpt* fragment so that *gpt* was transcribed in the same direction as *neo* to allow comparisons with our prior substrates and because most published systems use analogous configurations. Among 25 HR events from the *SVgpt* strain, 8 (32%) arose by gene conversion (Figure 4), 17 arose by deletion and no complex events were detected. Thus, the addition of a transcription unit between repeats had no effect on the conversion frequency, suggesting that the transcription machinery does not affect end-resection. Other factors that may control the relative frequencies of conversions and deletions are discussed below.

DISCUSSION

HR between direct repeats has been examined with a variety of substrates in plasmid or chromosomal contexts in a variety of cell types. Each system gives a characteristic conversion:deletion ratio, but because systems differ in more than one parameter, it has been difficult to pinpoint factors that control this ratio. Early studies of transfected plasmids with DSBs within or outside direct repeats revealed that essentially all HR in these substrates resulted in deletions, and that HR efficiency depended on the position of the DSB relative to the repeats. Thus, HR is very efficient when the DSB is equidistant from the two repeats, and it decreases as the DSB is moved to more asymmetric positions (21,22). This observation led to the model that these HR events arise by SSA which requires end processing to expose single-stranded regions in both repeats to allow complementary strands to anneal. In this model, dependence of SSA efficiency on DSB symmetry reflects the requirement for simultaneous exposure of complementary single strands in the two repeats. However, results with transfected plasmids cannot be extrapolated to chromosomal repeats because plasmid-borne repeats are linked by two segments, 5'→3' single strand resection eventually degrades both strands, and DNA ends in transfected (naked) plasmid DNA and chromatin are likely to be processed at different rates and/or to different extents. The first analysis of DSB-induced HR products in mammalian chromosomal direct repeats underscored these differences, with 97% of HR occurring by gene conversion (19).

Subsequent studies suggested that conversion frequencies are proportional to the distance between repeats and/or the length of the repeats themselves (Table 2). In yeast chromosomal direct repeats, the fraction of conversions increases with increasing distance between repeats (26). The present study is the first systematic examination of the effect of repeat separation on the conversion:deletion ratio in a mammalian chromosomal context. The decrease in deletions with longer distances between repeats (Figure 4) is consistent with the view that SSA requires simultaneous exposure of complementary single strands in the two repeats, as proposed for plasmid-borne repeats (21,22). Note, however, that even with widely spaced repeats or highly asymmetric DSBs, transfected plasmid

substrates yield essentially 100% deletions (albeit at low frequency). Thus, the chromosomal context appears to enhance conversion; this enhances genome stability, although the effect is minimal when repeats are separated by <1000 bp (Figure 4). The position of the DSB within a repeat may also control the conversion frequency as this too would influence the timing of complementary single-strand exposure.

In yeast, gene conversion requires Rad51, Rad52 and Rad54, and is facilitated by the Rad51 paralogs Rad55 and Rad57. In contrast, SSA is independent of all of these proteins except Rad52 (27,28), presumably because of Rad52's strand annealing activity (29). The role of mammalian RAD52 has been questioned because mouse *rad52* knockouts show minimal sensitivity to DNA-damaging agents, minimal HR defects, and do not show the serious cell growth defect characteristic of mouse *rad51* knockouts (30,31). It is presently unclear whether RAD52 is required for SSA in mammalian cells, but this can be addressed using the HR substrates described here coupled with RNAi knockdown of endogenous RAD52. In any case, the observed linear relationship between conversion rates and the distance separating repeats (Figure 4) suggests a dynamic competition between RAD51-dependent strand invasion leading to gene conversion and SSA. Both mechanisms depend on strand resection from broken ends. As the distance between repeats increases, the extent of strand resection required for SSA increases, and this in turn increases the likelihood that processed ends will invade a donor template and complete repair by gene conversion before complementary single-strands are exposed and annealed.

Although our data show that conversion increases with distance between repeats, it is clear that additional factors control the conversion:deletion ratio. An HR substrate with 1.4 kb *neo* repeats separated by 3.8 kb in CHO cells gave 97% conversions (19), but the frequency in a closely related substrate in human cells was only 34%. We ruled out the possibility that a transcription unit between repeats inhibited deletions. Although we did not test whether the orientation of a central transcription unit influences the conversion frequency, this cannot account for the distinct results in our CHO and human cell experiments as *SVgpt* was in the same orientation in these substrates. There are several other factors that may influence the conversion:deletion ratio, including cell species, cell type and structural/environmental differences. It is difficult to account for the distinct conversion rates on the basis of species differences because the HR substrate with a high conversion frequency in CHO cells had similarly high frequency when randomly integrated into human HT1080 cells (98%) (32). This does not rule out potential effects from other cell type differences, such as p53 status. p53 is known to suppress HR (33–38), and p53 is active in HT1080 cells (39,40) but inactive in human 293 cells (41). However, although a direct analysis of p53 effects on the conversion:deletion ratio has not yet been reported, it is unlikely that the low conversion frequency we observed in human 293 cells is due to the p53 defect, because wild-type p53 strongly suppresses conservative HR (conversion) (38). Thus, conversion would be predicted to be relatively efficient in p53 defective cells, yet the opposite was observed.

There are several structural differences between the HR substrates with high and low conversion rates that might account for their distinct behavior. The high conversion HR

substrate has 12 single-base heterologies in the donor *neo* but these are absent in the low conversion substrate. It is not clear how heterologies would preferentially inhibit SSA and/or promote conversion. In yeast, a single heterology affects spontaneous HR frequencies (42), but the efficiency of DSB-induced gene conversion is not affected by similar heterology densities (~ 1 single-base change per 100 bp) (43). Another difference is that the transcriptionally active *neo* genes in the high and low conversion substrates were regulated by mouse mammary tumor virus and SV40 promoters, respectively, but again, it is not clear how promoters might influence conversion frequencies. In fact, it is best to consider the promoter differences as part of a larger set of differences owing to the distinct chromosomal environments of these HR substrates. It is possible that differences in chromatin structure at different loci influence the rate of strand excision, which in turn would influence the frequencies of conversion and SSA. Differences in chromatin appear to influence spontaneous gene-conversion rates (44). Nonetheless, at any particular locus, conversion frequencies are likely to increase with distance between repeats, as observed in the present study.

Although the additional factors that regulate conversion rates in direct repeats remain obscure, it is clear that the conversion:deletion ratio is under genetic control. Thus, BRCA2 mutant cells, which display severe genome instability (5,45) and deficient HR-mediated DSB repair (5,46), also display a marked shift in direct repeat HR from conversion toward deletions (17). These results suggest the possibility that other HR proteins regulate conservative and non-conservative HR mechanisms, and underscore the importance of such control in tumor suppression.

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