

## Gene Conversion Tracts from Double-Strand Break Repair in Mammalian Cells

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**Mammalian cells are able to repair chromosomal double-strand breaks (DSBs) both by homologous recombination and by mechanisms that require little or no homology. Although spontaneous homologous recombination is rare, DSBs will stimulate recombination by 2 to 3 orders of magnitude when homology is provided either from exogenous DNA in gene-targeting experiments or from a repeated chromosomal sequence. Using a gene-targeting assay in mouse embryonic stem cells, we now investigate the effect of heterology on recombinational repair of DSBs. Cells were cotransfected with an endonuclease expression plasmid to induce chromosomal DSBs and with substrates containing up to 1.2% heterology from which to repair the DSBs. We find that heterology decreases the efficiency of recombinational repair, with 1.2% sequence divergence resulting in an approximately sixfold reduction in recombination. Gene conversion tract lengths were examined in 80 recombinants. Relatively short gene conversion tracts were observed, with 80% of the recombinants having tracts of 58 bp or less. These results suggest that chromosome ends in mammalian cells are generally protected from extensive degradation prior to recombination. Gene conversion tracts that were long (up to 511 bp) were continuous, i.e., they contained an uninterrupted incorporation of the silent mutations. This continuity suggests that these long tracts arose from extensive degradation of the ends or from formation of heteroduplex DNA which is corrected with a strong bias in the direction of the unbroken strand.**

DNA double-strand breaks (DSBs) compromise the genetic integrity of all organisms, since failure to repair a broken chromosome can result in loss of genetic information and inappropriate repair of DSBs can lead to defects such as chromosomal translocations. In striking contrast to the repair of DSBs in *Saccharomyces cerevisiae*, which occurs primarily by homologous recombination (11), repair of DSBs in mammalian cells has been assumed to occur primarily by nonhomologous recombination (12). The recent examination of DSB repair in mammalian cells, however, has contrasted with this view, demonstrating that repair of DSBs by homologous recombination can occur as a substantial proportion of repair events (15, 27, 28). Consistent with this, DSBs in mammalian cells can stimulate recombination to a high level (13), upwards of 1,000-fold or more, for allelic recombination (19a), intrachromosomal recombination (15, 28, 39), and gene targeting (6, 15, 27, 33).

Several models have been proposed for the repair of DSBs in mammalian cells and fungi, including the double-strand-break-repair model (38). In this model, the broken ends of chromosomal DNA are degraded to create a gap, which is repaired from an unbroken homologous template. The broken ends initiate recombination by invading the homologous template. As a result, heteroduplex DNA, consisting of paired strands from both the unbroken and broken recombination substrates, is present at the boundaries of the gap. Two Holliday junctions are formed at the boundaries of the heteroduplex and are subsequently resolved to give crossover or non-

crossover products. Evidence from yeast meiotic and mitotic recombination studies suggests a modification of this model in which double-strand gaps are not formed; instead, conversion involves mismatch repair of heteroduplex DNA formed upon strand invasion and branch migration or by annealing subsequent to repair synthesis (30, 34). DSB repair by this mechanism is conservative.

Alternative models for DSB repair have been proposed, including a migrating D-loop model in which only one of the broken strands invades the homologous repair template and where DSB repair is coupled to recombination-dependent replication (9, 14, 18, 25). Studies of extrachromosomal plasmid recombination in mammalian cells have led to the proposal of a nonconservative model of homologous recombination, termed single-strand annealing (16). In the single-strand annealing model, both recombination substrates must be cleaved at or near the region of homology. This is a nonconservative mechanism, since sequence information is lost.

Homologous recombination can be suppressed by sequence divergence between recombining substrates. Substrates with amounts of sequence divergence limited to a few percent or less recombine at reduced levels compared to fully homologous sequences, whereas sequence divergence of 10 to 20% can nearly abolish recombination in some systems (29, 31, 42). Both the overall amount of heterology and the longest stretch of uninterrupted homology contribute to the suppression of recombination, with the latter factor likely having a more pivotal role (43). This suppression has the outcome of preventing recombination between DNA of diverged species, as well as of limiting recombination between highly diverged repetitive elements in genomes. It has been shown in several studies that some of the barrier to recombination between homologous, but not identical, DNAs can be partially overcome in mismatch

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repair mutants (2, 5, 7, 22, 24), implicating this system as a guardian of the genome against unwanted exchanges.

Since DSBs are such potent inducers of recombination, we have designed a system to examine the effect of heterology on DSB-induced recombination in mammalian cells. In this system, a cleavage site for the rare-cutting endonuclease *I-SceI* is integrated into the genome of cells (27). As expression of this endonuclease *in vivo* is not toxic (27a), an expression vector for the endonuclease is introduced along with homologous substrates from which to repair the chromosomal DSB. The repair substrates contain various amounts of heterology, from 0.1 to 1.2%. We have examined the effects of sequence divergence on both the frequency of recombination and the gene conversion tracts that result from DSB-induced recombination.

#### MATERIALS AND METHODS

**Plasmid constructs and DNA manipulations.** The multiply marked *pneo* substrates *pneo-5mu*, *pneo-7mu*, and *pneo-8mu* were derived from *pneo12* and intermediates produced during construction of *pneo12* (39). To construct the *pneo-mu* plasmids, the 745-bp *EagI/XbaI neo* gene fragment from *pneo12* or an intermediate plasmid was subcloned into the *EagI/XbaI* sites of BluescriptII KS+. For the homologous wild-type *neo* fragment in the control plasmid *pneo-WT*, the *EagI/RsrII* fragment from *pMClneoA2* (41) replaced the corresponding *EagI/RsrII* fragment in *pneo-5mu*. The mouse embryonic stem (ES) cell line clone 12 contains a randomly integrated single copy of the *S2neo* gene (33). Integrated with the *S2neo* gene in the genome of this ES cell line are vector sequences which are homologous to the vector sequences in the *pneo* substrates. This homology extends for at least 700 bp downstream from the *neo* gene and is separated from the *neo* gene homology by approximately 100 bp of unrelated sequence, which is composed of 3' *neo* gene sequences for *S2neo* and vector sequences for *pneo*.

**Cell culture and transfections.** ES cells were cultured on gelatin-coated dishes, as previously described (26), in the presence of  $10^3$  U of leukemia inhibitory factor (ESGRO; Gibco Life Science) per ml. For each sample of cells for transfection,  $2 \times 10^7$  cells in 1 ml of phosphate-buffered saline were electroporated with 25  $\mu$ g of each uncut plasmid DNA in a 0.4-cm-electrode-gap cuvette (250 V, 960  $\mu$ F). Electroporated cells were aliquoted into five 10-cm-diameter dishes. Colonies were selected in 200  $\mu$ g of G418 (Geneticin; Gibco Life Science) per ml beginning 20 to 24 h after electroporation and were grown in selective medium for 10 to 14 days before colony counts or colony expansion.

**PCR analysis.** A region of the chromosomal *neo* gene in the ES clone 12 cells and in *neo*<sup>+</sup> colonies was PCR amplified with primers Neo1 and Neo2 (27, 33). Amplified products were digested with *I-SceI* and appropriate restriction enzymes and electrophoresed on 0.8 to 1.3% agarose gels (25% agarose/75% Nusieve agarose).

#### RESULTS

**Gene targeting with diverged repair substrates.** To analyze DSB-induced recombination between diverged sequences, we developed a gene-targeting assay in which the target chromosomal locus contains an *I-SceI* endonuclease cleavage site that can be repaired by diverged sequences in circular plasmids following an *I-SceI*-induced DSB at the locus. The chromosomal locus contains a *neo* gene (termed *S2neo*) which has been mutated by insertion of the 18-bp *I-SceI* cleavage site (Fig. 1A). The *I-SceI* site was inserted into an *NcoI* restriction site, with the mutation resulting in a 4-bp deletion of *neo* gene sequence, as well as the insertion of the 18-bp cleavage site. Restoration of a functional *neo* gene has been found to be dependent upon recombination with a correcting *neo* gene fragment (15, 33). The *I-SceI* endonuclease is transiently expressed in cells from a phosphoglycerate kinase 1 promoter in the expression vector *pPGK3xnlsl-SceI* (8).

The circular repair plasmids that were introduced contain internal *neo* gene fragments to restrict repair to a defined recombination pathway. With this design, selected recombination events are limited to conservative gene conversion events that do not involve crossing-over (Fig. 1B). Recombination with an associated crossover would result in *neo* gene disruption by plasmid sequences. We were particularly interested in

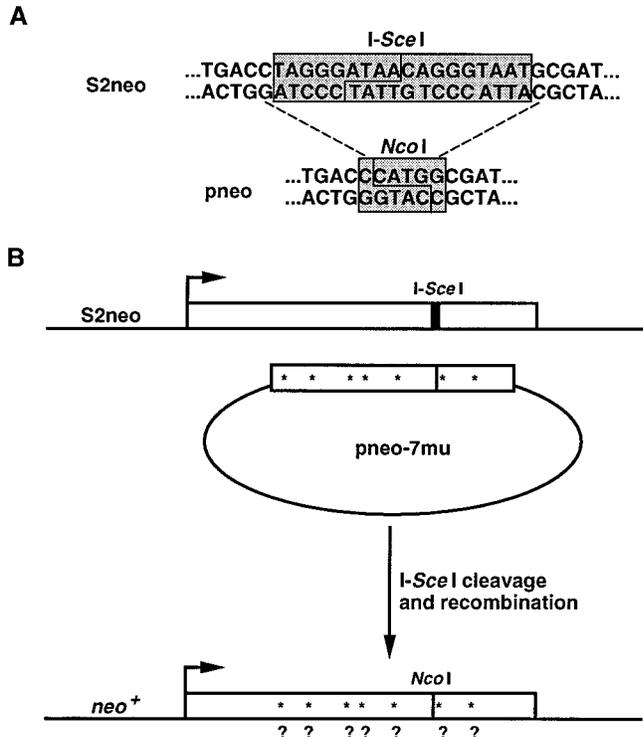


FIG. 1. DSB-induced gene targeting with diverged repair substrates. (A) Sequences of the chromosomal *S2neo* gene and of the correcting *pneo* plasmids at the position of the *I-SceI* cleavage site. The *I-SceI* endonuclease recognizes an 18-bp nonpalindromic site and cleaves to produce four-base 3' overhangs, as indicated. The first three bases of the *I-SceI* site create a stop codon (TAG) in the *neo* coding sequence, making the *S2neo* gene a nonfunctional *neo* gene. The *pneo* plasmids have a wild-type *neo* gene sequence at this position, which is located at an *NcoI* restriction site. Note that the *S2neo* gene has a deletion of the four-base overhangs of the *NcoI* site which creates a nonrevertable deletion mutation. (B) DSB-induced gene targeting occurs when the chromosomal *S2neo* gene integrated in the genome of ES cells is cleaved *in vivo* at the *I-SceI* site (thick vertical bar) and is repaired from a transfected *pneo* plasmid. As an example, recombination is shown with plasmid *pneo-7mu*, which contains the correcting *neo* gene sequence at the *NcoI* site (thin vertical bar). The *pneo-7mu* repair substrate also contains seven silent mutations which create new restriction sites (asterisks). Recombination yields a functional *neo*<sup>+</sup> gene which can be analyzed for the conversion of the silent mutations (?).

gene conversion unassociated with crossing-over since this pathway has been predicted to be a major recombinational repair pathway for chromosomal DSBs in mitotically growing cells (20).

Each of the repair substrates, the *pneo* plasmids, contains a 745-bp internal *neo* gene fragment that is homologous with the chromosomal *S2neo* gene (Fig. 2). The diverged sequences in the repair substrates are base substitutions in the *neo* gene that are phenotypically silent mutations at third base codon positions, with each mutation creating a new restriction site (Fig. 2). In addition to the correcting sequence at the *NcoI* site, five to eight silent mutations were incorporated in the 745-bp *neo* gene fragment. As larger numbers of mutations are incorporated in the repair substrates, the stretches of perfect homology in the *neo* fragment decrease and the overall percent divergence of the fragment increases. Thus, in *pneo-5mu*, which contains five silent mutations, the longest stretch of perfect homology is 206 bp and there is an overall divergence of 0.8%. *neo* gene fragments in *pneo-7mu* and *pneo-8mu* contain seven and eight silent mutations, respectively, with the overall divergence of *pneo-7mu* being 1.1% and that of *pneo-8mu* being

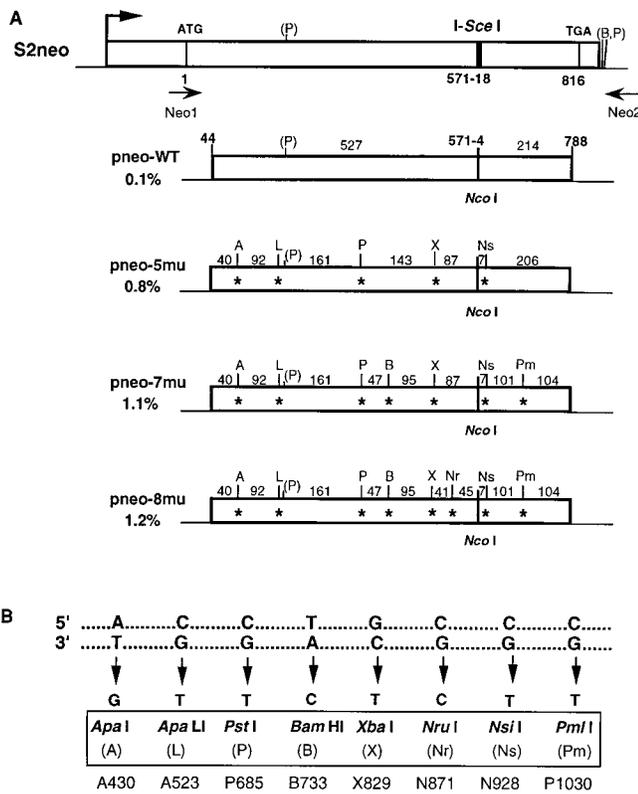


FIG. 2. Silent mutations in the diverged repair substrates. (A) Positions of the silent mutations and corresponding new restriction sites in the donor pneo substrates. The length of homology between the *neo* fragment in the pneo plasmid and the chromosomal S2neo gene is 745 bp. The translation start, I-SceI cleavage site, and termination codon in the S2neo gene are also indicated. The percentages of divergence of the internal *neo* gene fragments in pneo-WT, pneo-5mu, pneo-7mu, and pneo-8mu relative to the same region in S2neo are shown. For simplicity, the heterology at the NcoI/I-SceI sites is considered to be one change within the 745-bp segment, although recombination at this position results in loss of the 18-bp cleavage site and gain of 4 bp at the NcoI site. The distances in base pairs between the 1-bp silent mutations (asterisks) are indicated. Neo1 and Neo2 are the primers that were used for PCR amplification of the chromosomal *neo* gene. Abbreviations for the new restriction sites that are generated by the silent mutations are shown in panel B. Note that there is a naturally occurring *PstI* site (P) within the *neo* gene fragment as well as *BamHI* and *PstI* sites (B, P) downstream of the *neo* gene. The promoter (arrow) for the S2neo gene is derived from polyomavirus (strain F441) and the herpes virus thymidine kinase gene (41). (B) Silent mutations in the diverged pneo substrates which create new restriction sites. The top duplex indicates the sequence of the wild-type *neo* gene at the positions where silent mutations were introduced. The sequence of the mutations is given for the bottom strand. Restriction sites created as a result of the introduced mutations are indicated, with abbreviations used for the sites shown in parentheses. The designations given by Taghian and Nikoloff (39) for the mutations are shown at the bottom.

1.2%. The longest stretch of perfect homology is 161 bp for both pneo-7mu and pneo-8mu, although in pneo-8mu another stretch of perfect homology adjacent to the cleavage site is shortened from 87 to 45 bp by the additional point mutation. Plasmid pneo-WT serves as a control in these experiments, differing from the S2neo gene only by having a wild-type sequence at the NcoI site.

In addition to the *neo* gene homology, the pneo plasmids also have homology with the chromosome downstream of the S2neo gene. This homology is at least 700 bp of bacterial vector sequences. Because there is approximately 100 bp of unrelated sequence separating the two homologous segments (composed of 3' *neo* gene sequences for S2neo and vector sequences for

pneo), it is not clear if the downstream homology affects DSB repair.

**Effect of heterology on the frequency of DSB-induced chromosomal recombination.** To determine the effect of heterology on the frequency of DSB-induced gene targeting, we used a mouse ES cell line which has a single copy of S2neo integrated into the genome (33). This ES cell line, termed clone 12, was cotransfected with each of the pneo substrates and the I-SceI expression vector. Cells were selected in G418 starting 1 day after transfection. G418<sup>r</sup> (*neo*<sup>+</sup>) recombinant colonies were counted 10 to 14 days later.

In the cotransfection of pneo-WT and the I-SceI expression vector, a frequency of up to  $4.7 \times 10^{-4}$  *neo*<sup>+</sup> colonies was obtained from the transfected cell population (Table 1). In each of five independent experiments with the diverged repair substrates and the I-SceI expression vector, the frequency of *neo*<sup>+</sup> colonies relative to the pneo-WT control was found to decrease as the amount of heterology of the pneo substrate increased (Table 1; Fig. 3). With 0.8% divergence in pneo-5mu and 1.1% divergence in pneo-7mu, mean 2.5-fold and 4-fold decreases in recombination were observed, respectively. The largest effect, seen with 1.2% divergence in pneo-8mu, was a sixfold decrease.

In these experiments, no *neo*<sup>+</sup> colonies were obtained from cotransfections of the pneo plasmids with a control pPGKlacZ vector (Table 1 and data not shown). Similarly, we did not detect *neo*<sup>+</sup> clones from cells which were transfected with the pneo substrates alone (data not shown). These results show that gene targeting of the pneo plasmid is inefficient in the absence of a chromosomal DSB ( $<10^{-7}$  of the electroporated cells) but is stimulated more than 3 orders of magnitude by a DSB in the target locus. No *neo*<sup>+</sup> colonies resulted from transfection of cells with the I-SceI expression vector alone, indicating that restoration of a *neo*<sup>+</sup> gene is dependent upon a correcting *neo* gene fragment. Taken together these data clearly demonstrate that all of the recombinants detected from cotransfection of the pneo plasmids and the I-SceI expression vector were derived from DSB-induced recombination. In addition to the ES clone 12 cell line, we have performed similar experiments in another ES cell line which has a single S2neo gene integrated at a different position in the genome. Similar results have been obtained with this clone (data not shown), demonstrating that our results are independent of chromosomal context of the DSB site.

**Gene conversion tracts from the pneo-8mu repair substrate.**

For insight into the mechanism of gene conversion, we examined the incorporation of the silent mutations into the chromosome of the recombinants. Genomic DNA was isolated from *neo*<sup>+</sup> colonies, and PCR amplification was performed with *neo* gene primers that would hybridize to the chromosomal *neo* gene but not to the pneo plasmid DNA (Fig. 2A) (27, 33). This strategy would prevent amplification of plasmid DNA that had integrated randomly in the genome. Endonuclease digests were performed on the amplified products to verify that the chromosomal S2neo gene had undergone DSB-promoted recombination and to determine the extent of gene conversion beyond the position of the I-SceI cleavage site.

To restore *neo* gene function, each *neo*<sup>+</sup> colony was expected to have lost the I-SceI site and incorporated an NcoI site at that position. We analyzed 40 *neo*<sup>+</sup> colonies derived from cotransfection of the pneo-8mu plasmid with the I-SceI expression vector. As expected, in each of the *neo*<sup>+</sup> colonies the I-SceI site was lost from the chromosomal S2neo locus and replaced with the NcoI site (Fig. 4 and 5A), verifying that the *neo*<sup>+</sup> colonies resulted from gene targeting. The incorporation of the 4 bp at the NcoI site occurred with the loss of the

TABLE 1. Gene targeting in ES cells with diverged repair substrates

Transfected plasmid(s)	Frequency of <i>neo</i> <sup>+</sup> colonies <sup>a</sup>				
	Expt 1 (% WT) <sup>b</sup>	Expt 2 (% WT)	Expt 3 (% WT)	Expt 4 (% WT)	Expt 5 (% WT)
pneo-WT + pPGK3xnlS- <i>SceI</i>	$1.40 \times 10^{-4}$ (100)	$1.44 \times 10^{-4}$ (100)	$1.15 \times 10^{-4}$ (100)	$4.69 \times 10^{-4}$ (100)	$4.63 \times 10^{-4}$ (100)
pneo-5mu + pPGK3xnlS- <i>SceI</i>	$0.39 \times 10^{-4}$ (28)	$0.71 \times 10^{-4}$ (49)	$0.61 \times 10^{-4}$ (53)	$1.77 \times 10^{-4}$ (38)	$1.90 \times 10^{-4}$ (41)
pneo-7mu + pPGK3xnlS- <i>SceI</i>	$0.28 \times 10^{-4}$ (20)	$0.58 \times 10^{-4}$ (40)	$0.33 \times 10^{-4}$ (29)	$1.14 \times 10^{-4}$ (24)	$1.22 \times 10^{-4}$ (26)
pneo-8mu + pPGK3xnlS- <i>SceI</i>	$0.18 \times 10^{-4}$ (13)	$0.27 \times 10^{-4}$ (19)	$0.27 \times 10^{-4}$ (23)	$0.72 \times 10^{-4}$ (15)	$0.73 \times 10^{-4}$ (16)
pneo-WT + pPGKlacZ	$<1 \times 10^{-7}$	ND <sup>c</sup>	ND	$<1 \times 10^{-7}$	ND
pPGK3xnlS- <i>SceI</i>	$<1 \times 10^{-7}$	ND	ND	$<1 \times 10^{-7}$	ND
pMC1neo <sup>d</sup>	$5.4 \times 10^{-4}$	ND	$4.8 \times 10^{-4}$	$7.8 \times 10^{-4}$	$1.4 \times 10^{-3}$

<sup>a</sup> The frequency of *neo*<sup>+</sup> colonies was determined by dividing the number of *neo*<sup>+</sup> colonies obtained by the number of electroporated cells ( $2 \times 10^7$ ), correcting for 50% viability of cells generally observed following electroporation under these conditions.

<sup>b</sup> % WT, percent frequency of *neo*<sup>+</sup> colonies derived from transfection of the diverged pneo repair substrate with pPGK3xnlS-*SceI* divided by the frequency of *neo*<sup>+</sup> colonies derived from transfection of pneo-WT with pPGK3xnlS-*SceI*.

<sup>c</sup> ND, not determined.

<sup>d</sup> Total number of colonies from pMC1neo transfection were estimated by plating dilutions of electroporated cells (1:6 to 1:60).

four-base I-*SceI* overhangs, as well as the loss of 5 bp upstream and 9 bp downstream of the I-*SceI* site (Fig. 1A).

If silent mutations from the donor pneo plasmids had been coconverted with the *NcoI* site, the amplified products were expected to be digested by restriction enzymes recognizing the corresponding new restriction sites. The incorporation of the silent mutations in the 40 recombinants examined was found to inversely correlate with the distance from the *NcoI* site (Fig. 5A). The mutation nearest to the *NcoI* site, located 8 bp downstream at the *NsiI* site, was found to be incorporated at a high frequency, i.e., in 83% of the recombinants. The next closest mutation, at the *NruI* site (46 bp upstream), was found to be incorporated in 40% of the recombinants, while the mutation at the *XbaI* site (88 bp upstream) was found in 25% of the recombinants. Each of the remaining mutations was incorporated into 20% or fewer of the recombinants, with the mutation at the *ApaI* site (394 bp upstream) being incorporated into just two of the clones. The farthest mutation, at the *ApaI* site (487 bp upstream), was not found in any of the 40 recombinants examined. When checked, Southern blot analysis of *neo*<sup>+</sup> recombinant clones gave the same results to those obtained by PCR analysis for the incorporation of the silent mutations (data not shown).

All of the gene conversion tracts were found to be continuous from the cleavage site, such that each of the mutations between the outermost converted restriction site and the *NcoI* site was converted. The longer gene conversion tracts ( $\geq 58$  bp) were mostly bidirectional, extending both 5' and 3' from the cleavage site. The ability of one side to recombine did not appear to interfere with the ability of the other side to recombine. For example, the clones which have the longest gene conversion tracts (i.e., the class 9 and 10 recombinants) are bidirectional. A few of the gene conversion tracts were unidirectional, for example, the gene conversion tract in the class 4 recombinant extended 88 bp 5' to the *NcoI* site while the *NsiI* mutation, which is only 8 bp 3' to the *NcoI* site, was not converted. Similarly, in class 6 recombinants, the gene conversion tract extended 110 bp 3' to the *NcoI* site, while the *NruI* mutation 46 bp 5' to the *NcoI* site was not converted.

In most of these recombinants, there was no ambiguity as to the incorporation of the silent mutations. The amplified product appeared either completely cleaved or completely un-cleaved when analyzed by agarose gel electrophoresis (Fig. 4). However, for four recombinants, the amplified products were found to be approximately half cleaved by one or more restriction enzymes (Fig. 4E and data not shown). The partial cleavage was reproducible from independent PCR amplifications of

genomic DNA and may reflect either segregation of unrepaired mismatches in heteroduplex DNA after recombination or independent repair events in daughter cells of a cell that had been transfected with the I-*SceI* expression vector. For one mixed recombinant clone (i.e., a mixed class 1 and 2 recombinant) the amplified product was only partially cleaved by *NsiI*. For two other mixed recombinant clones (mixed class 2 and 3 recombinants) the amplified product was only partially cleaved by *NruI*, although for both of these clones the product was completely cleaved by *NsiI*. The amplified product of another mixed recombinant clone (a mixed class 0 and 2 recombinant) was partially cleaved by *NsiI*; however, it was also partially cleaved by I-*SceI* and *NcoI*, suggesting that the clone may contain a duplication of the *neo* gene in the chromosome with one of the genes being converted.

**Gene conversion tracts from other pneo repair substrates.** *neo*<sup>+</sup> colonies were examined that were derived from cotrans-

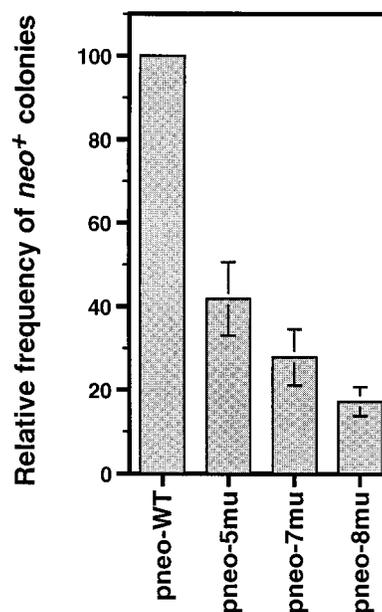


FIG. 3. Relative gene-targeting efficiencies of diverged repair substrates. The frequency of *neo*<sup>+</sup> colonies was plotted for the cotransfections of each of the pneo substrates with pPGK3xnlS-*SceI* relative to the pneo-WT with pPGK3xnlS-*SceI* control. In each case, the mean of five independent experiments is shown with the standard deviation indicated by an error bar.

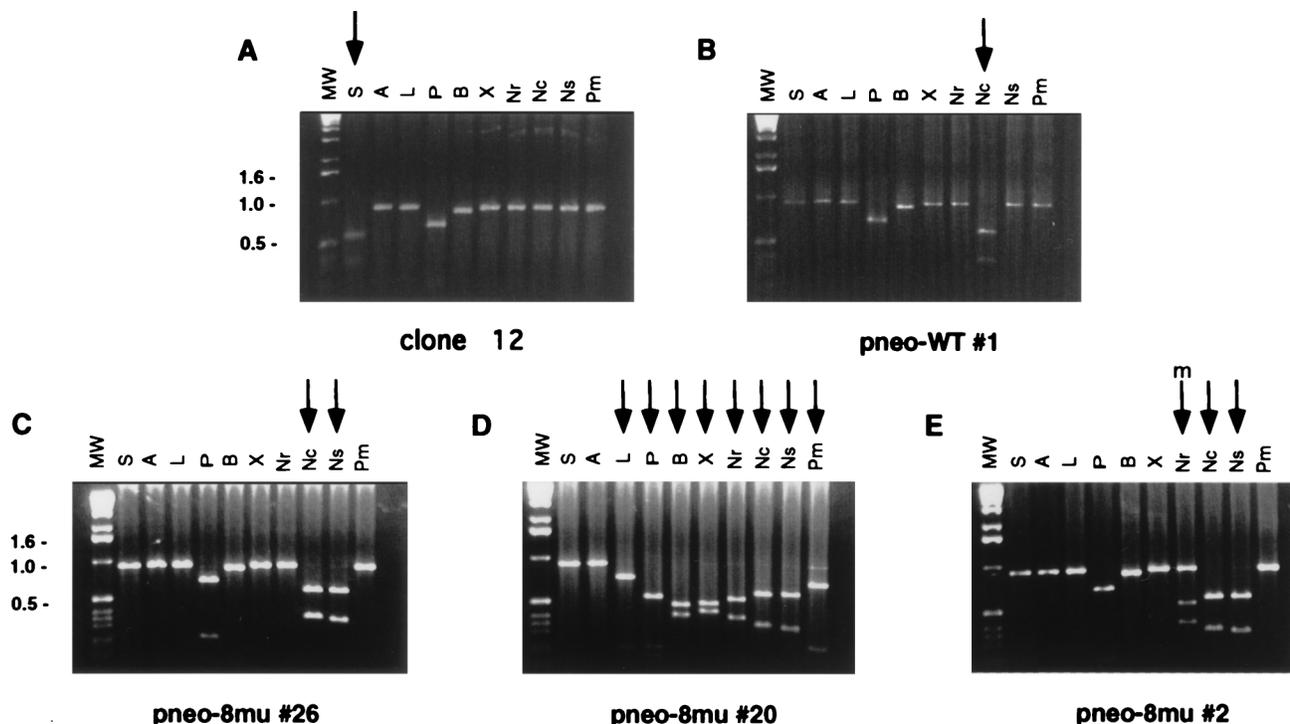


FIG. 4. PCR analysis of parental ES cells and *neo*<sup>+</sup> clones derived from cotransfection of pneo substrates with the I-*SceI* expression vector. The *neo* gene coding region was amplified by PCR as a 0.9-kb fragment from genomic DNA with primers Neo1 and Neo2 and electrophoresed on agarose gels following digestion by the indicated endonucleases. Endonucleases are abbreviated as follows: I-*SceI*, S; *ApaI*, A; *ApaLI*, L; *PstI*, P; *BamHI*, B; *XbaI*, X; *NruI*, Nr; *NcoI*, Nc; *NsiI*, Ns; and *PmlI*, Pm. The 1-kb ladder molecular weight marker used is also indicated (MW). Note that due to naturally occurring *PstI* and *BamHI* sites (Fig. 2), the amplified fragments in each of the clones are shifted when cut with these enzymes. (A) Amplified fragment from the S2neo gene in the parental ES clone 12 cells. The fragment is cleaved by I-*SceI*, as expected, but it is not cleaved by *NcoI* or the restriction enzymes whose sites were created in the pneo plasmids. (B) Amplified *neo* fragment from a *neo*<sup>+</sup> colony from cotransfection of pneo-WT and the I-*SceI* expression vector. The fragment is cleaved by *NcoI* but not by I-*SceI* or the other restriction enzymes. (C to E) Amplified *neo* gene fragments from selected *neo*<sup>+</sup> colonies from cotransfection of the pneo-8mu substrate and the I-*SceI* expression vector, showing a short gene conversion tract (C), a long tract (D), and a mixed tract (E). The amplified fragment from each of the *neo*<sup>+</sup> clones is not cleaved by I-*SceI* but is cleaved by *NcoI* and various restriction enzymes, as indicated by the arrows. In clone pneo-8mu #20 (D), the small amount of DNA not cleaved by *PmlI* is not reproducible. The "m" over the arrow in panel E indicates partial cleavage by the restriction enzyme.

fection of the I-*SceI* expression vector with the other pneo plasmids. Colonies derived from cotransfection of the I-*SceI* expression vector and pneo-WT were examined as a control. Except for the incorporation of the *NcoI* site and loss of the I-*SceI* cleavage site, no other restriction site change to the chromosomal locus was observed (Fig. 4B and data not shown).

For each of the *neo*<sup>+</sup> colonies derived from cotransfection of the I-*SceI* expression vector and either pneo-7mu (20 recombinants) or pneo-5mu (20 recombinants), the I-*SceI* site in the chromosomal *neo* locus was converted to an *NcoI* site (Fig. 5B and C). As observed with the recombinants derived from transfection of the pneo-8mu plasmid, the frequency of incorporation of the silent mutations on either side of the *NcoI* site for these clones was dependent on the distance of the mutation from the I-*SceI* cleavage site. The mutation at the *NsiI* site 8 bp from the *NcoI* site was incorporated into 83% of the recombinants (19 of 20 from pneo-7mu and 14 of 20 from pneo-5mu), whereas the most distant mutations were incorporated in 10% or less of the recombinants. All gene conversion tracts derived from these plasmids were also continuous.

As observed with transfections with pneo-8mu, a few recombinants, one from pneo-5mu and one from pneo-7mu, had amplified products that were only partially cleaved by some restriction enzymes. The amplified product from the mixed clone generated by pneo-5mu transfection was only partially cleaved by *NsiI* (Fig. 5C), and the amplified product from the

mixed clone generated by pneo-7mu was partially cleaved by five enzymes, *PstI*, *BamHI*, *XbaI*, *NsiI*, and *PmlI* (Fig. 5B). Cleavage by *NcoI* in both cases was complete. As with the mixed clones described above, these mixed clones likely arose either from heteroduplex formation with inefficient mismatch repair or from a cell that had been transfected with the I-*SceI* expression vector and whose daughter cells had independent repair events.

## DISCUSSION

We have examined the effect of heterology on recombinational repair of chromosomal DSBs in mammalian cells. We find that sequence divergence in the range of 0.8 to 1.2% in the 745-bp homologous fragment in our gene-targeting plasmid decreased the frequency of DSB-induced recombinational repair, with a divergence of 1.2% resulting in an approximately sixfold decrease in gene targeting. A substantial majority (80%) of the 80 recombinants examined had observed gene conversion tracts of 58 bp or less. All of the gene conversion tracts were continuous, incorporating all of the mutations between the cleavage site and the outermost converted site. Gene conversion tracts 58 bp or longer were mostly bidirectional.

A summary of the gene conversions in the recombinant clones from the three heterologous substrate plasmids is shown in Fig. 6. We found that the majority of recombinants from each plasmid transfection had gene conversion tracts of at least

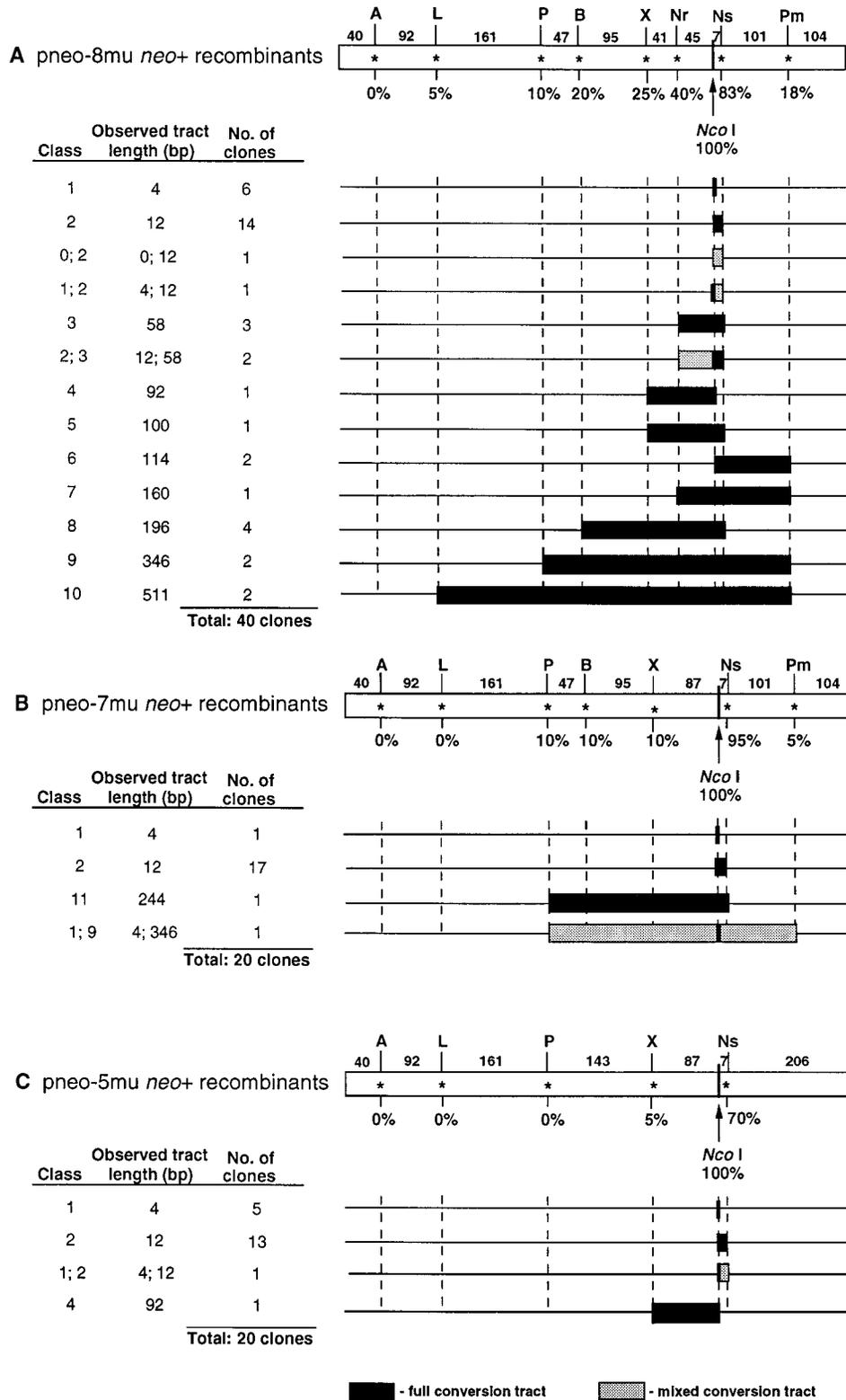


FIG. 5. Gene conversion tracts from *neo*<sup>+</sup> recombinants. Observed gene conversion tracts from *neo*<sup>+</sup> clones derived from cotransfection of ES clone 12 cells with the I-SceI expression vector and repair substrates pneo-8mu (A), pneo-7mu (B), and pneo-5mu (C). The recombinants were derived from two experiments. The full-length homology region in the respective *neo* fragment is shown (open rectangles). The positions of the silent mutations for each pneo plasmid are shown above the homology regions with the distance between the mutations indicated in base pairs. When gene conversion tracts are calculated, an additional base pair is added for the conversion of the silent mutation. Below each silent mutation is the percent conversion of the mutation in the gene conversion tracts. The correcting *Nco*I site, shown below the tract, occurs in 100% of *neo*<sup>+</sup> clones, since it restores a functional *neo*<sup>+</sup> gene. Gene conversion tracts extending to the last incorporated mutation and mixed conversion tracts (along with the two classes constituting them) are shown for each of the recombinants. The 0 class indicates no conversion with retention of the I-SceI site.

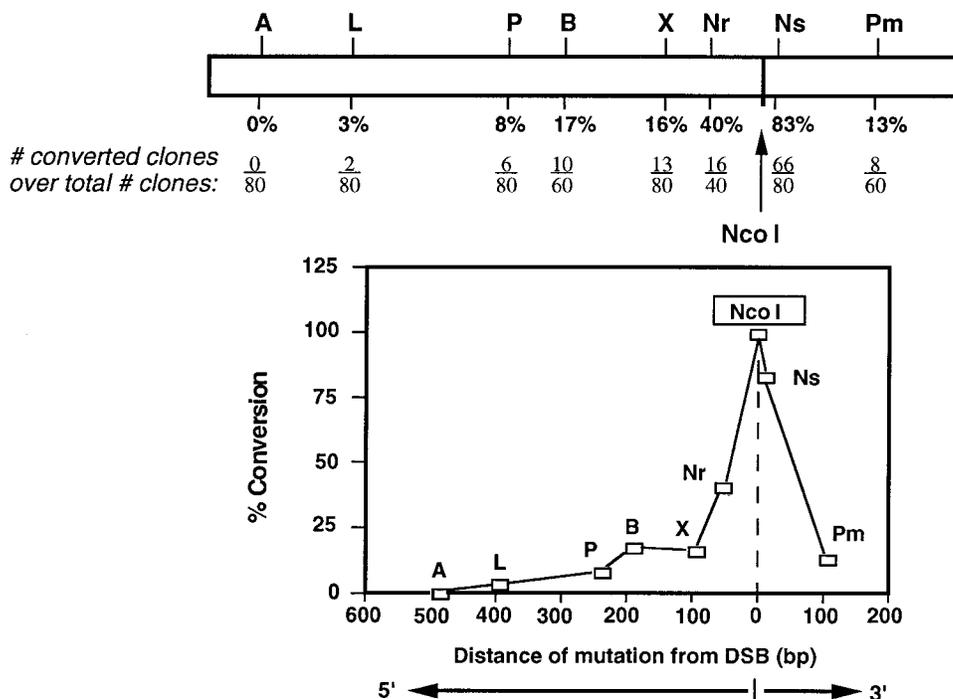


FIG. 6. Summary of gene conversion frequencies for each silent mutation. Below each silent mutation is the percent conversion from the pneo-5mu, pneo-7mu, and pneo-8mu substrates. The number of clones converted at each mutation is divided by the total number of clones analyzed for each mutation. For the *Nco*I site, there is a conversion tract of at least 4 bp. Using these data, the conversion frequency for each mutation was plotted as a function of distance from the DSB.

12 bp, incorporating both the *Nco*I and *Nsi*I sites. In the 17% of recombinants (14 of 80) that did not incorporate the mutation at the *Nsi*I site, the endpoint of the conversion tract was located within the 7 bp between these two sites. In these recombinants there was either no or very little degradation of the chromosome ends prior to or during recombination. The next mutation from the cleavage site, located 46 bp upstream at the *Nru*I site in the pneo-8mu substrate, was converted in 40% recombinants examined. Clones which have converted no more than the three mutations at the *Nco*I, *Nsi*I, and *Nru*I sites (i.e., the class 1, 2, and 3 clones) (Fig. 5A) comprise 68% of the recombinants repaired from pneo-8mu, giving observed gene conversion tracts of 58 bp or less. The lack of conversion at the next mutations (at the *Xba*I and *Pml*I sites) thus demarcates a maximum gene conversion tract of 200 bp (i.e., for the class 3 clones). The longest observed gene conversion tract was 511 bp and was seen in just 2 of the 80 clones. This gene conversion tract contained seven of the eight silent mutations, with the farthest mutation, located 93 bp upstream (at an *Apa*I site), not being converted. Although the distance likely affects the incorporation of this mutation, its location only 40 bp from the homology border also may have had an impact. Reduced conversion for markers near homology borders has been seen for spontaneous and DSB-induced recombination in yeast (1, 37).

Plotting the percent conversion at each of the silent mutations against the distance of the mutation from the cleavage site gives the distribution shown in Fig. 6. There is a steep decline in the amount of conversion close to the cleavage site. At greater distances from the cleavage site (around 100 bp), the degree of incorporation of the mutations has a much shallower decline. There does not appear to be a marked directional preference or end effect, as mutations located at approximately similar distances on opposite sides of the cleavage site (i.e., at the *Xba*I and *Pml*I sites) are incorporated at similar

frequencies. In a recent study, Taghian and Nickoloff report that conversion by chromosomal DSBs between direct repeats gave almost identical bimodal and symmetric distribution (39).

Two possible mechanisms can be invoked to account for the gene conversion tracts from the DSB site. One is mismatch correction following heteroduplex formation between one strand of the *neo* gene on the cleaved chromosome and the complementary strand of the *neo* fragment on the donor plasmid. In *S. cerevisiae* it is likely that the 3' strand of the cleaved chromosome would form heteroduplex DNA with an uncleaved partner, since DSBs in yeast are processed to form 3' single-stranded tails (4, 35, 36). The occurrence of a few clones with partially converted mutations is consistent with the idea that heteroduplex DNA is formed in at least some instances. (It cannot be excluded, however, that these clones arose from two independent repair events that occurred after DNA replication in cells expressing *I-Sce*I.) An alternative mechanism involves conversion in a double-stranded gap, in which both strands of the DNA duplex on each side of the break are removed by nucleases. In this case, information available for repair must derive entirely from the *neo* fragment on the donor pneo plasmid. Formation of a double-strand gap at the *I-Sce*I break site would be consistent with the continuous nature of the gene conversion tracts we observed. With gap formation, longer tracts would reflect extensive degradation and shorter tracts would reflect limited amounts of degradation.

The observation that heterology affects recombination frequencies, however, argues for a significant contribution of heteroduplex formation to gene conversion events. Since we find that at least 17% of the recombinants have maintained the parental sequence to within 7 bp of the *I-Sce*I recognition site, at least a portion of the chromosome ends are protected from any more than a few base pairs of degradation. If all the chromosome ends are protected from degradation, formation

of heteroduplex DNA between the two substrates would also give rise to continuous tracts if it were coupled with a strong bias to correct mismatches in the direction of the donor substrate. Such a bias would be signaled by the chromosomal break (19, 22, 23). The bias would have to overcome the usual directionality in the repair of mismatched bases. For example, the G/T mismatch at the *Xba*I site would be expected to be rarely converted to A/T ( $\leq 10\%$  of repairs [3]), the mutation in the donor plasmid, yet it is converted in 16% of the recombinants. Since most or all DSB-induced conversion in yeast reflects mismatch repair of heteroduplex DNA (20, 23) and many of the features of yeast conversion tracts are similar to those we observe here (see below and reference 37), mismatch repair of heteroduplex DNA may predominate over double-strand gap formation in mammalian cells as well.

We cannot rule out the possibility that conversion occurs by both mechanisms, i.e., heteroduplex correction and double-strand gap formation, with shorter tracts arising from heteroduplex correction of preserved chromosome ends and longer tracts from chromosome breaks which have eluded end protection mechanisms. The bimodal nature of the conversion tracts would be consistent with two distinct mechanisms, although it does not exclude the possibility that only one mechanism (i.e., heteroduplex correction, see above) is occurring. Examination of gene conversion in mismatch repair mutants will be necessary to address this question.

The 2.5- to 6-fold decrease in the frequency of recombination with the diverged substrates demonstrates the effect of sequence divergence on the efficiency of DSB-induced recombination. In previously described gene-targeting experiments, 0.6% sequence divergence was found to reduce spontaneous recombination 20-fold (40), a much greater reduction than seen here. In studies examining spontaneous intrachromosomal recombination, 1% sequence divergence was found to have a much less dramatic effect, at least in some cases (43). In these experiments, the minimal length of uninterrupted homology was determined to be a more important factor governing the frequency of recombination than the absolute amount of sequence divergence (43). The minimal length of uninterrupted homology required for efficient recombination was estimated to be between 232 and 134 bp, since a decrease from 232 bp of uninterrupted homology to 134 bp resulted in a 20-fold reduction in recombination. In our experiments, the length of uninterrupted homology was also within this range, e.g., 206 bp (pneo-5mu) and 161 bp (pneo-7mu and pneo-8mu). By contrast, we see only a two- to threefold reduction in recombination between pneo-5mu and pneo-8mu, indicating that DSBs may partially overcome the barrier to homology length requirements in recombination between diverged sequences.

In each of our experiments, we found a decrease in the frequency of recombination as the divergence of the repair substrates increased (Fig. 3). This trend suggests that additional mutations both close to and far away from the cleavage site can incrementally impact on recombination frequencies. The one additional mutation in plasmid pneo-8mu is 46 bp from the DSB and interrupts the 87-bp tract of perfect homology upstream of the *I-Sce*I cleavage site, implying that the length of perfect homology adjacent to a DSB may be an important factor governing the efficiency of DSB-promoted recombination.

Interestingly, the DSB-induced gene conversion tracts we obtained in ES cells are similar to those found in DSB-induced recombination in *S. cerevisiae*. In yeast, gene conversion tracts are short (200 to 300 bp) and the majority of tracts are continuous, although one primary difference is that gene conver-

sion tracts in yeast are primarily unidirectional (37). The similarity to DSB-induced recombination in yeast may be thought surprising, since nonhomologous repair contributes significantly to DSB repair in mammalian cells (10, 15, 17, 21, 27) yet is rare in yeast. However, the recent identification of mammalian homologs to genes involved in recombination in yeast (32), along with the work presented here, suggests that recombination pathways between the organisms may be highly conserved.

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#### REFERENCES

- Ahn, B.-Y., K. J. Dornfeld, T. J. Fagrelus, and D. M. Livingston. 1988. Effect of limited homology on gene conversion in a *Saccharomyces cerevisiae* plasmid recombination system. *Mol. Cell. Biol.* **8**:2442-2448.
- Bailis, A. M., L. Arthur, and R. Rothstein. 1990. A defect in mismatch repair in *Saccharomyces cerevisiae* stimulates ectopic recombination between homologous genes by an excision repair dependent process. *Genetics* **126**:535-547.
- Brown, T. C., and J. Jiricny. 1988. Different base/base mispairs are corrected with different efficiencies and specificities in monkey kidney cells. *Cell* **54**:705-711.
- Cao, L., E. Alani, and N. Kleckner. 1990. A pathway for generation and processing of double-strand breaks during meiotic recombination in *S. cerevisiae*. *Cell* **61**:1089-1101.
- Chambers, S. R., N. Hunter, E. J. Louis, and R. H. Borts. 1996. The mismatch repair system reduces meiotic homologous recombination and stimulates recombination-dependent chromosome loss. *Mol. Cell. Biol.* **16**:6110-6120.
- Choulika, A., A. Perrin, B. Dujon, and J.-F. Nicolas. 1995. Induction of homologous recombination in mammalian chromosomes by using the *I-Sce*I system of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **15**:1968-1973.
- de Wind, N., M. Dekker, A. Berns, M. Radman, and H. te Riele. 1995. Inactivation of the mouse Msh2 gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer. *Cell* **82**:321-330.
- Donoho, G., M. Jasin, and P. Berg. Unpublished results.
- Ferguson, D. O., and W. K. Holloman. 1996. Recombinational repair of gaps in DNA is asymmetric in *Ustilago maydis* and can be explained by a migrating D-loop model. *Proc. Natl. Acad. Sci. USA* **93**:5419-5424.
- Godwin, A. R., R. J. Bollag, D.-M. Christie, and R. M. Liskay. 1994. Spontaneous and restriction enzyme-induced chromosomal recombination in mammalian cells. *Proc. Natl. Acad. Sci. USA* **91**:12554-12558.
- Haber, J. E. 1995. *In vivo* biochemistry: physical monitoring of recombination induced by site-specific endonucleases. *BioEssays* **17**:609-620.
- Jackson, S. P., and P. A. Jeggo. 1995. DNA double-strand break repair and V(D)J recombination: involvement of DNA-PK. *Trends Biochem. Sci.* **20**:412-415.
- Jasin, M. 1996. Genetic manipulation of genomes with rare-cutting endonucleases. *Trends Genet.* **12**:224-228.
- Kogoma, T. 1996. Recombination by replication. *Cell* **85**:625-627.
- Liang, F., P. J. Romanienko, D. T. Weaver, P. A. Jeggo, and M. Jasin. 1996. Chromosomal double-strand break repair in Ku80 deficient cells. *Proc. Natl. Acad. Sci. USA* **93**:8929-8933.
- Lin, F.-L., K. Sperle, and N. Sternberg. 1990. Intermolecular recombination between DNAs introduced into mouse L cells is mediated by a nonconservative pathway that leads to crossover products. *Mol. Cell. Biol.* **10**:103-112.
- Lukacovich, T., D. Yang, and A. S. Waldman. 1994. Repair of a specific double-strand break generated within a mammalian chromosome by yeast endonuclease *I-Sce*I. *Nucleic Acids Res.* **22**:5649-5657.
- Malkova, A., E. L. Ivanov, and J. E. Haber. 1996. Double-strand break repair in the absence of RAD51 in yeast: a possible role for break-induced replication. *Proc. Natl. Acad. Sci. USA* **93**:7131-7136.
- Modrich, P. 1991. Mechanisms and biological effects of mismatch repair. *Annu. Rev. Genet.* **25**:229-253.
- Moynahan, M. E., and M. Jasin. 1997. Loss of heterozygosity induced by a chromosomal double-strand break. *Proc. Natl. Acad. Sci. USA* **94**:8988-8993.
- Petes, T. D., R. E. Malone, and L. S. Symington. 1991. Recombination in yeast, p. 407-521. In J. R. Broach, J. R. Pringle, and E. W. Jones (ed.), *The molecular and cellular biology of the yeast Saccharomyces: genome dynam-*

- ics, protein synthesis, and energetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
21. **Phillips, J. W., and W. F. Morgan.** 1994. Illegitimate recombination induced by DNA double-strand breaks in a mammalian chromosome. *Mol. Cell. Biol.* **14**:5794–5803.
  22. **Priebe, S. D., J. Westmoreland, T. Nilsson-Tillgren, and M. A. Resnick.** 1994. Induction of recombination between homologous diverged DNAs by double-strand gaps and breaks and role of mismatch repair. *Mol. Cell. Biol.* **14**:48024814.
  23. **Ray, B. L., C. I. White, and J. E. Haber.** 1991. Heteroduplex formation and mismatch repair of the “stuck” mutation during mating-type switching in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **11**:5372–5380.
  24. **Rayssiguier, C., D. S. Thaler, and M. Radman.** 1989. The barrier to recombination between *Escherichia coli* and *Salmonella typhimurium* is disrupted in mismatch-repair mutants. *Nature* **342**:396–401.
  25. **Resnick, M.** 1976. The repair of double-strand breaks in DNA; a model involving recombination. *J. Theor. Biol.* **59**:97–109.
  26. **Robertson, E. J.** 1987. Embryo-derived stem cell lines, p. 71–112. *In* E. J. Robertson (ed.), *Teratocarcinomas and embryonic stem cells: a practical approach*. IRL Press, Washington, D.C.
  27. **Rouet, P., F. Smih, and M. Jasin.** 1994. Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. *Mol. Cell. Biol.* **14**:8096–8106.
  - 27a. **Rouet, P., F. Smih, and M. Jasin.** 1994. Expression of site-specific endonuclease stimulates recombination in mammalian cells. *Proc. Natl. Acad. Sci. USA* **91**:6064–6068.
  28. **Sargent, R. G., M. A. Brennehan, and J. H. Wilson.** 1997. Repair of site-specific double-strand breaks in a mammalian chromosome by homologous and illegitimate recombination. *Mol. Cell. Biol.* **17**:267–277.
  29. **Schneider, W. P., B. P. Nichols, and C. Yanofsky.** 1981. Procedure for production of hybrid genes and proteins and its use in assessing significance of amino acid differences in homologous tryptophan synthetase  $\alpha$  polypeptides. *Proc. Natl. Acad. Sci. USA* **78**:2169–2173.
  30. **Schwacha, A., and N. Kleckner.** 1995. Identification of double Holliday junctions as intermediates in meiotic recombination. *Cell* **83**:783–791.
  31. **Shen, P., and H. V. Huang.** 1986. Homologous recombination in *Escherichia coli*: dependence on substrate length and homology. *Genetics* **112**:441–457.
  32. **Shinohara, A., and T. Ogawa.** 1995. Homologous recombination and the role of double-strand breaks. *Trends Biochem.* **20**:387–391.
  33. **Smih, F., P. Rouet, P. J. Romanienko, and M. Jasin.** 1995. Double-strand breaks at the target locus stimulate gene targeting in embryonic stem cells. *Nucleic Acids Res.* **23**:5012–5019.
  34. **Stahl, F.** 1996. Meiotic recombination in yeast: coronation of the double-strand-break repair model. *Cell* **87**:965–968.
  35. **Sugawara, N., and J. E. Haber.** 1992. Characterization of double-strand break-induced recombination: homology requirements and single-stranded DNA formation. *Mol. Cell. Biol.* **12**:563–575.
  36. **Sun, H., D. Treco, N. P. Schultes, and J. W. Szostak.** 1989. Double-strand breaks at an initiation site for meiotic gene conversion. *Nature* **338**:87–90.
  37. **Sweetser, D. B., H. Hough, J. F. Whelden, M. Arbuckle, and J. A. Nickoloff.** 1994. Fine-resolution mapping of spontaneous and double-strand break-induced gene conversion tracts in *Saccharomyces cerevisiae* reveals reversible mitotic conversion polarity. *Mol. Cell. Biol.* **14**:3863–3875.
  38. **Szostak, J. W., T. L. Orr-Weaver, R. J. Rothstein, and F. W. Stahl.** 1983. The double-strand-break repair model for recombination. *Cell* **33**:25–35.
  39. **Taghian, D. G., and J. A. Nickoloff.** Chromosomal double-strand breaks induce gene conversion at high frequency in mammalian cells. *Mol. Cell. Biol.* **17**:6386–6393.
  40. **te Riele, H., E. R. Maandag, and A. Berns.** 1992. Highly efficient gene targeting in embryonic stem cells through homologous recombination with isogenic DNA constructs. *Proc. Natl. Acad. Sci. USA* **89**:5128–5132.
  41. **Thomas, K. R., and M. R. Capecchi.** 1987. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* **51**:503–512.
  42. **Waldman, A. S., and R. M. Liskay.** 1987. Differential effects of base-pair mismatch on intrachromosomal versus extrachromosomal recombination in mouse cells. *Proc. Natl. Acad. Sci. USA* **84**:5340–5344.
  43. **Waldman, A. S., and R. M. Liskay.** 1988. Dependence of intrachromosomal recombination in mammalian cells on uninterrupted homology. *Mol. Cell. Biol.* **8**:5350–5357.