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Paul Hasty
Baylor College of Medicine

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Gene conversion during vector insertion in embryonic stem cells

Paul Hasty¹*, Jaime Rivera-Pérez¹ and Allan Bradley¹,²

¹Department of Human and Molecular Genetics and ²Howard Hughes Medical Institute, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA

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ABSTRACT

Recombination of an insertion vector into its chromosomal homologue is a conservative event in that both the chromosomal and the vector sequences are preserved. However, gene conversion may accompany homologous recombination of an insertion vector. To examine gene conversion in more detail we have determined the targeting frequencies and the structure of the recombinant alleles generated with a series of vectors which target the hprt gene in embryonic stem cells. We demonstrate that gene conversion of the introduced mutation does not significantly limit homologous recombination and that gene conversion occurs without a sequence specific bias for five different mutations. The frequency of the loss of a vector mutation and the gain of a chromosomal sequence is inversely proportional to the distance between the vector mutation and the double-strand break. The loss of a chromosomal sequence and the gain of a vector mutation occurs at a low frequency.

INTRODUCTION

Homologous recombination between introduced and chromosomal DNA is referred to as gene targeting in mammalian cells and the models used to describe gene targeting mechanisms were principally derived from fungal systems (1–3). In mammalian cells the mechanism and final structure of the recombinant allele depends upon the configuration of the targeting vector. Replacement vectors (4) will effectively replace host sequences by either double reciprocal recombination (an exchange of sequences between the two substrates) or by gene conversion (loss of sequences from one substrate and gain of sequences from the other substrate). The observable consequence of either mechanism results in identical mutant alleles because the non-integrated components are degraded; therefore, the recombination mechanism cannot be determined. By contrast, the recombinant allele generated with an insertion vector (4,5) can yield considerably more information because the recombination substrates are preserved. Insertion vectors are characterized by a double-strand break (DSB) inside the target homology and integrate into the host target by a mechanism which is similar to the double-strand break repair (DSBR) model (3–12).

In this report we examined gene conversion during homologous recombination of an insertion vector into the hypoxanthine phosphoribosyltransferase (hprt; 17) locus in embryonic stem (ES) cells. The insertion vector contains a disruption in the hprt homology when compared to the chromosomal sequence. This disruption is referred to as a vector mutation when describing the vector sequence. The chromosomal sequence is considered to be wild-type. Gene conversion was not found to significantly reduce the targeting frequency and occurred without a sequence specific bias for five different mutations. A progressively higher frequency of gene conversion that resulted in loss of the vector mutation (gain of the chromosomal sequence) was observed as the distance between the mutation and the DSB decreased. Gene conversion that resulted in loss of the wild-type chromosomal sequence (gain of the vector mutation) was observed at a low frequency. We also demonstrate that large gaps present in the vector sequence were accurately repaired during gene targeting.

MATERIALS AND METHODS

Vector construction

All vectors consist of a SacI–EcoRI hprt fragment which contains exons 2 and 3 inserted into a pTZ cloning vector (Pharmacia) containing MC1neopA (Stratagene). IV6.8 (10) contains the hprt fragment without modifications which was mutated to make all the other vectors (Fig. 1).

IV6.8N: A NolI site was generated by ligating an oligonucleotide (5'-TTGCGGCCGCAA-3') into an Accl site, previously filled in with nucleotides which divides the homologous sequences into a 6.2 kb long arm and a 0.7 kb short arm. NolI linearizes in the mutation; Xhol linearizes 472 bp 5' to the mutation and Nhel linearizes 3.2 kb 5' to the mutation.

IV6.8ΔA86 has an 86 bp deletion that destroys the Xhol site in exon 3. The mutation divides the homologous sequences into a 5', 5.6 kb long arm and a 3', 1.2 kb short arm. The deletion was constructed by utilizing the 3' to 5' exonuclease activity of Klenow (Boehringer Mannheim) in the presence of limiting dNTPs for 30 min at room temperature. To generate a unique linearization site in the vector two of the three existing EcoRI sites were destroyed. The site in the MC1neopA promoter was

* To whom correspondence should be addressed at present address: The University of Texas, M. D. Anderson Cancer Center, Department of Biochemistry and Molecular Biology, 1515 Holcombe Boulevard, Houston, TX 77030, USA
generates a BamHl kb EcoKL sequences (18) which destroys the site, EcoRl (5'-AATTGCCGGATCCGGCXXrGATCX:GGC-3') into the arm and a 3', 1.2 kb short arm. BamtU-Notl-BamVU Notl site serves as the linearization (5'-TCGATGGATCCGCGGCCGCGGATCCA-3') into the Xhol and blunt end ligation. The remaining £coRI site is the site at the 3' end of hprt was destroyed by end filling EcoRl site. CCGGGCCGGATCCGGC-3' which also generates a BamHl destroyed by inserting the oligonucleotide 5'-AATTGCCGGAT- 26 bp oligonucleotide (5'-TCGATGGATCCGCGGCCGCGGATCCA-3') into the hprt sequences into the Xmnl site in the hprt sequences (9) which destroys the Xmnl site and generates Notl–Nhel–Notl sites and divides the homology into a 5', 1.2 kb short arm and a 3', 5.6 kb long arm. The linearization site is Xhol, 4.3 kb, 3' to the mutation on the long arm.

IV6.8G0.5 has a 0.5 kb gap. To ensure that ends of the vector DNA which constitute the gap were homologous with the chromosome, a piece of 'non-homologous filler DNA' with compatible ends was utilized. To construct the 0.5 kb gap it was necessary to destroy two additional HindIII sites by end filling and blunt end ligation. These sites were the most 5' HindIII site in the hprt sequences and a HindIII site in the plasmid polylinker. The filler DNA was a 2.3 kb fragment. The linearization sites were HindIII and Xhol which liberates the filler DNA from the vector. The gap in this vector lies 4.4 kb, 3' to the filled HindIII site in hprt. No attempt was made to purify the filler DNA from the vector prior to the electroporation.

IV6.8G2.5 has a 2.5 kb gap between the endogenous Nhel and Xhol sites separated by a 600 bp fragment of filler DNA. The vector was cut with Nhel and Xhol restriction enzymes for the electroporation.

IV6.8G4.2 has a gap of 4.2 kb between the two endogenous HindIII sites. An existing HindIII site in the polylinker was destroyed by end filling and blunt end ligation. The vector was linearized with HindIII for the electroporation.

**Electroporation and tissue culture**

The experiments described in Table 1 have been grouped into experiments performed on the same day (experiments A–F). This is necessary since there is some variation in the relative targeting frequencies from experiment to experiment. However, within a group the ratios of the targeting frequencies from one vector to the next are always consistent (9).

Twenty-five μg of cut DNA was electroporated into 10² AB1 cells (19) at 575V/cm as previously described (10) and cultured as previously described (20). A single electroporation was plated onto a 90 mm SNL76/7 feeder plate (19).

Two methods were used to determine the ratio of 6-thioguanine (6-TG) resistant clones as a fraction of G418 resistant clones. In both methods the cells were not replated before selection so that each clone represents a single targeting event as previously described (10). In the first procedure 180 μg/ml of G418 (active ingredient) was added to each 90 mm plate 24 h after electroporation. After 10 days the G418 resistant clones were counted. Then 6-TG was added to each plate at a final concentration of 10 μM. After 21 days the 6-TG + G418 resistant colonies were counted. In the alternative protocol the plates were initially selected with a concentration of 10 μM TG was added. The selection was maintained for another 10 days. The first procedure was used for 10–20% of the plates to control for transfection efficiencies and the alternative for 80–90% of the plates. The different protocols always gave the same absolute targeting frequency for each construct.

**Southern blot analysis of targeted colonies**

The DNA from the 6-TG + G418 resistant clones was digested with a restriction enzyme, separated through a 0.7% TAE gel by

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**Figure 1. Vectors which target the hprt locus.** The thick line represents the homologous hprt sequences and the thin line represents the plasmid. All of the vectors were derived from the same 6.8 kb SacI–EcoRI fragment of hprt with exons 2 and 3 (labeled rectangles) as described in the experimental procedures. IV, insertion vector (neo outside the hprt homology). The checkbox indicates the neo selection cassette, MC1neoPA (Stratagene), and the arrow illustrates the direction of transcription. The striped box represents 'filler' DNA for IV6.8G2.5. The stippled box represents filler DNA for IV6.8G4.2. For the selection cassette, MC1 neoPA (Stratagene), and the arrow illustrates the neo the vectors were derived from the same 6.8 kb hprt fragment of hprt sequences and the thin line represents the plasmid. All of the homologous sequence and the thin line represents the plasmid. All of the homologous sequence and the thin line represents the plasmid.
electrophoresis, transferred to Gene Screen filters (DuPont) and hybridized to a probe internal to the vector, hprt exons 2 and 3 from the cDNA cut with HincII and HpaII (17). Some Southern blots were re-probed with neo sequences. The use of internal probes for these vectors has been shown to be reliable and result in an accurate determination of the recombinant allele structure (10,18,21). A BamHI digest was performed on DNA from 6-TG + G418 resistant clones generated from all the vectors. Other digests were performed from clones generated with the following vectors: IV6.8AN, AccI digest; IV6.8BN and IV6.8Δ86, BamHI + XhoI digest; IV6.8XN3, XmnI and Nhel digests (re-probed with neo); IV6.8G0.5, HindIII and EcoRI digests; IV6.8G2.5, HindIII digest; IV6.8G4.2, Nhel and a HindIII digest. The probes were labeled with random primed labeling using manufacturer’s conditions (Boehringer Mannheim).

PCR and oligonucleotide hybridization

The clones targeted with IV6.8Δ86 were analyzed by PCR with primer combinations specific for the 5’ duplicate. The upstream sense primer was 5’-CCTGAATTTAATTTCTAGG-3’ (specific for the intron 2/exon 3 junction). The downstream antisense primer was 5’-TGCTGACAAGGCTATTAGTT-3’ and is complementary to the plasmid and amplifies the 5’ duplicate. The following conditions were used in 20 μl total volume: 150 ng genomic DNA; 1 U AmpliTaq Polymerase (Perkin-Elmer Cetus); 50 ng of each primer; 250 μM of each dNTP; 1 x Cetus buffer (Perkin-Elmer Cetus), 5% dimethylsulfoxide (DMSO); 16.7 mM MgCl2. Amplification was performed in a DNA Thermal Cycler (Perkin-Elmer Cetus) for 1 cycle at 93°C for 6 min followed by 30 cycles of denaturing at 93°C for 30 s, annealing at 37°C for 30 s and extending at 70°C for 1 min. The final cycle was extended at 70°C for 15 min.

Oligonucleotide hybridization. The 1.2 and 1.1 kb PCR products were separated by electrophoresis through a 1% TAE gel at 50 V for 4 h. The DNA was transferred on to a Gene Screen Plus filter and hybridized to an oligonucleotide within the 86 bp deletion, y (5’-CCACAATGTGATGGCCTCCC-3’) by standard conditions. After exposure the filter was stripped and hybridized to a probe internal to the vector, 38 cycles of denaturing at 93°C for 30 s, annealing at 37°C for 30 s and extending at 70°C for 1 min. The final cycle was extended at 70°C for 15 min.

RESULTS

The affect of the position of the double-strand break and vector mutation on the targeting frequency

This study focuses on gene conversion during homologous recombination of an insertion vector. The gene targeting frequency and the integration pattern after homologous recombination was analyzed for a series of vectors that contain the same homologous sequences; however, each vector has a unique mutation. The targeting frequency was determined after the vector DNA was transfected into ES cells by electroporation and selected in G418 for neomycin phosphotransferase activity and in 6-TG for the absence of Hprt function. Southern analysis was performed to confirm that the 6-TG + G418 resistant clones were targeted and to observe gene conversion. The relative targeting frequencies are expressed as the ratio of 6-TG + G418 resistant clones to G418 resistant clones. Absolute targeting frequencies (6-TG + G418 resistant clones per electroporation) are not presented but may be derived from Table 1.

A series of vectors were electroporated into AB1 ES cells and the number of G418 resistant and 6-TG + G418 resistant clones was scored. Since there is some variation in the targeting frequencies between experiments, each experimental group indicates an experiment in which all electroporations were performed at the same time and with a common batch of ES cells under the same conditions. Under these conditions the relative relationship between the targeting frequencies obtained with each vector are constant (9). Linearization refers to the restriction enzyme used to linearize the construct before the electroporation. All of the constructs are linearized in the homolog. Each electroporation consists of 25 μg DNA transfected into 10^7 ES cells. A representative sample of the 6-TG resistant clones were analyzed to ascertain if the recombinant allele had the expected structure. Modified insertion events, where the exact structure of the targeted allele has not been elucidated were previously described (5,8).

<table>
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<tr>
<th>Exp Vector</th>
<th>Linearization</th>
<th>Electro-positions</th>
<th>Total G418</th>
<th>Total TQ + G418</th>
<th>Integration atsite of TG resistant Clones</th>
<th>Vector insertion modified</th>
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<tr>
<td>A IV6.8</td>
<td>Xhol</td>
<td>3</td>
<td>3240</td>
<td>110 1/29</td>
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<td>ND</td>
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<td>110 1/68</td>
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<td>6066</td>
<td>421 1/14</td>
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<tr>
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<td>511 1/33</td>
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<td>126 1/159</td>
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<td>233 1/74</td>
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A series of vectors were electroporated into AB1 ES cells and the number of G418 resistant and 6-TG + G418 resistant clones was scored. Since there is some variation in the targeting frequencies between experiments, each experimental group indicates an experiment in which all electroporations were performed at the same time and with a common batch of ES cells under the same conditions. Under these conditions the relative relationship between the targeting frequencies obtained with each vector are constant (9). Linearization refers to the restriction enzyme used to linearize the construct before the electroporation. All of the constructs are linearized in the homolog. Each electroporation consists of 25 μg DNA transfected into 10^7 ES cells. A representative sample of the 6-TG resistant clones were analyzed to ascertain if the recombinant allele had the expected structure. Modified insertion events, where the exact structure of the targeted allele has not been elucidated were previously described (5,8).
The IV.68 derivative constructs (Fig. 1) with mutations (between 12 and 86 bp) that include both the addition and the deletion of nucleotides compared to the chromosomal template were linearized at different distances from the vector mutation and introduced into ES cells. The targeting frequency of the vectors IV6.8BN, IV6.8AN, IV6.8RB and IV6.8ANΔ was reduced by about 1.5–2-fold compared with IV6.8 linearized at a comparable location in the homology (Table 1A–D); however, a reduction in the targeting efficiency was not always observed (Table 1C).

Southern analysis of 6-TG + G418 resistant clones demonstrated that loss of the vector mutation and gain of the chromosomal sequence occurred at a high frequency when the mutation was close to the DSB for all mutations observed (Table 2). The loss of the vector mutation occurred at a frequency inversely proportional to the distance between the mutation and the DSB. Loss of the vector mutation occurred in: 88 and 69% of the clones with terminal heterologies (IV6.8BN and IV6.8AN cut in the mutation with NotI, respectively); 62 and 54% of the clones when the mutation was 0.123 kb away from the DSB (IV6.8RB cut with XhoI and IV6.8ANΔ cut with EcoRI, respectively); 40% of the clones when the mutation was 0.472 kb away from the DSB (IV6.8AN cut with XhoI). A detailed analysis of the clones generated with IV6.8ANΔ is presented in Figure 3. Detailed analysis of the targeted clones generated with IV6.8BN and IV6.8RB is presented elsewhere (9,18).

The frequency of loss of the vector mutation and gain of the chromosomal sequence dropped sharply when the vector mutation was further away from the DSB. Loss of the vector mutation occurred in just 7% of the clones when the mutation was 2.6 and 3.2 kb away from the DSB (IV6.8RB and IV6.8AN cut with NheI) and in 16% of the clones when the mutation was 4.3 kb away from the DSB (IV6.8ANΔ cut with XhoI). In one experiment loss of the vector mutation was not observed (IV6.8G0.5 with 4.4 kb between the DSB and the vector mutation).

Gain of the vector mutation and loss of the chromosomal sequence occurred at a low frequency (Table 2). Gain of the vector mutation was not observed in most of the experiments; however, it was observed in 1 out of 72 clones targeted by a vector with terminal heterologies (IV6.8BN cut in the mutation with NotI), 12 out of 175 clones targeted by a vector with the mutation 0.123 kb away from the DSB (IV6.8RB and IV6.8AN cut with NheI) and in 16% of the clones when the mutation was 4.3 kb away from the DSB (IV6.8ANΔ cut with XhoI). Southern analysis that demonstrates gene conversion during homologous recombination with IV6.8ANΔ is presented in Figure 4.

Gaps of up to 4.2 kb are accurately repaired

To determine if there were any length constraints in the repair mechanism, a set of vectors with large double-strand gaps in the hprt DNA were constructed and linearized at the location of the deletion. Each gapped construct was cloned so that the missing target DNA was not co-electroporated into the ES cells; thus, eliminating the possibility of the two fragments being ligated back together before targeting (22). Filler DNA was inserted into vectors where the sites delimiting the gaps were non-compatible to ensure the ends were perfectly homologous. The vectors IV6.8G0.5, IV6.8G2.5 and IV6.8G4.2 have gaps of 0.536, 2.5 and 4.2 kb, respectively (Fig. 1).
The targeting frequency (Table 1F) and integration pattern (Fig. 5) were determined for each vector. The targeting efficiency for IV6.8G0.5, IV6.8G2.5 and IV6.8G4.2 was reduced by 2.6-, 3.5- and 20-fold compared to IV6.8 linearized in the homology (Xhol), respectively (Table 1). The reduction in the targeting frequencies are due to either rate limiting constraints of repair or the reduced length of homology (4,21,23). The gaps were shown to be repaired for 12/12, 11/12 and 8/8 6-TG' clones targeted with IV6.8G0.5, IV6.8G2.5 and IV6.8G4.2, respectively (Table 1F and Fig. 5). The single clone that gave an alternative pattern did not resemble recombination without repair; we classify this as a modified insertion event (9). Repair was demonstrated by restoration of the fragment length and restriction enzyme sites so that the final recombinant allele resembles one generated with the parental IV6.8 vector.

In control experiments, the same vectors were linearized at the edge of the homologous sequences. Although 6-TG resistant clones were generated at lower frequencies than when linearized in the homology, none of the clones repaired the deletion and the filler DNA integrated into the recombinant structure (data not shown).

## DISCUSSION

In this report we examined gene conversion during homologous recombination of insertion vectors into a chromosomal target in ES cells. Gene conversion was scored by the presence of either the vector mutation or chromosomal sequence in both duplicates. We made the following observations. (i) The presence of the vector mutation had only a marginal impact on the targeting frequency and the distance between the mutation and the DSB did not affect the targeting frequency. This indicates that gene conversion is not rate limiting for the vector mutation since gene conversion was frequently observed in some cases. (ii) A sequence specific bias in gene conversion was not observed. The same pattern of gene conversion was observed for five different vector mutations that included the addition of nucleotides and the deletion of nucleotides. (iii) Loss of the vector mutation and gain of the chromosomal sequence was bi-directional and occurred with an increased frequency as the distance between the vector mutation and DSB decreased. (iv) Gain of the vector mutation and loss of the chromosomal sequence occurred at a low frequency. (v) Repair of up to 4.2 kb gaps is accurate.

What are the gene conversion mechanisms responsible for these observations? Two forms of gene conversion have been proposed: gap formation and repair and mismatch repair of heteroduplexes. Gap formation and repair describes removal of nucleotides from the strand with a DSB and subsequent repair on a homologous DNA template (3,7). Mismatch repair of heteroduplexes describes the repair of a mismatch after the formation of a heteroduplex during strand invasion or after branch migration of the Holliday junctions (3).

Why was loss of the vector mutation more common when it was close to the DSB? Both forms of gene conversion could explain this observation. Gap formation and repair predicts removal of vector sequences adjacent to the DSB and repair that utilizes an intact chromosomal template. A vector mutation within the range of the gap is replaced by chromosomal sequence. If this mechanism was used, then gaps of ≥0.472 kb are commonly formed but rarely extend to 2.6 kb. Mismatch repair of heteroduplexes may also explain the data. Both vector ends invade the homologous template without significant degradation of the ends. We have previously presented data that shows the ends of the vector do not always undergo gap formation (9). After strand invasion a heteroduplex would be formed with a nick present at the end of the vector strand. The mismatch could be resolved to either the vector mutation or chromosomal sequence; however, the nick may stimulate removal of the vector mutation. A nick was shown to stimulate bidirectional repair preferentially directed toward the nicked strand (25). If this mechanism was used, then it is likely that heteroduplexes of 0.472 kb are frequently formed during strand invasion but rarely extend to 2.6 kb. By either mechanism, loss of the vector mutation in favor of the chromosomal sequence increases as the distance between the vector mutation and the DSB decreases.
Why was there gain of the vector mutation and loss of the chromosomal sequence in a few clones when the vector mutation was close to the DSB? Gap formation and repair predicts sequence removal from only one strand with the DSB (the vector) and therefore, can not be responsible for removal of the chromosomal sequence. Mismatch repair of heteroduplexes may form during strand invasion and may then be resolved to the chromosomal sequence. Mismatch repair of heteroduplexes may reflect ligation of the nicked strand prior to mismatch repair. Alternatively, there may be competing gene conversion mechanisms. Gap formation may frequently remove the vector mutation but not in every instance. For these rare events a heteroduplex may form during strand invasion and may then be resolved to either the vector mutation or chromosomal sequence.

A small percentage of clones contain only the vector mutation or chromosomal sequence in both duplicates when the vector mutation was >2.6 kb away from the DSB. There was not a strong preference but not an absolute requirement to repair the vector mutation when Xmn1 did not cut (not shown). Either the vector mutation or the chromosomal sequence was always present. Exons 2 + 3 are shown as numbered rectangles and were used as the probe. The thick line represents *aprt* DNA of vector origin, the thin line represents *aprt* DNA of chromosomal origin and the line of intermediate thickness indicates plasmid sequences. The MC1 neoA cassette is shown as a box labeled neo. The length of DNA is shown in kb. WT, wild type (AB1 cell DNA). Clones 11 and 13 are +/+, where + indicates presence of the vector mutation. There is an additional band in clone 11 that does not match any expected band which is probably the result of a random integration of a second vector unit elsewhere in the genome. Clones 9 and 10 are +/-, where - indicates absence of the vector mutation. Clone 2 is +/—. Clones 1, 3-8, 14 and 15 are -/+.

Figure 3. Southern analysis for TG *G* + G418 clones targeted with IV6 Δ86

Analysis of 24 clones showed that a single unit of the vector integrated into the target locus for 22 clones and multiple units for two clones. (A) Correction of the deletion was analyzed by the restoration of an XhoI site in both duplicates with a BamHI and a BamHI + XhoI digest hybridized to exons 2 and 3. 54% corrected the deletion (representative clones 18 and 20). Two additional clones contained an integration of at least two units in the target locus in which at least one unit was corrected and the others was not (representative clone 17). The panel to the right of the predicted fragments indicates the presence (+) or absence (-) of the vector mutation in exon 3 of both duplicates. The thick line represents *aprt* DNA of vector origin, the thin line represents *aprt* DNA of chromosomal origin and the line of intermediate thickness represents plasmid DNA. MC1 neoA is shown as a box labeled neo. Exons 2 and 3 are numbered rectangles. The Δ86 represents the 86 bp deletion. B, BamHI; R, EcoRI; X, XhoI. (B) Southern analysis. B, BamHI and B+X, BamHI + XhoI digests were done on TG + G418 clones (clones 17, 18, 20 and 21 are represented). The presence (+) or absence (-) of the vector mutation for both 5' and 3' duplicates is indicated in the upper panel. WT, wild type (AB1 cell DNA). The length of DNA is in kb. (C) Primers A and B, small arrow heads, were used to amplify exon 3 (large box) of the 5' duplicate by the polymerase chain reaction (PCR) and hybridized to oligo y, inside the deletion and oligo z, outside the deletion. The sizes of the PCR products are in kb. The 1.2 kb fragment represents correction of the deletion and hybridized to both oligonucleotides (representative clones 18 and 20). The 1.1 kb fragment represents the deletion and hybridizes to only oligo Z (representative clone 21). Clones with multiple units in the target locus (some corrected and some not) or clones which segregated without repair of the heteroduplex, amplified a 1.2 kb fragment which hybridized to both oligonucleotides and a 1.1 kb fragment which hybridized just to oligonucleotide z (representative clone 17).
The targeting frequency by limiting either strand invasion or prior to the transfection, were accurately repaired. The gaps were never transferred to the chromosomal sequences. The reduced targeting frequencies for these vectors indicates the gaps inhibit the targeting frequency by limiting either strand invasion or repair. However, the shorter length of homology will contribute to a reduction in the targeting frequency (21); therefore, the affect the gaps have on the gene targeting efficiency is difficult to determine.

Gene targeting by vector insertion is considered to be a conservative recombination event since both the vector and chromosomal DNA components are retained in the recombined allele. However, there is a non-conservative component that can be scored when there are sequence differences between the two partners. In this study we showed that gene conversion was frequent when the vector mutation was close to the DSB. However, gene conversion occurs at sites as distant as 4.3 kb from the DSB.

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