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Efficiency of Insertion versus Replacement Vector Targeting Varies at Different Chromosomal Loci

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We have analyzed the targeting frequencies and recombination products generated with isogenic vectors at the *fah* and *fgr* loci in embryonic stem cells. A single vector which could be linearized at different sites to generate either a replacement or an insertion vector was constructed for each locus. A replacement event predominated when the vectors were linearized at the edge of the homologous sequences, while an insertion event predominated when the vectors were linearized within the homologous sequences. However, the ratio of the targeting frequencies exhibited by the different vector configurations differed for the two loci. When the *fgr* vector was linearized as an insertion vector, the ratio of targeted to random integrations was four- to eightfold greater than when the vector was linearized as a replacement vector. By contrast, the ratio of targeted to random integrations at the *fah* locus did not vary with the linearization site of the vector. The different relationships between the targeting frequency and the vector configuration at the *fgr* and *fah* loci may indicate a DNA sequence or chromatin structure preference for different targeting pathways.

The frequency of homologous recombination between an introduced vector and chromosomal DNA sequences is affected by many factors. One variable that is known to increase the gene targeting frequency in both *Saccharomyces cerevisiae* and some experiments with mammalian cells is the generation of a double-stranded break (DSB) or gap within the sequences which are homologous between the vector and the chromosomal target (1, 12–14, 39). Vectors with a DSB in the homologous counterpart of the chromosomal sequences have been termed insertion vectors and usually recombine through a single reciprocal pathway (25).

The DSB repair (DSBR) model, originally proposed to explain the repair of breaks in DNA, has been invoked to explain the elevation of targeting frequencies in *S. cerevisiae* observed by the provision of a DSB in the homologous sequences (24, 27, 35). Although this model was originally developed with *S. cerevisiae*, it is supported by data for vector-chromosome recombination in mammalian cells (12, 14, 39). A vector that targets via this pathway will integrate the entire vector to form a duplication of homology (a vector insertion product) or will give rise to heteroduplex DNA, which can be experimentally scored only if a unique sequence in the targeting vector is transferred to the targeted cell (a gene replacement product) (25).

A vector with a DSB outside the region of homology (a replacement vector) may recombine with the host chromosomal sequences by double-reciprocal recombination or gene conversion. This results in a gene replacement product (24) which can be scored only when chromosomal sequences are altered. Recently, we have described a unique class of insertion-type recombination products generated at a high frequency with replacement vectors (13, 41). These insertion-like products suggest a preference for the integration of replacement vectors via an insertion pathway after end-end joining, possibly via a mechanism similar to that described by the DSBR model (13, 38).

The presence of free ends of a vector, either homologous or heterologous, may be important for directing specific targeted integration pathways which in turn may affect both the targeting frequencies and the integration products. Previously, it has been shown that at the *Aprt* locus in CHO cells and at an integrated simian virus 40 genome in COS1 cells, when a DSB is made in the homologous sequences of the vector, the gene targeting frequency increases relative to the frequencies observed when the DSB is made outside these elements (1, 14). However, at the immunoglobulin μ heavy-chain constant region in hybridoma cells, replacement and insertion vectors target at about the same frequency (15). We have reported previously that at the *hprt* locus in embryonic stem (ES) cells insertion vectors target more efficiently than replacement vectors (11–13), yet the Capecchi laboratory (9, 37) has reported that the two vector types target at about the same efficiency.

Free DNA ends are also important for extrachromosomal homologous recombination in mammalian cells. DSBs in the homologous sequences in either of the substrates involved in the exchange increase the recombination frequency (5, 6, 17). As predicted by the DSBR pathway, both insertion and replacement products are observed. However, other reports have shown that intermolecular extrachromosomal homologous recombination is increased if both introduced DNAs are linearized within or just close to the homologous sequences. To explain this observation, the single-strand annealing (SSA) model is invoked. In the SSA model, the breaks appear to be substrates for exonuclease or helicase activity which generates complementary single-stranded DNA ends. These homologous single-stranded regions can anneal to each other and result in a replacement product (2, 19, 30, 40). The location of the break does not need to be flanked by homology as long as single-strand homology is exposed after exonuclease digestion or

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helicase unwinding (2, 19, 20, 30). A similar pathway has been observed in *Xenopus* oocytes (21, 22) and during intramolecular extrachromosomal recombination (8, 18). This pathway seems to predominate over the DSBR pathway for extrachromosomal recombination.

In vector-chromosome recombination, a DSB in the homology can result in a higher targeting frequency than a DSB at the edge of or outside the homologous region at some chromosomal loci but apparently not at others. Does this imply that chromatin structure or specific DNA sequences influence the efficiency of homologous recombination with replacement and insertion vectors? During meiosis or a mating type switch in *S. cerevisiae*, chromosomal breaks appear at specific sites and increase the frequencies of recombination at these specific loci (29, 33, 34). If similar sequences are present in the mammalian genome and can be activated by homologous pairing in nonmeiotic cells, then it might be anticipated that a vector homologous to such a region will target efficiently even without a break in the homologous sequences of the vector.

Replacement vectors exhibit a wide range of targeting frequencies in ES cells. One of the variables which can influence these frequencies is the degree of homology (length and identity) between the participating sequences (11, 31, 36, 37). The degree of homology might affect the targeting frequency at several levels, including the search for the chromosomal partner and the formation of recombination intermediates. If the search is not limiting, then the frequency of targeting could be enhanced by the inclusion of sites in the homologous DNA which are subject to chromosomal breaks or the formation of single strands. If such sequences were randomly dispersed in the genome, this would explain the wide variation in targeting frequencies from locus to locus. The probability of such a sequence being included in a targeting vector would increase with added homologous sequences.

We have examined how the targeting frequency is influenced by the provision of a DSB in the vector, using vectors which target two different autosomal loci: *fgr*, a tyrosine kinase gene of the *src* family (7), and the fumarylacetoacetate hydrolase (*fah*) gene (10). The vectors designed to target these loci have been designed so that they are very similar; each can be linearized to generate either insertion- or replacement-style vectors, and both were constructed with DNA which is isogenic with respect to the stem cells. We have compared the relative targeting frequencies of these vectors and the structure of the recombinant alleles which are generated by the different vector configurations at the respective loci. We have found that a DSB within the homologous sequences elevated the targeting frequency by four- to eightfold for one locus but did not change the frequency for the other locus. Thus, different DNA sequences or their immediate chromosomal environment can influence the relative efficiencies of different gene targeting pathways.

MATERIALS AND METHODS

Vector construction. (i) *fgr* (7). A 6.0-kb *Kpn*I-*Nhe*I fragment that contains exons 2 to 4 was cloned into the *Kpn*I and *Xba*I sites of pBluescriptIIKS (Stratagene). The *Nhe*I and *Xba*I sites have compatible sticky ends, and both sites are destroyed after the ligation, leaving a unique *Nhe*I site in the homologous region. The *fgr* sequences were obtained from a 129 mouse library. Pol2sneobpA (32) was cloned into a *Tth*111I site in exon 2. The *neo* cassette separates the homologous sequence into a 2.0-kb upstream short arm and a 4.0-kb downstream long arm. The unique *Nhe*I site was used to linearize the vector in the long arm of homology. This site was located 1.2 kb from the *neo* cassette and 2.8 kb from the 3′ end. *Kpn*I was used to linearize the vector at the edge of the homologous sequences.

(ii) *fah* (10). A 6.0-kb *Apa*I-*Not*I fragment (the *Not*I site was engineered) that contains exons 3 to 5 was cloned into the *Apa*I and *Not*I sites of pBluescriptIIKS (Stratagene). The *fah* sequences were obtained from a mouse 129 library which is isogenic with the cell line to be targeted. Pol2sneobpA (32) was cloned into an *Sph*I site in exon 5 which had previously been blunted with deoxynucleoside triphosphates and Klenow fragment. The *neo* cassette separates the homologous region into a 1.4-kb downstream short arm and a 4.6-kb upstream long arm. A unique *Hin*dIII site was used to linearize the vector in the long arm of homology. The *Hin*dIII site was located 2.6 kb from the *Apa*I site (5′ end) and 2.0 kb from the *neo* cassette. *Apa*I was used to linearize the vector at the 3′ edge of the homologous region.

Electroporation and tissue culture. Comparisons of targeting frequencies for *fah* and *fgr* cut at the edge of or inside the homologous region have been grouped into electroporations performed under the same conditions on the same day with a common batch of ES cells. This is necessary since there is variation in the relative targeting frequencies from experiment to experiment. Within a group, the relative ratios from one vector to the next are consistent (12).

The DNA for an electroporation was prepared by Triton lysis and banded once by ultracentrifugation in a CsCl density gradient. DNA was cut to completion with the specified restriction enzyme. Electroporations were performed on AB1 cells (23), which were cultured as previously described (28). For the electroporation, the AB1 cells were resuspended in phosphate-buffered saline at a density of 10^7 cells per ml. DNA (25 μg) was electroporated in 1 ml at 575 V/cm and 500 μF with a Bio-Rad gene pulser. After each electroporation, the DNA and cells were incubated for 10 min at room temperature and then 10^7 cells were plated onto four 9-cm-diameter SNL76/7 feeder plates (23).

Twenty-four hours after the electroporation, 180 μg of G418 (active ingredient) per ml was added to each 9-cm-diameter plate, and cultures were maintained for 12 days in order to select and count $G418^r$ colonies. Colonies were then picked and plated onto 96-well plates. The cells were grown on the plates and passaged 5 to 7 days later; half of each colony was frozen in a 96-well plate, and the other half was plated onto a gelatinized plate without feeder cells for Southern analysis and grown for an additional 5 to 7 days (26).

Southern blot analysis of targeted colonies. Southern analysis was performed on clones grown in 96-well plates as previously described (26). The subset of clones identified as targeted by this screen were thawed and grown for more extensive Southern analysis. The last passage for these clones was grown on gelatinized plates without feeder cells to minimize contamination with feeder cell DNA.

For *fgr* targeting, genomic DNA from G418-resistant clones was digested with *Bam*HI and hybridized to a *Bam*HI-*Eco*RV external probe located 5′ to the vector sequences. The filter was stripped and rehybridized to the *neo* cassette to confirm the targeting pattern.

For *fah* targeting, genomic DNA from G418-resistant clones was digested with *Eco*RV and hybridized to a 0.5-kb *Eco*RI fragment found immediately 3′ to the homologous sequences in the vector. The same probe was also hybridized to a *Kpn*I or *Hin*dIII digest to further analyze the integration events.

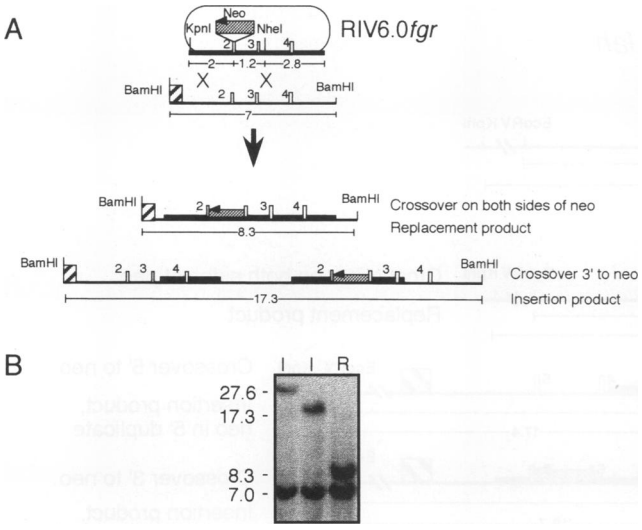

FIG. 1. (A) Vector chromosome recombination between RIV6.0*fgr* and the *fgr* locus. *fgr* DNA of vector origin (thick line), the vector backbone (thin line), *fgr* DNA of chromosomal origin (line of intermediate thickness), Pol2sneobpA (shaded box), the external probe (hatched box), and crossover points (X) are indicated. The length of DNA is shown in kilobases. (B) Southern blot analysis of G418^r clones generated with RIV6.0*fgr*. A *Bam*HI digest was used to analyze both insertion (I) and replacement (R) events. The 27.6-kb fragment demonstrates the integration of two vector units.

RESULTS

Targeting frequency and pattern of integration at the *fgr* locus. The replacement-insertion targeting vector RIV6.0*fgr* (Fig. 1A) was constructed to evaluate the differences in the targeting frequency and the integration pattern of insertion and replacement vectors at an autosomal locus. This vector contains 6.0 kb of sequences homologous to the *fgr* locus and is isogenic with the ES cell line. A *neo* cassette, which serves to select for the transfected clones, was cloned into exon 2. This selectable marker is retained in the locus following replacement and insertion events; therefore, RIV6.0*fgr* may be cut with different enzymes and used as either an insertion or a replacement vector. RIV6.0*fgr* was cut with *Nhe*I in the homologous sequences to generate an insertion vector. Alternatively, the vector was cut with *Kpn*I at the edge of the region of homology to generate a replacement vector (Table 1, experiments A and C).

Both forms of the linearized vector were introduced into a common batch of ES cells by electroporation, and clones were selected in G418 for 12 days. The types and proportions of the various recombinant classes among the G418-resistant clones were scored by Southern analysis (Fig. 1). RIV6.0*fgr* linearized with *Nhe*I (insertion vector) was found to target at a four- to eightfold-higher frequency than when linearized with *Kpn*I (replacement vector) (Table 1). Thus, the linearization site of this vector greatly influences the targeting frequency.

Southern analysis identified the different classes of recombinant clones (insertion and replacement events [1, 3, 11–13, 25, 37–39]). A *Bam*HI digest hybridized to a 5′ external probe detected a 7.0-kb wildtype fragment, while a replacement product showed a 1.3-kb increase in the fragment size and an insertion product showed a 10.3-kb increase in the same fragment. Concatemeric integration into the target locus increased the size of the wild-type fragment by a multiple of the vector's length (Fig. 1). The identities of these fragments were also confirmed by rehybridization with a *neo* probe and produced the 8.3- and 17.3-kb fragments expected for replacement and insertion events, respectively (data not shown). Analysis of 182 G418-resistant clones generated by RIV6.0*fgr* with a DSB in the homology (*Nhe*I) generated 51 insertion events (17 of the clones contained more than one unit) and 3 replacement events (Table 1, experiments A and C). RIV6.0*fgr* linearized at the edge of the homologous sequence (*Kpn*I) generated 8 replacement events in 186 G418^r colonies screened, and no insertion events were observed (Table 1, experiments A and C).

Targeting frequency and pattern of integration at the *fah* locus. To determine if insertion or replacement vectors would show the same variation in the targeting frequency at another locus, the vector RIV6.0*fah* was constructed (Fig. 2A). This vector was made from isogenic DNA and is similar in design to RIV6.0*fgr*. It may be linearized with different enzymes which cut at unique sites to generate either an insertion vector (*Hin*dIII) or a replacement vector (*Apa*I).

The linearized vectors were introduced by electroporation into a common batch of ES cells which were subsequently selected in G418. After 12 days, the G418^r colonies were

TABLE 1. Targeting frequency and integration pattern

Expt[a]	Vector	Linearization site	Vector type	No. of clones			Targeting frequency		Integration pattern		
				Total G418^r	G418^r screened	Total targeted	Absolute (targeted clones/cells electroporated)	Relative (targeted clones/ G418^r clones analyzed)	Vector insertions		No. of gene replacements
									Total	VILA/VISA[b]	
A	RIV6.0*fgr*	*Nhe*I	Insertion	2,032	91	43	9.7×10^{-5}	1/2.1	40[c]	ND	3
	RIV6.0*fgr*	*Kpn*I	Replacement	1,672	92	5	9.1×10^{-6}	1/18.4	0	NA	5
B	RIV6.0*fah*	*Hin*dIII	Insertion	1,331	93	10	1.4×10^{-5}	1/9.3	9[d]	6/3	1
	RIV6.0*fah*	*Apa*I	Replacement	1,611	92	7	1.2×10^{-5}	1/13.1	0	NA	7
C	RIV6.0*fgr*	*Nhe*I	Insertion	2,988	91	11	3.6×10^{-5}	1/8.3	11[e]	ND	0
	RIV6.0*fgr*	*Kpn*I	Replacement	3,586	94	3	1.1×10^{-5}	1/31.3	0	NA	3
	RIV6.0*fah*	*Hin*dIII	Insertion	1,103	73	6	9.1×10^{-6}	1/12.1	6	5/1	0
	RIV6.0*fah*	*Apa*I	Replacement	1,527	66	4	9.2×10^{-6}	1/16.5	0	NA	4

[a] Each experiment was performed with a single batch of ES cells. One electroporation (25 μg of DNA and 10^7 ES cells) was performed for each vector linearized with the indicated restriction enzyme for each experiment.
[b] VILA, vector insertion long arm; VISA, vector insertion short arm; NA, not applicable; ND, not determined.
[c] Fourteen of these targeted clones contain more than one vector unit.
[d] Two of these targeted clones contain more than one vector unit.
[e] Three of these targeted clones contain more than one vector unit.

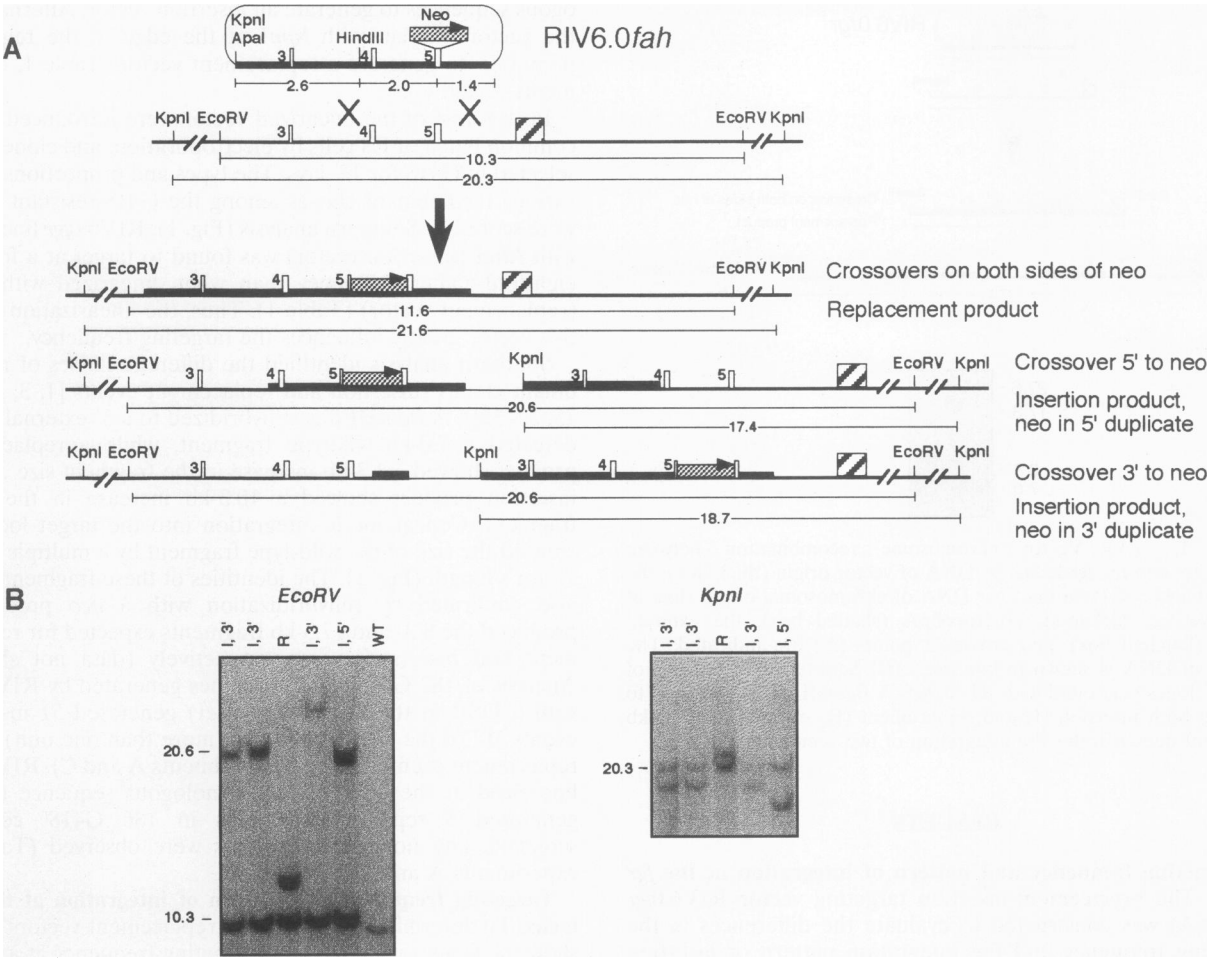

FIG. 2. (A) Vector chromosome recombination between RIV6.0*fah* and the *fah* locus. *fah* DNA of vector origin (thick line), the vector backbone (thin line), *fah* DNA of chromosomal origin (line of intermediate thickness), Pol2sneobpA (shaded box), the external probe (hatched box), and crossover points (X) are indicated. The length of DNA is shown in kilobases. (B) Southern blot analysis of G418^r clones generated with RIV6.0*fah*. An *Eco*RV digest was used to determine both insertion (I) and replacement (R) events. A *Kpn*I digest was used to analyze the integration pattern for G418^r clones transfected with RIV6.0*fah* linearized with *Hin*dIII. The insertion (I) integration products that resulted from a crossover on the 3′ or 5′ vector arm were differentiated. The Southern blot analysis represents individual clones in the same order from left to right for the *Eco*RV and *Kpn*I blots. WT, wild type.

isolated and expanded in 96-well plates for Southern analysis. RIV6.0*fah* linearized to make an insertion vector was found to target at the same frequency as when linearized to make a replacement vector (Table 1, experiments B and C). Thus, in contrast to the finding for RIV6.0*fgr*, the targeting frequency of RIV6.0*fah* was found to be independent of the linearization site (Table 1, experiments B and C; $P < 0.05$).

To identify the various categories of recombination events, Southern analysis was performed on genomic DNA from the G418^r clones (Fig. 2). An *Eco*RV digest hybridized to a 3′ external probe discriminated between the various types of recombination events, since there is not an *Eco*RV site in the vector. The wild-type fragment is 10.3 kb; a replacement recombination product resulted in a 1.3-kb increase in the fragment size (the size of the *neo* cassette), while an insertion recombination product resulted in a 10.3-kb increase in the fragment size for each vector unit present. RIV6.0*fah* linearized as an insertion vector generated 15 insertion events (two of the clones contained more than one unit) and 1 replacement event in 166 G418^r colonies screened (Table 1, experiments B and C). RIV6.0*fah* linearized as a replacement vector generated 11 replacement events and no insertion events in 158 G418^r colonies screened (Table 1, experiments B and C).

Further analysis of the targeted clones generated with RIV6.0*fah* linearized as an insertion vector was performed with *Kpn*I (Fig. 2) or *Hin*dIII (data not shown) to determine the position of the crossover. The wild-type *Kpn*I fragment is 20.3 kb and the vector backbone introduces an additional *Kpn*I site so that a 17.4-kb fragment is generated if the vector recombines on the long arm (5′ crossover) to place the *neo* cassette in the 5′ duplicate or an 18.7-kb fragment is generated if the vector recombines on the short arm (3′ crossover) to place the *neo* cassette in the 3′ duplicate. Of the analyzed clones with insertion events, 11 had integrated the *neo* cassette in the 5′ duplicate while the remaining 4 had the *neo* cassette in the 3′ duplicate.

DISCUSSION

To critically evaluate the significance of a DSB in the homologous sequences of a targeting vector, we sought to perform experiments controlled for the many variables which can affect the targeting frequency (11, 31, 36, 37). These

variables were minimized so that meaningful comparisons between the targeting efficiencies of various vectors could be made. In particular, the DNAs are isogenic and of similar lengths, and the vectors were designed to undergo both replacement (DSB at the edge of the homologous sequences) and insertion (DSB within the homologous sequences) events. The same selection cassette was inserted into similar positions with respect to the homologous sequences to yield vectors with asymmetric arms. One other major variable, the significant fluctuation in the targeting frequency of a locus from experiment to experiment (12, 16), was controlled by transfecting the differently linearized vectors at the same time into a common batch of ES cells. The targeting frequency was analyzed for two vectors cut at different sites. One of the vectors described here, RIV6.0*fgr*, exhibited a four- to eightfold increase in the targeting frequency when linearized as an insertion vector compared with the frequency when linearized as a replacement vector. However, the linearization site of the *fah* vector did not detectably alter the targeting frequency.

In *S. cerevisiae*, a DSB in the homologous sequences of the vector increased the gene targeting frequency (24). In mammalian cells, targeting frequencies can also be increased with a DSB in the homologous sequences of the vector (1, 12–14). However, not all reports have described a change in the targeting frequency when a DSB is made in the homologous sequences of the vector (9, 15, 37). Indeed, targeting of the same region of the *hprt* locus by different groups has resulted in different conclusions (9, 12, 13, 41), although some of the variables controlled in the comparisons reported here could have contributed to the difficulty in reconciling these two sets of data (9, 13). In particular, the comparisons made by Thomas and Capecchi for the *hprt* locus (37) were not made with identical vectors. The discordancy between the studies might be a reflection of the precise DNA sequences present in the vectors; even though the vectors are overlapping, they are not identical. In addition, the Capecchi group constructed their insertion vectors in a configuration different from those described here, and the configuration of insertion vectors has been shown to influence the targeting frequency (12). The Capecchi laboratory has reinvestigated the reported difference in the targeting frequency between insertion and replacement vectors, using isogenic DNA, and has shown that the two vector types can target at the same frequency when an insertion vector configuration similar to ours is used (9). The discrepancy between these observations has been suggested to result from polymorphic variation between the nonisogenic vectors and the chromosome.

The recombinant alleles generated with the *fgr* and *fah* replacement vectors were shown to have the predicted replacement recombinant alleles in all cases; this contrasts with our previous observations for the *hprt* locus which had shown a predominance of insertion-like products (13). The RIV6.0*fgr* vector might be anticipated to show correct targeting because there was sufficient homologous sequence on the 2-kb short vector arm (38). However, the short arm for RIV6.0*fah* was only 1.4 kb long, which may be too short to reliably generate replacement events (13, 38). The isogenic sequences used here may also enhance the frequency of replacement events compared with insertion-like events.

In contrast to the predicted recombination events shown by these replacement vectors, the recombinant alleles generated with one of the insertion vectors were unusual. Both insertion vectors were linearized on the long arm of the homologous sequences, and, as expected, the majority of targeted clones had insertion events, with one of the clones showing a replacement event. This is predicted by the DSBR model (25, 35). More detailed analysis of clones generated with the RIV6.0*fah* insertion vector showed that in 25% of insertion events the crossover had resolved on the short arm, even though the break was at the midpoint on the long arm. Analogous vectors at the *hprt* locus have never shown short-arm resolution when the vector was linearized on the long arm (184 clones analyzed) (reference 12 and unpublished data). The analysis of these unusual clones does not reveal exactly how this occurred. But this observation, coupled with the fact that a DSB in this vector does not elevate the targeting frequency, suggests that the 1.4-kb short arm of RIV6.0*fah* is more recombinogenic than the long arm.

Thus, DSBs within the homologous region can increase the targeting frequency in ES cells but do not always do so. Why should one region of the genome be stimulated by a DSB in the homologous sequences of the vector while another is not? Why should the presence of adjacent homologous chromosomal ends be rate limiting for insertion events with one locus and not another? The DNA ends are probably critical in the strand exchange once the homologous locus has been found (12). It is, we believe, unlikely that one locus should be different from another with respect to the mechanics of the actual strand exchange.

One explanation for why targeting frequencies at one locus might be insensitive to a DSB is that breaks or single strands are generated in the chromosomal locus at a significant frequency. From the perspective of the recombination event, it should make no difference whether the broken ends or single strands are in the vector or the genome; the recombinant alleles which are generated would be indistinguishable. The different results observed with the two vectors may define a region of DNA in or near the *fah* sequences that was the site for frequent DSBs or single-strand formation which stimulates homologous pairing. This site would not serve to influence the frequency of targeting events via the insertion pathway (since a break is already present in the vector) but would serve as a site to initiate strand exchange for the replacement vectors, thereby elevating the relative frequency with which this vector class targets. One extension of this hypothesis is that the *fah* locus might target more efficiently than the *fgr* locus if uncut DNA was used as the targeting substrate.

The possibility of breaks being generated in the homologous sequences in the chromosomal partner of a targeting vector allows alternative models for this category of replacement recombination events to be considered. Although gene conversion by heteroduplex mismatch repair and double-crossover events is possible, the SSA pathway may also operate if a vector is linearized outside the homologous sequences and a DSB or single strand is present in the chromosomal target. This event could occur during S phase as a mimic of a DNA exchange between sister chromatids and in response to DNA repair. Intrachromosomal homologous recombination has been shown to be an active process during S phase in mammalian cells (4), and its occurrence may be the critical time for gene targeting.

The *fah* locus was shown to be targeted with a replacement vector at a high frequency, which, if the SSA model is applicable, suggests that the locus contains or is located close to DNA sequences that are frequently converted to single strands or ends that are exposed for annealing. There are sequences found in yeast cells that are highly recombinogenic because of frequent breaks and the generation of 3′ single-strand overhangs (29, 34). If DNA sequences that are similarly predisposed to breaks or single strands are found in mammalian DNA, then the SSA pathway with a replacement vector may predominate over the DSBR pathway with an insertion vector at that specific DNA region. A replacement vector

might then target at a frequency similar to or greater than that of an insertion vector at such a site.

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