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Genetic Exchange Between Homeologous Sequences in Mammalian Chromosomes Is Averted by Local Homology Requirements for Initiation and Resolution of Recombination

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ABSTRACT

We examined the mechanism by which recombination between imperfectly matched sequences (homeologous recombination) is suppressed in mammalian chromosomes. DNA substrates were constructed, each containing a thymidine kinase (*tk*) gene disrupted by insertion of an *Xho*I linker and referred to as a "recipient" gene. Each substrate also contained one of several "donor" *tk* sequences that could potentially correct the recipient gene via recombination. Each donor sequence either was perfectly homologous to the recipient gene or contained homeologous sequence sharing only 80% identity with the recipient gene. Mouse L*tk*$^-$ fibroblasts were stably transfected with the various substrates and *tk*$^+$ segregants produced via intrachromosomal recombination were recovered. We observed exclusion of homeologous sequence from gene conversion tracts when homeologous sequence was positioned adjacent to homologous sequence in the donor but not when homeologous sequence was surrounded by homology in the donor. Our results support a model in which homeologous recombination in mammalian chromosomes is suppressed by a nondestructive dismantling of mismatched heteroduplex DNA (hDNA) intermediates. We suggest that mammalian cells do not dismantle mismatched hDNA by responding to mismatches in hDNA *per se* but rather rejection of mismatched hDNA appears to be driven by a requirement for localized homology for resolution of recombination.

MAMMALIAN cells have evolved numerous mechanisms to protect genomic integrity. One such mechanism is the exquisite sensitivity that the recombination machinery displays toward small degrees of sequence divergence (Waldman and Liskay 1987, 1988; Lukacsovich and Waldman 1999). This sensitivity serves to block genetic exchanges between similar but imperfectly matched sequences, often referred to as "homeologous recombination." The importance of restraining homeologous recombination is evident when one considers the abundance of repeated homeologous sequences, such as *Alu* family members, in a mammalian genome. Efficient recombination among dispersed homeologous genomic elements would destabilize the genome by producing a variety of potentially deleterious chromosomal rearrangements.

Protection against homeologous recombination is achieved in part by the requirement for a significant length of perfect homology in order for recombination to initiate. In their studies on recombination in bacteria, Shen and Huang (1986, 1989) coined the term minimal efficient processing segment (MEPS) to describe the minimal length of continuous homology needed for efficient recombination. The value for MEPS for RecBCD-mediated recombination in *Escherichia coli* was determined by Shen and Huang (1986) to be ~30 bp. We previously estimated the value of MEPS for intrachromosomal recombination in mammalian cells to be substantially greater, with a value between 134 and 232 bp (Waldman and Liskay 1988). We further demonstrated that recombination in mammalian chromosomes is exquisitely sensitive to small degrees of sequence divergence and that a single nucleotide mismatch is sufficient to functionally disrupt MEPS and reduce recombination rate (Lukacsovich and Waldman 1999).

Evidence has been reported that in mammalian cells, yeast, and bacteria the DNA mismatch repair (MMR) machinery is involved in the genesis of the sensitivity of recombination to mismatches (Rayssiguier *et al.* 1989; Alani *et al.* 1994; Selva *et al.* 1995; Harfe and Jinks-Robertson 2000; Nicholson *et al.* 2000; Elliott and

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Jasin 2001; Schofield and Hsieh 2003; Surtees *et al.* 2004). Several general models for how MMR proteins collaborate to impede homeologous recombination have been proposed (Surtees *et al.* 2004). In one model, heteroduplex DNA (hDNA) intermediates may be formed between homeologous sequences but then are rejected by multiple overlapping long-patch MMR repair tracts that destroy the recombination intermediate and abort recombination. In a second model, MMR proteins block migration of Holliday junctions (or other recombination intermediates) into homeologous sequences thereby precluding extensive hDNA formation and preventing formation of a stable recombination intermediate. In a third model, recombination intermediates are initially formed between homeologous sequences but the mismatched hDNA is unwound by a helicase and/or reversal of strand exchange. In a variation of this latter model, a fourth model proposes that mismatched hDNA is not unwound but is successfully repaired in a way that restores the original sequences. The latter three models are "nondestructive" in that they do not involve wholesale degradation of recombination intermediates and abortion of recombination altogether. Although these various potential mechanisms are not mutually exclusive, recent reports favor a nondestructive hDNA unwinding mechanism in yeast (Spell and Jinks-Robertson 2004; Sugawara *et al.* 2004; Goldfarb and Alani 2005). In particular, evidence suggests that the heterodimer of Msh2-Msh6 (MutS homologs) may serve as a sensor of mispaired bases in hDNA and recruit the Sgs1 helicase (a yeast homolog of the *E. coli* RecQ helicase) to unwind hDNA formed between homeologous sequences. Our previous work (Lukacsovich and Waldman 1999) demonstrating sensitivity of recombination in mammalian cells to single nucleotide mismatches did not allow a determination of which of the broad models described above is most likely operative. In general, there has been little investigation into the mechanism for rejection of homeologous recombination in mammalian cells.

In previous investigations we found not only that small degrees of sequence divergence can suppress intrachromosomal recombination but also that cells can potentially carry out accurate exchanges between homeologous sequences if homeologous sequences are located adjacent to a region of high homology (Waldman and Liskay 1988; Yang and Waldman 1997). Such findings previously led us to conclude that stringent homology requirements are involved almost exclusively in the initiation of recombination and, once initiated, a recombination event can propagate into sequences displaying a high degree of divergence. Three caveats in our earlier work left several issues unresolved. We could not formally distinguish the "beginning" from the "end" of gene conversion tracts and so, although we deemed it unlikely, it remained a possibility that resolution (rather than initiation) of recombination required high homology. Another matter involved the possibility that the palindromic *Xho*I linker insertion mutation used as a marker in genetic selection for recombinants may have played a role in facilitating recovery of homeologous recombination events. Finally, although placement of a region of homology adjacent to homeologous sequence did allow recovery of recombination events within the homeologous interval, the frequency of recombination among the homeologous sequences remained about fivefold lower than that for recombination between homologous sequences. This latter result raised the possibility that more than one step in recombination may be sensitive to mismatches. These unresolved issues motivated us to reexamine the homology dependency of recombination in mammalian cells.

In this article we present evidence that a nondestructive dismantling of mismatched hDNA operates in mammalian chromosomes to remove homeologous sequences from recombination intermediates. Our data suggest that both initiation and resolution of recombination require substantial homology and that hDNA rejection can be evaded by the presence of certain DNA motifs or sequences or by expanses of homology surrounding an interval of homeologous sequence. Collectively, our data lead us to conclude that mammalian cells do not reject spans of mismatched hDNA *per se*, but rather, dismantling of mismatched hDNA is a consequence of a search for localized homology needed for resolution of recombination.

MATERIALS AND METHODS

Cell culture and generation of stably transfected cell lines: All cell lines used were derived from thymidine kinase-deficient mouse L cells (L*tk*$^-$ cells). Cells were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, minimum essential medium nonessential amino acids (GIBCO), and 50 μg/ml of gentamicin sulfate (Sigma). Cells were maintained at 37° in a humidified incubator under an atmosphere of 5% CO_2.

To generate stably transfected cell lines, plasmid DNAs were linearized with *Cla*I and introduced into L*tk*$^-$ cells by either electroporation (Lukacsovich *et al.* 1994) or syringe-mediated transfection (Waldman and Waldman 1998). Cell lines that contained one or more stably integrated copies of plasmid DNA were recovered by selection in G418 (200 μg/ml active drug), and transfected cell lines containing one or two copies of integrated plasmid DNA were identified by Southern blotting analysis of genomic DNA isolated from transfected clones as described (Waldman and Liskay 1987).

Recombination substrates: All plasmids used as recombination substrates are illustrated in Figure 1. All plasmids are based on the vector pJS-1, which is identical to pSV2neo (Southern and Berg 1982) except for several restriction site modifications previously described (Liskay *et al.* 1984). Each recombination substrate contains a herpes simplex type 1 (HSV-1) thymidine kinase (*tk*) gene rendered nonfunctional by insertion of an 8-bp *Xho*I linker after nucleotide position 1215 or after nucleotide position 1035 of the *tk* gene [nucleotide numbering according to Wagner *et al.* (1981)].

The latter mutant *tk* gene is referred to as mutant 28 while the former mutant *tk* gene is referred to as mutant 8 (Waldman and Liskay 1987).

Plasmid pHYB12-8 contains *tk* mutant 8 on a 2.5-kb *Bam*HI fragment inserted into the unique *Bam*HI site of the vector as well as a 1224-bp *tk* sequence inserted into the unique *Hin*dIII site. This latter *tk* sequence is referred to as a "hybrid donor" and contains 818 bp of HSV-1 *tk* sequence (nucleotides 445–1262) joined to 407 bp of HSV-2 *tk* sequence (nucleotides 1263–1670) at a common *Pst*I site to produce a continuous in-frame *tk* coding sequence. Plasmid pHYB21A is identical to pHYB12-8 except that it contains at the *Hin*dIII site a 612-bp hybrid donor consisting of 320 bp of HSV-2 *tk* sequence (nucleotides 848–1167) joined to 292 bp of HSV-1 *tk* sequence (nucleotides 1168–1459) at a common *Bal*I site. Plasmid pHYB21A-28 is identical to pHYB21A except it contains *tk* mutant 28 inserted into the *Bam*HI site of the vector. Control plasmids p1-8 and p1A contain only the HSV-1 *tk* portions of the hybrid donors from pHYB12-8 and pHYB21A, respectively, but are otherwise identical to pHYB12-8 and pHYB21A. Plasmid pHYB121 is identical to pHYB21A except that it contains at the *Hin*dIII site a 612-bp sequence consisting of nucleotides 848–1096 of HSV-1 *tk* sequence followed by nucleotides 1097–1167 of HSV-2 *tk* sequence followed by nucleotides 1168–1459 of HSV-1 *tk* sequence.

Determination of intrachromosomal recombination rates: Recombinants were recovered from cell lines containing integrated recombination substrates by selecting for tk^+ segregants using medium supplemented with hypoxanthine–aminopterin–thymidine (HAT) (GIBCO). Fluctuation analysis and rate calculations were carried out as previously described (Waldman *et al.* 1999). Rates are expressed in terms of recombination events per cell per generation per copy of integrated substrate.

PCR amplification: A segment of recipient *tk* sequence was PCR amplified from genomic DNA isolated from HAT^R segregants using the primers AW85 (5′-TAATACGACTCACTAT AGGGCCAGCGTCTTGTCATTGGCG-3′) and AW90 (5′-GGA TAACAATTTCACACAGGCGGTGGGGTATCGACAGAGT-3′) to produce a PCR product whose sequence spans the original location of the *Xho*I linker insertion in the recipient *tk* gene. AW85 is composed of nucleotides 308–327 from the coding strand of the HSV-1 *tk* gene with a T7 forward universal priming site appended to the 5′ end of the primer. AW90 is composed of nucleotides 1786–1767 from the noncoding strand of the HSV-1 tk gene with an M13 reverse universal priming site appended to the 5′ end of the primer. PCR reactions were carried out using 600 ng of genomic template DNA in a final volume of 25 μl. PCR was carried out using Ready-To-Go PCR beads (GE Healthcare) and a touchdown PCR protocol. The annealing temperature was initially set to 72° and was progressively decreased in steps of 2° down to 62°, with two cycles at each temperature. An additional 20 cycles were run at an annealing temperature of 60°.

Slot blot hybridization analysis: PCR products (1 fmol) amplified as described above from HAT^R recombinants recovered from cell lines containing pHYB12-8 were applied to nitrocellulose filters through a slot-blot template (Minifold II, Schleicher & Scheull) and hybridized with ^{32}P-labeled oligonucleotides specific for HSV-1 or HSV-2 *tk* sequence. Hybridization conditions were the same as those used for Southern blot hybridization (Waldman and Liskay 1987) except that filters were hybridized and washed at 50°. Two 15-bp oligonucleotides were used as probes: probe 1 (5′-AGgGCGGCGGG tcGT-3′), which consists of nucleotides 1264–1278 of the HSV-1 *tk* gene, and probe 2 (5′-AGcGCGGCGGGagGT-3′), which consists of nucleotides 1264–1278 of the HSV-2 *tk* gene. There are three nucleotide differences between the HSV-1 and HSV-2 tk genes in the range covered by these oligonucleotides; these heterologous bases are indicated in lowercase letters in the oligonucleotide sequences. Under the hybridization conditions used, probe 1 hybridizes specifically to HSV-1 *tk* sequence while probe 2 is specific for HSV-2 *tk* sequence. Probes 1 and 2 map just downstream from the junction between HSV-1 and HSV-2 tk sequence on the hybrid donor of pHYB12-8.

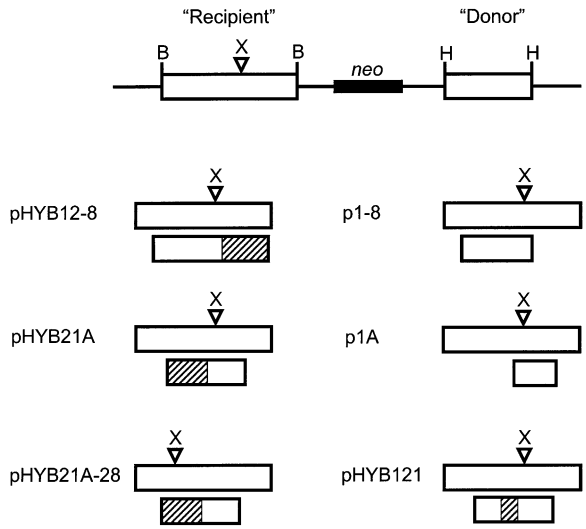

Figure 1.—Recombination substrates. (Top) A schematic of a generic recombination substrate. The DNA construct is shown as if linearized at the unique *Cla*I site in the vector. Inserted between two *Bam*HI sites (B) is a 2.5-kb fragment containing an HSV-1 *tk* gene disrupted by insertion of an *Xho*I (X) linker and referred to as a "recipient." Inserted between two *Hin*dIII (H) sites is a truncated *tk* sequence referred to as a "donor." The direction of transcription of recipient and donor *tk* sequences is from left to right, and the two *tk* sequences are separated by ~4.4 kb. Below the generic substrate are schematics of the recipient and donor *tk* sequences contained in the six specific recombination substrates used. For each substrate, the recipient gene is shown on top with the donor gene aligned beneath it. Open rectangles represent HSV-1 *tk* sequences while stippled rectangles represent HSV-2 *tk* sequences. For pHYB21A-28 the recipient *tk* gene is mutant 28, while for all other substrates the recipient *tk* gene is mutant 8 (see materials and methods).

DNA sequencing: When possible, nucleotide sequences were determined by direct sequencing of uncloned PCR products. Prior to sequencing, PCR products were treated with shrimp alkaline phosphatase and exonuclease I (USB). PCR products were then sequenced from a T7 primer or an M13 reverse primer using a Licor 4000L at the DNA Sequencing and Synthesis Core Facility in the Department of Biological Sciences at the University of South Carolina. For recombinants that contained more than a single copy of the recombination substrate, the PCR product to be sequenced was first isolated by cloning into the pCR2.1 vector using a TA cloning kit (Invitrogen) and then sequenced.

RESULTS

Homeologous sequence does not reduce the recovery of recombination events within an adjacent tract of homologous sequence: We designed several recombination substrates (Figure 1), each of which contained

TABLE 1

Rates of intrachromosomal recombination

Substrate	Cell line[a]	Copy no.[b]	Cells tested ($\times 10^{-6}$)	HAT^R colonies	Recombination frequency ($\times 10^7$)[c]	Recombination rate ($\times 10^8$)[d]
pHYB12-8	12CE8	1	142	38	2.70	10.0
	12CE8	1	139	6	0.43	3.8
	12CE10	2	169	70	2.10	6.6
	12CE10	2	221	8	0.41	1.9
	Mean				1.41	5.6
p1-8	1CE8	1	180	40	2.20	8.2
	1CE8	1	175	49	2.80	9.8
	1CM1	1	189	16	0.85	4.1
	Mean				1.95	7.4
pHYB21A	D2	1	571	123	2.20	7.1
	K3	1	300	100	1.70	4.9
	H3	2	382	36	0.47	1.8
	Mean				1.46	4.6
p1A	10	1	292	28	0.94	3.9
	5	1	277	13	0.46	2.5
	Mean				0.70	3.2
pHYB21A-28	1	1	256	14	0.55	2.8
	2	1	210	3	0.14	1.4
	Mean				0.35	2.1
pHYB121	1	1	224	12	0.54	2.9
	2	1	294	33	1.12	4.4
	Mean				0.83	3.7

[a] For each cell line, data from independent fluctuation tests are presented in individual rows.
[b] Number of copies of stably integrated recombination substrate in the particular cell line.
[c] Calculated as the number of HAT^R colonies per number of cells tested divided by the number of copies of integrated substrate.
[d] Recombination rate is expressed as number of recombination events per cell per generation per copy of integrated substrate.

a complete HSV-1 *tk* gene rendered nonfunctional due to the insertion of an *Xho*I linker. We refer to this disrupted gene as a "recipient" *tk* gene. Each substrate also contained a second truncated *tk* sequence we refer to as a "donor." The donors on pHYB12-8 and pHYB21A were hybrids composed of both HSV-1 and HSV-2 *tk* sequences, while the donors on p1-8 and p1A consisted of HSV-1 *tk* sequence alone. HSV-1 and HSV-2 *tk* sequences display ~80% sequence identity, with fairly evenly scattered nucleotide mismatches, and therefore can be described as being homeologous to one another. The donor on p1-8 was precisely the HSV-1 *tk* portion of the pHYB12-8 donor and, likewise, the donor on p1A was precisely the HSV-1 *tk* portion of the pHYB21A donor. For each substrate, the *Xho*I linker insertion mutation in the recipient *tk* gene could potentially be corrected through recombination with the donor *tk* sequence; such events could be recovered by selecting for HAT^R segregants arising from cell lines stably transfected with the substrate. Because the coding region of each donor was truncated at the 5′ and 3′ ends, the only recoverable recombination products were gene conversions or double crossovers. For pHYB12-8 and pHYB21A, when the recipient *tk* gene was aligned with the hybrid donor the *Xho*I linker insertion mutation was paired opposite the homologous portion of the donor (see Figure 1). The homology provided on the donors of all substrates was critical to enable recovery of recombinants, since we determined that substrates containing an 800-bp HSV-2 *tk* donor that is entirely homeologous to the recipient gene produced recombinants at an undetectably low rate ($<1 \times 10^9$) (Waldman and Liskay 1987; Waldman and Liskay 1988).

For the substrates pHYB12-8, p1-8, pHYB21A, and p1A, two or more L*tk*$^-$ cell lines were isolated that were stably transfected with each particular substrate. The rate of appearance of HAT^R segregants for each cell line was determined by fluctuation analysis (Table 1). Although the recombination rates for cell lines containing

any individual construct varied somewhat, the data in Table 1 reveal no significant differences among the rates associated with the various constructs. Notably, the mean rate for cell lines containing pHYB12-8 (5.6×10^{-8}) was similar to the mean rate for cell lines containing p1-8 (7.4×10^{-8}). Likewise, the mean rate for cell lines containing pHB21A (4.6×10^{-8}) was similar to the mean rate for cell lines containing p1A (3.2×10^{-8}).

Southern blotting analysis of HAT^R clones recovered from all cell lines revealed the expected restriction patterns for gene conversions with no evident gross rearrangements (data not shown).

Homeologous sequence is effectively excluded from gene conversion tracts: For both pHYB12-8 and pHYB21A, the distance between the position of the *Xho*I linker insertion in the recipient HSV-1 *tk* gene and the position of the junction between HSV-1 and HSV-2 *tk* sequences in the hybrid donor was ~50 bp. It was possible for both initiation and resolution of recombination events to occur within HSV-1 *tk* sequence, which would result in exclusion of homeologous HSV-2 *tk* sequence from the conversion tract. We were interested in learning whether homeologous sequence was indeed excluded from gene conversion tracts.

Recipient *tk* gene sequences were PCR amplified from genomic DNA samples isolated from HAT^R segregants recovered from cell lines containing pHYB12-8 or pHYB21A. For cell lines containing pHYB12-8, each PCR product was hybridized on slot blots with an HSV-1 *tk*-specific probe (Figure 2, I) and, in parallel, with an HSV-2 *tk*-specific probe (Figure 2, II). As illustrated in Figure 2, III, the probes mapped immediately downstream from the position of the junction between the HSV-1 *tk* and HSV-2 *tk* sequences in the donor from pHYB12-8 and therefore were expediently placed to determine whether gene conversion tracts in the corrected recipient contained any homeologous sequence. Of a total of 44 HAT^R segregants analyzed as depicted in Figure 2, none harbored a gene conversion tract containing HSV-2 *tk* sequence as indicated by lack of hybridization of the PCR products to the HSV-2 *tk* probe. PCR products generated from six HAT^R clones were sequenced and were found to contain only HSV-1 tk sequence, confirming the slot blot hybridization analysis (data not shown). We also determined the nucleotide sequences of PCR products amplified from the corrected recipient *tk* genes from 37 HAT^R segregants recovered from cell lines containing pHYB21A. All samples sequenced showed accurate correction of the *Xho*I linker insertion, with no introduced mutations. Sequence analysis also revealed that only a single clone, named K3-1, harbored a gene conversion tract containing any HSV-2 tk sequence. Approximately 60 bp of homeologous sequence was encompassed in this exceptional gene conversion tract recovered from cell line K3 (see Figure 3, clone K3-1). Thus, remarkably, 80 of 81 gene conversion tracts analyzed did not include even a single heterologous nucleotide from the homeologous portion of the hybrid donor.

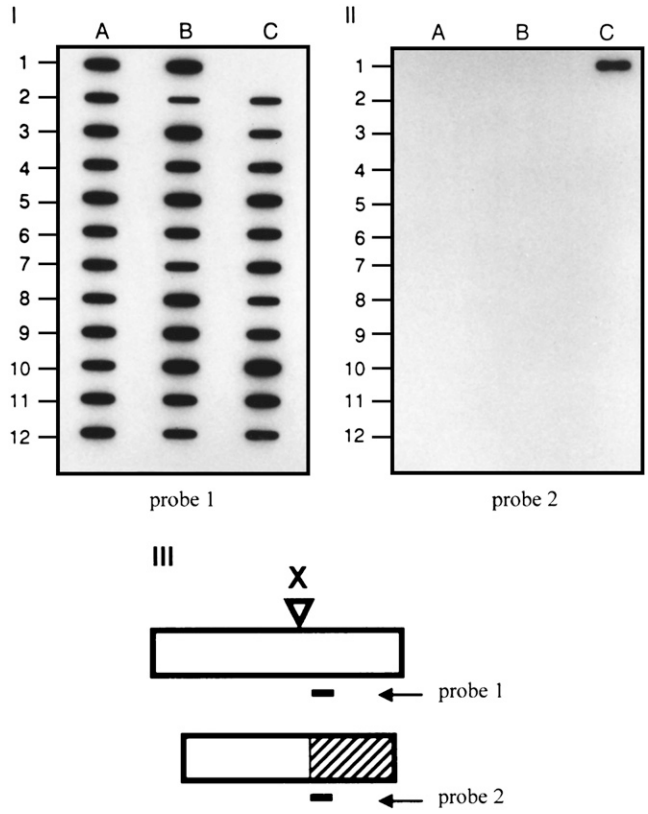

FIGURE 2.—Slot blot analysis of recombination events recovered from cell lines containing pHYB12-8. Recipient *tk* gene sequence was PCR amplified from genomic DNA isolated from HAT^R segregants from cell lines containing pHYB12-8 and each PCR product was applied to two slot blots. One blot (I) was hybridized with probe 1, which is specific for HSV-1 *tk* sequence, while the other blot (II) was hybridized with probe 2, which is specific for HSV-2 *tk* sequence. Both probes mapped immediately downstream from the position of the junction between HSV-1 and HSV-2 *tk* sequences in the pHYB12-8 donor, as illustrated in III. (In III, open rectangles represent HSV-1 *tk* sequence and the stippled rectangle represents HSV-2 *tk* sequence.) The first row on each slot blot contains hybridization controls, with samples 1A and 1B containing HSV-1 *tk* sequence and sample 1C containing HSV-2 *tk* sequence. All other samples in rows 2–12 were derived from independent HAT^R segregants. In addition to the 33 HAT^R clones analyzed in the blots shown, an additional 11 HAT^R clones were analyzed in a similar fashion.

Strand exchange can penetrate homeologous sequences: In considering how the fastidious exclusion of homeologous sequence from recombination products may be brought about, we bore in mind our earlier studies in which substrates that contained hybrid donors (and were similar to pHYB12-8 and pHYB21A) yielded gene conversion tracts that in some cases included >300 bp of homeologous sequence (YANG and WALDMAN 1997). The exclusion of homeologous sequence from gene conversion tracts in our current work seemed at odds with those earlier studies. The contrasting observations suggested a testable hypothesis,

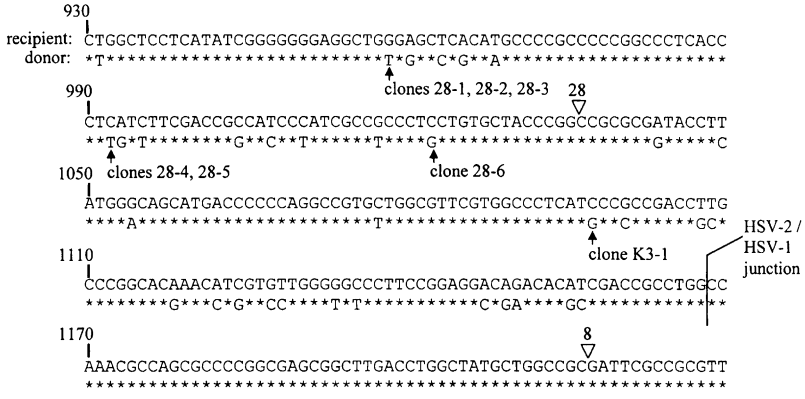

FIGURE 3.—Nucleotide sequences of gene conversion tracts containing homeologous HSV-2 *tk* sequence. Nucleotide numbering is according to WAGNER *et al.* (1981). The upper line of sequence is HSV-1 *tk* sequence from a portion of the recipient *tk* genes in pHYB21A and pHYB21A-28. The locations of the *Xho*I linker insertion in *tk* mutant 8 (the recipient gene in pHYB21A) and in *tk* mutant 28 (the recipient gene in pHYB21A-28) are indicated by labeled inverted triangles. The lower line of sequence is from a portion of the common hybrid donor *tk* sequence in both pHYB21A and pHYB21A-28, with the junction between HSV-2 and HSV-1 sequence shown. An asterisk is present at each position in the donor sequence where the donor is identical to the recipient HSV-1 *tk* sequence. In the HSV-2 *tk* portion of the donor, each nucleotide difference between donor HSV-2 *tk* and recipient HSV-1 *tk* sequence is indicated. Beneath the donor sequence is indicated the upstream-most HSV-2 *tk* marker in the gene conversion tracts of recovered clones. Clone K3-1 was recovered from cell line K3 containing pHYB21A. The gene conversion tract from clone K3-1 contains the HSV-2 "G" nucleotide indicated by the arrow as well as every downstream HSV-2 nucleotide marker through the position of the HSV-2/ HSV-1 junction. Clones 28-1–28-6 were recovered from lines containing HYB21A-28. The gene conversion tract from each clone contains the HSV-2 nucleotide indicated by the appropriate arrow as well as every downstream HSV-2 nucleotide marker through the position of the HSV-2/HSV-1 junction. Every gene conversion tract appeared to be continuous except for the tract from clone 28-5. Clone 28-5 displays a "C" (equal to HSV-1 sequence) at position 995 despite displaying 2 upstream and 25 downstream HSV-2 markers.

namely, that the position of the *Xho*I linker insertion in the recipient *tk* gene may influence the outcome of recombination events. The *Xho*I linker insertion in the recipient HSV-1 *tk* gene in previous studies was positioned opposite the homeologous HSV-2 *tk* portion of the hybrid donor rather than opposite the homologous HSV-1 *tk* portion as in pHYB12-8 and pHYB21A. We therefore engineered substrate pHYB21A-28 (Figure 1), which contains the very same hybrid donor *tk* sequence as does pHYB21A but contains a recipient *tk* gene (mutant 28) with an *Xho*I linker insertion positioned opposite the homeologous HSV-2 *tk* sequence of the donor, 132 bp upstream from the position of the junction between HSV-2 *tk* and HSV-1 *tk* sequences.

Two cell lines were established that contained stably integrated copies of pHYB21A-28. As presented in Table 1, HAT^R segregants were recovered from cell lines containing pHYB21A-28 at an average rate of 2.1×10^{-8}, about twofold lower than the rate of 4.6×10^{-8} obtained with substrate pHYB21A (Table 1). Southern blotting analysis revealed that HAT^R segregants arose from correction of the *Xho*I linker insertion mutation, with no apparent gross rearrangements (data not shown).

To further characterize recombinants derived from pHYB21A-28, seven HAT^R clones were analyzed by PCR and DNA sequencing. Quite markedly, sequencing revealed that six of seven HAT^R clones displayed gene conversion tracts containing HSV-2 *tk* sequences, with as much as 208 bp of homeologous HSV-2 tk sequence recovered in a conversion tract (Figure 3). All clones, with the exception of clone 28-5 (Figure 3), displayed apparently continuous gene conversion tracts. All gene conversion tracts were accurate with no mutations observed. The difference in the number of conversion tracts recovered from pHYB21A-28 *vs.* pHYB12-8 or pHYB21A that contained homeologous sequence was highly significant ($P = 0.000012$, by a Fisher exact test). These results confirmed that positioning the *Xho*I linker insertion opposite the homeologous portion of the hybrid donor indeed enables the recovery of homeologous sequences in gene conversion tracts. We suggest a possible explanation for these results in the DISCUSSION. Regardless of the precise mechanism by which the *Xho*I linker insertion exerts its influence, a salient point is that the recombinants recovered with pHYB21A-28 demonstrated that strand exchange can propagate into and through a considerable length of homeologous sequence.

Homology requirements for both initiation and resolution of recombination exclude homeologous sequence from gene conversion tracts: Our experiments produced the curious findings that gene conversions recovered using pHYB21A-28 included homeologous sequence while virtually all recombination events recovered using pHYB12-8 or pHYB21A did not include even a single mismatched base. To reconcile these observations, we inferred that a significant portion of recombination events recovered from cell lines containing pHYB12-8 or pHYB21A likely initially involved strand exchange between homeologous sequences, but any mismatched hDNA produced in recombination intermediates involving pHYB12-8 or pHYB21A was ostensibly dismantled prior to resolution. Such a scenario suggested two possibilities for how homeologous recombination may be avoided and these possibilities are not mutually exclusive. One possibility is that mismatched hDNA is rapidly recognized by the cellular MMR system, which responds to eliminate the mismatches. A second possibility is that when strand

exchange enters into homeologous sequence, recombination machinery (which may include components of the MMR system) acting at the site of active strand exchange executes a search for homology required for resolution. This search for homology may be coupled to a mechanism for signaling a reversal of strand exchange to "back out" of homeology. Importantly, in this latter mechanism survival or rejection of the hDNA intermediate is driven by a search for localized homology and not by a response against mismatched hDNA *per se*.

To further investigate how mismatched hDNA is dismantled, two cell lines were established that each contained an integrated copy of pHYB121 (Figure 1). The donor on pHYB121 was similar to the donor on pHYB21A, except that the only segment of homeology on the pHYB121 donor was a 60-bp segment of HSV-2 *tk* sequence identical to that present in the gene conversion tract of clone K3-1 (see Figure 3). Significantly, the 60-bp homeologous sequence in the pHYB121 donor was flanked on both sides by segments sharing perfect homology with the recipient *tk* gene. These flanking segments were intended to provide localized regions of homology for recombination initiation and resolution. If stringent homology requirements are tied to initiation and resolution, while mismatched hDNA intermediates are tolerated, we reasoned that gene conversion tracts containing the 60-bp homeologous sequence in the pHYB121 donor should be readily recoverable.

Fluctuation tests were conducted on the cell lines containing pHYB121 and HAT^R segregants were recovered at an average rate of 3.7×10^{-8} (Table 1). PCR and Southern blot analysis of genomic DNA isolated from HAT^R clones showed that these clones arose from gene conversions in the absence of any unexpected rearrangements (data not shown). DNA sequence analysis of 39 recombinants revealed that 11 clones contained the entire 60-bp HSV-2 *tk* segment of the pHYB121 donor within the gene conversion tract; the corrected recipient *tk* gene in these 11 clones had the same sequence as the corrected *tk* recipient in clone K3-1 (see Figure 3). The remaining 28 clones displayed accurate correction of the *Xho*I linker insertion and contained only HSV-1 *tk* sequence. The difference between this outcome *vs.* the outcome for cell lines containing pHYB21A, in which only 1 of 37 conversion tracts contained HSV-2 *tk* sequence, was highly statistically significant ($P = 0.0032$, by a Fisher exact test). The results obtained using pHYB121 provided strong evidence that homeologous sequence can be incorporated into a gene conversion tract if local homology requirements for initiation and resolution are met.

DISCUSSION

Earlier studies by us (Waldman and Liskay 1987, 1988; Lukacsovich and Waldman 1999) and other groups (Rayssiguier *et al.* 1989; Alani *et al.* 1994; Selva *et al.* 1995; Harfe and Jinks-Robertson 2000;Nicholson *et al.* 2000; Elliott and Jasin 2001; Schofield and Hsieh 2003; Surtees *et al.* 2004) established that recombination among chromosomal sequences in mammalian cells is very sensitive to sequence heterology. Our current work was aimed at providing insight into the mechanism by which such exquisite sensitivity to mismatch is engendered. From our current work, we can make four inferences. First, the mechanism for preventing an exchange within homeologous sequences in mammalian chromosomes does not typically involve the wholesale destruction of a recombination intermediate. Second, following initiation of recombination within an interval of homology, incorporation of adjoining homeologous sequence into a gene conversion tract can be prevented by dismantling mismatched hDNA that is transiently formed between homeologous sequences. Third, dismantling of a mismatched hDNA recombination intermediate is driven by a search for localized homology needed for resolution of recombination and not by a general intolerance of the mismatches in the hDNA. Fourth, certain DNA motifs or sequences may alter homology requirements for recombination. These conclusions and associated issues are considered in more detail below.

Recombination rates measured with constructs pHYB12-8 and pHYB21A were comparable to rates measured with control substrates p1-8 and p1A (Table 1) despite the striking observation that homeologous sequences were almost entirely excluded from gene conversion tracts recovered from pHYB1-8 and pHYB21A. From these findings alone, one might speculate that strand exchange never proceeds past the very first mismatched base within homeologous sequence. However, this possibility is contradicted by the results obtained with pHYB21A-28, which demonstrate that by merely moving the position of the *Xho*I linker insertion it is possible to recover gene conversion tracts encompassing homeologous sequence. This latter finding indicates that strand exchange can proceed well into homeologous sequence in pHYB21A-28 and so we deduce that strand exchange very likely proceeds into homeologous sequence in pHYB12-8 and pHYB21A as well. In the cases of pHYB12-8 and pHYB21A, however, we surmise that prior to resolution mismatched hDNA is dismantled in a manner that does not involve the complete degradation of the recombination intermediate. Work by Sugawara *et al.* (2004) provides strong evidence that mismatched hDNA in yeast is also rejected in a nondestructive fashion.

Experiments with pHYB121, in which hDNA rejection was overcome by surrounding a homeologous sequence by regions of high homology, argue against an outright cellular response to mismatches in hDNA and suggest that the availability of localized homology suitable for initiation and resolution is the critical factor in determining whether or not hDNA will be dismantled.

Said plainly, where recombination begins and ends is most important. In total, 80 of 81 gene conversion tracts recovered from pHYB12-8 or pHYB21A and all 39 gene conversion tracts recovered from pHYB121 had both ends situated within regions of localized high homology. Indeed, none of the gene conversion tracts recovered from pHYB121 had an end located within the 60-bp homeologous segment. A requirement that recombination begins and ends within regions of high homology should ordinarily prevent exchange between homeologous sequences. It seems unlikely that a situation such as exists in pHYB121, in which homeology is surrounded by substantial intervals of high homology, would be encountered very often in nature.

How is dismantling of mismatched hDNA achieved? Numerous studies in prokaryotes and eukaryotes have demonstrated that MMR proteins play important roles in blocking homeologous recombination (reviewed in Harfe and Jinks-Robertson 2000; Schofield and Hsieh 2003; Surtees *et al.* 2004). Our current work does not directly address the role of MMR in hDNA rejection, but our experiments with pHYB121 led us to conclude that rapid action of MMR on mismatches in hDNA is not a suitable explanation for dismantling hDNA. It has been shown that MMR in bacteria reduces the rate of strand exchange in the presence of mismatches (Worth *et al.* 1994, 1998; Bazemore *et al.* 1997; Zahrt and Maloy 1997; Fabisiewicz and Worth 2001) but, importantly, MMR does *not* target preformed mismatched hDNA (Worth *et al.* 1994; Westmoreland *et al.* 1997). Work done in yeast also provides evidence that MMR proteins play a role in impeding branch migration through mismatches (Chen and Jinks-Robertson 1998, 1999). These studies in yeast and bacteria are consistent with our inference that the mammalian machinery responsible for dismantling mismatched hDNA is coupled to strand exchange.

Two types of processes, both involving MMR proteins, have been described that can potentially dismantle hDNA formed between homeologous sequences. One such process is restorative MMR, which appears to be a viable explanation for some gene conversion gradients seen near double-strand breaks (DSBs) in yeast (reviewed in Surtees *et al.* 2004). To accommodate our observation that dismantling of hDNA can be overcome by surrounding a homeologous interval by regions of high homology, restorative MMR would seemingly have to be delayed to allow time for a search for homology needed for resolution. Restorative repair would also have to be shut down if suitable homology is encountered. A second way that mismatched hDNA may be dismantled is via an unwinding process generally referred to simply as "hDNA rejection." MMR proteins, acting in association with strand exchange machinery, may serve as sensors of mispairs in hDNA to help position the strand exchange machinery in an expanse of homology needed for resolution. If strand exchange progresses into a region lacking a suitable stretch of homology, the MMR proteins may signal to other proteins to reverse strand exchange, which would dismantle mismatched hDNA and bring the recombination machinery back into a region of suitable homology. Candidate proteins for possible involvement in catalyzing a reversal of strand exchange include BLM helicase (a RecQ homolog and a homolog of yeast Sgs1), Rad51C [implicated in Holliday junction processing (Liu *et al.* 2004)], and Rad51D [interacts with and stimulates the Holliday structure unwinding activity of BLM (Braybrooke *et al.* 2003)].

We have described above two ways in which MMR proteins may be involved in recombination in the dismantling of mismatched hDNA in mechanisms that prevent homeologous recombination. Later in recombination, following resolution, there must be a shift to a positive role for MMR proteins in correcting mismatches (in surviving hDNA) in the direction of conversion since gene conversion is classically viewed as often stemming from repair of hDNA. These considerations evoke discrete functional activities for MMR proteins. In fact, separation-of-function mutations have been described for MMR proteins regarding roles in the repair of mismatches *vs.* antirecombination activity (Welz-Voegele *et al.* 2002; Hoffmann *et al.* 2003; Calmann *et al.* 2005; Goldfarb and Alani 2005).

Experiments with pHYB21A-28 in conjunction with our earlier studies (Yang and Waldman 1997; Waldman *et al.* 1999) suggest that certain DNA motifs or sequences might influence recombination. In pHYB21A-28, the *Xho*I linker in the recipient *tk* gene is positioned opposite the homeologous portion of the donor when recipient and donor are paired (Figure 1). One possibility is that recombination initiates in pHYB21A-28 within the region of perfect homology shared by the recipient and donor. Following strand exchange into the homeologous region, mismatched hDNA is presumably dismantled as usual except, perhaps, in cases in which strand exchange proceeds past the *Xho*I linker insertion. Strand exchange past the *Xho*I linker would create mismatched hDNA that encompasses a palindromic insertion loop heterology. The palindromic insertion loop heterology may serve as a target for preferential cleavage, which might impede dismantling of mismatched hDNA by disrupting either restorative MMR or hDNA unwinding. Alternatively, the *Xho*I linker may serve as a site for initiation of recombination, perhaps by serving as a site for formation of a DSB that may enlarge into a gap. In this scenario, recombination events might be able to propagate to the right, through homeology, and resolve within the downstream homologous interval. Regardless of whether the *Xho*I linker in pHYB21A-28 exerts its effect early or late in recombination, it is clear that the *Xho*I linker indeed influences recombination. Evidence for a novel palindrome loop

mismatch repair pathway in mammalian cells has been reported recently (Miller *et al.* 2004), and several motifs, including palindromes and quasipalindromes, have been shown to stimulate recombination in a variety of organisms (Gordenin and Resnick 1998; Bacolla and Wells 2004). Further investigation into the potential for various DNA motifs and/or sequences to affect recombination is warranted.

With regard to mechanisms for strand exchange, we have intentionally kept our discussion general since we do not know by what mechanism recombination is carried out in our experimental system. One possibility is that gene conversions in our system are produced predominantly by a synthesis-dependent strand-annealing (SDSA) mechanism, which is frequently used to explain gene conversions initiated at DSBs in eukaryotic genomes (Prado *et al.* 2003; Schofield and Hsieh 2003; Puchta 2005). In SDSA, a 3′ end of a broken recipient DNA sequence invades a recombination donor sequence and then uses the donor sequence as a template for DNA synthesis to extend the 3′ end. The newly synthesized DNA strand is released from its template, anneals to a 3′ DNA tail from the other side of the DSB, and following single-strand gap filling, trimming of flaps, and ligation, the DSB is healed. To explain how SDSA might involve a homology search for resolution, we must assume that DNA synthesis during SDSA is not very processive and that the nascent strand of DNA is frequently released from its template and transiently annealed with a DNA strand from the other side of the DSB. Such annealing would form a span of hDNA that can in theory be tested for homology. If homology is high, then the event may resolve. If the homology test fails—that is, there is too high a degree of mismatch in the hDNA—then the hDNA would be unwound, the template would again be invaded, and DNA synthesis would resume. After multiple rounds of failed homology tests, mismatched hDNA would be dismantled by degrading or correcting (via restorative repair) the nascent strand of DNA all the way back to a region of homology between recipient and donor. It is also conceivable that initial strand invasion in SDSA is not very sensitive to homeology, whereas the rejoining step is. This latter possibility would be consistent with the above-mentioned speculation about initiation of recombination by breakage at the *Xho*I linker in pHYB21A-28.

As an alternative to SDSA, gene conversions in our system may be produced by a mechanism that involves the formation of a symmetrical Holliday junction, or a half-Holliday junction (produced as a single-end invasion intermediate). In such mechanisms, strand exchange occurs via branch migration and hDNA is produced as a strand from the donor displaces a strand of the recipient. It seems plausible to conjecture that recombination in our system proceeds in such a fashion and that hDNA rejection is accomplished by a reversal of branch migration to unwind mismatched hDNA. This view is in line with current thinking about hDNA rejection in yeast (Spell and Jinks-Robertson 2004; Sugawara *et al.* 2004; Goldfarb and Alani 2005).

In our previous work (Waldman and Liskay 1988; Lukacsovich and Waldman 1999), we discussed the concept of MEPS in terms of the amount of homology needed for initiation of recombination in mammalian chromosomes. Our current work illustrates that substantial homology is normally required for resolution as well. It is possible that initiation and resolution have distinct MEPS values and that the value of 134–232 bp we reported for mammalian cells (Waldman and Liskay 1988) may actually define the homology requirements for resolution. Reassessing the MEPS value for initiation *vs.* resolution is a difficult issue that would require further investigation. Our current work puts us in a position to revisit our previous conclusion that a single mismatch can reduce recombination rate in light of our conclusion that rejection of mismatched hDNA does not typically lead to wholesale destruction of a recombination intermediate. In our previous work, we used substrates with short donors in which mismatches effectively reduced the length of uninterrupted homology to <160 bp. In the current work, all substrates contained minimally 240 bp of perfect homology. We surmise that in our earlier work the amount of uninterrupted homology may have been sufficiently low so as to affect the initiation of recombination. Alternatively, formation of a certain amount of hDNA may be necessary to stabilize a recombination intermediate and dismantling of mismatched hDNA in the constrained homology interval in the previous substrates may have led to destabilization and breakdown of a joint molecule.

Our work provides a springboard for additional questions. It is of particular interest to gain a better understanding of which proteins play critical roles in hDNA rejection and how certain DNA motifs, sequences, or lesions might alter the homology requirements for initiation and/or resolution of recombination. Insight into such issues will contribute to a more complete understanding of how stability is maintained, and sometimes compromised, in mammalian genomes.

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