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Retrovirus Recombination Depends on the Length of Sequence Identity and Is Not Error Prone

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Retroviruses, as a result of the presence of two identical genomic RNA molecules in their virions, recombine at a high rate. When nonhomologous RNA is present in the dimer RNA molecules, nonhomologous recombination can occur, although the rate is very low, only 0.1% of the rate of essentially homologous recombination (J. Zhang and H. M. Temin, Science 259:234–238, 1993). We found, as is found in naturally occurring highly oncogenic retroviruses (J. Zhang and H. M. Temin, J. Virol. 67:1747–1751, 1993), that the crossovers usually occur at a short region of sequence identity. We modified the previously studied vectors to study the effect of different lengths of short regions of sequence identity in the midst of otherwise nonidentical sequences. We found that the efficiency of recombination depends on the length of this sequence identity. However, the highest rate in such molecules remained lower than for recombination between essentially homologous molecules, even when there was extensive sequence identity. Junction sequences of the recombinants indicated that retrovirus recombination is not an error-prone process as was reported for human immunodeficiency virus reverse transcriptase by using a cell-free system (J. A. Peliska and S. J. Benkovic, Science 258:1112–1118, 1992).

Retroviruses, as a result of the presence of two identical genomic RNA molecules in their virions, recombine at a high rate (2, 10, 11). When nonhomologous RNA is present in the dimer RNA molecules, nonhomologous recombination can occur, although the rate is very low—only 0.1% of the rate of essentially homologous recombination (4, 12). The formation of the 3′ proto-oncogene–virus junctions of highly oncogenic retroviruses involves such nonhomologous recombination (13).

Analysis of these 3′ junctions and the junctions in experimental systems shows that the nonhomologous recombination usually involves a short region of sequence identity in the midst of otherwise nonidentical sequences (9, 12, 13). We modified our previously studied vectors to study quantitatively the effect of different lengths of such sequence identity on the rate recombination.

We found that the efficiency of recombination depended on the length of the sequence identity but that the highest rate remained lower than for recombination between essentially homologous molecules even when there was extensive sequence identity. Sequence analysis of junctions of recombination indicates that switching of the growing point of reverse transcription of Moloney murine leukemia virus (MoMuLV) did not add an additional base as reported for strand transfers by human immunodeficiency virus (HIV) reverse transcriptase in a cell-free system (7).

MATERIALS AND METHODS

Nomenclature. Plasmids have small p's before their names (for example, pJZ211); viruses made from these plasmids do not (for example, JZ211).

Vector construction. (i) Construction of pLN20A, pLN20B, pLN20C, pLN40AB, pLN60ABC, pLN20D, pLN20E, pLN20F, pLN40DE, and pLN60DEF. The region of sequence identity between the infectious and chimeric vectors was derived from the 60-bp sequence from the 3′ end of the *hyg* gene. The 60-bp region was divided into three contiguous 20-bp regions designated 20A, 20B, and 20C. These regions were inserted into a site downstream of the *neo* gene of the infectious vector. To insert 20- to 60-bp sequences from the 3′ end of *hyg* into pLN 3′ to the functional domain, we made a *Hin*dIII-*Bam*HI DNA fragment by annealing two oligonucleotides. For example, for LN20B, we synthesized two oligonucleotides, 5′-GATCCA GCTTATCAGGGATCAGCTA-3′ and 5′-AGCTTAGCTGA TCCCTGATAAGCTG-3′. After annealing these two oligonucleotides, we designated the resulting DNA fragment sequence B (CAGCTTATCAGGGATCAGCT) with *Bam*HI and *Hin*dIII ends. This DNA fragment was inserted into the *Bam*HI and *Hin*dIII sites of pLNCX (6). All inserted sequences in the resulting vectors were confirmed by sequencing. Only pLN60ABC contained a G-for-C substitution that was not in JZ211, as a result of a mistake in the synthesis of the oligonucleotide (see Fig. 3). This 60-bp sequence is not in the *hyg* functional domain (see below) and should not interfere with hygromycin resistance.

(ii) Construction of other vectors. Other vectors were constructed by standard DNA-cloning procedures. The 290-bp sequence from the 3′ end of the *hyg* sequence in pLN290 (see Fig. 2D) was cloned as the *Sac*II-*Hin*dIII fragment from pJZ211 (12). The 830-bp sequence from the 3′ end of the *hyg* sequence in pLN830 (see Fig. 2F) was the *Eco*RI-*Hin*dIII fragment from pJZ211. A detailed description of the cloning steps is available on request.

Cells, transfection, and infection. The handling of D17 cells, and PA317 and PG13 helper cells and the performance of DNA transfections, virus harvesting, and virus infections were as previously described (12).

RESULTS

Experimental approach. The retrovirus vectors and protocol have been described previously (12). Briefly, to study recombination between chimeric and infectious MoMuLV RNAs, we

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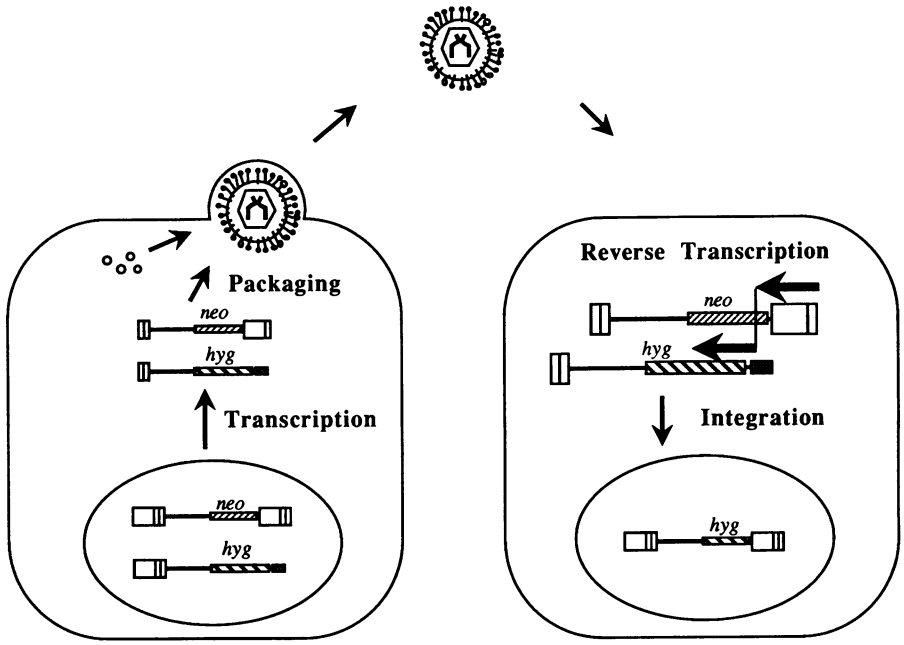

FIG. 1. Outline of the experimental approach for determination of the rate of recombination between two partially identical retrovirus RNAs during a single cycle of retroviral replication. Chimeric RNA vector JZ211 or JZ248 and an infectious vector were introduced into the helper cell line PG13 as previously described (12). PG13 helper cells containing these two vector proviruses were designated step 3 cells. Viral RNAs were transcribed from the proviruses and packaged into virions. Viruses from step 3 cells were used to infect D17 target cells. Hyg^r colonies form only when recombination occurs between the chimeric and infectious vector genomes so that the *hyg* gene is between two LTRs.

prepared a chimeric RNA (JZ211, JZ248) (Fig. 1 and 2A and B) consisting of a truncated MoMuLV in which the hygromycin resistance gene (Hyg^r) was expressed from a MoMuLV long terminal repeat (LTR) and a herpes simplex virus thymidine kinase termination sequence replaced the 3′ MoMuLV LTR, which was completely deleted. The infectious MoMuLV vectors contained a *neo* gene between two MoMuLV LTRs (6) (Fig. 1 and 2C to P). Vectors expressing the chimeric RNAs and an infectious MoMuLV vector were introduced into the helper cell line PG13 (5). PG13 clones containing a single proviral copy of the expression vector for the chimeric RNA and a single proviral copy of the infectious MoMuLV vector were selected and designated step 3 cells (12) (Fig. 1). In each experiment, 4 to 10 step 3 clones resulting from two or three step 2 clones were studied. The freshly harvested supernatant medium of step 3 clones was used to infect D17 cells (the target cells) (3), and the infected cells were selected for Hyg^r and for Neo^r. Hyg^r colonies, designated step 4 cells, form only when recombination occurs between the chimeric and infectious vector genomes so that the *hyg* gene is between two LTRs (12) (Fig. 1). The target cells do not contain viral *gag-pol* and *env* gene products for retrovirus replication, and no progeny virus is released from them (12). Therefore, the recombinant vector virus had undergone only one cycle of replication.

More frequent recombination occurs when a sequence identity exists between the chimeric and infectious viral RNAs. Many perfect matches are found between two randomly chosen sequences of sufficient length. Nonhomologous recombination usually occurs at a small region of sequence identity (2- to 8-bp sequence identity in our experimental system [12] and 1- to 11-bp sequence identity in naturally occurring highly oncogenic retroviruses [13]). To test the effect of a larger region of sequence identity on the rate of recombination between these constructs, we constructed an infectious MuLV vector, pLN290 (Fig. 2D), which contained a complete *neo* gene and 290 bp from the 3′ end of the *hyg* gene at the 3′ end of the *neo* gene of pLN (Fig. 2C). When this vector was introduced into PG13 cells with the chimeric RNA vector JZ211 (Fig. 2A), the ratio of Hyg^r to Neo^r CFU produced was $(1.7 \pm 0.5) \times 10^{-3}$, about 50 times higher than the ratio for recombination between JZ211 and LN (Wilcoxon rank sum test, $P < 0.006$). This result indicates that more frequent recombination occurs when a larger region of sequence identity (290 bp) exists between the chimeric and infectious viral RNAs in the midst of otherwise nonidentical sequences. However, this rate remained lower than that for recombination between essentially homologous molecules (Table 1).

Another infectious vector, pLN830, provided 830 bp from the 3′ end of the *hyg* gene positioned at the 3′ end of the *neo* gene (Fig. 2F). The ratio of Hyg^r to Neo^r CFU was $(1.9 \pm 1.3) \times 10^{-3}$, again lower than that of recombination between essentially homologous molecules (Table 1). Thus, the rate of recombination between two partially identical RNAs did not reach that of recombination between essentially homologous molecules even when there was extensive sequence identity (830 bp).

Insertion of an antisense sequence does not increase the rate of recombination. To study recombination between a 290-bp sequence in *hyg* and the same sequence in antisense orientation, we constructed pJZ273 (Fig. 2E). pJZ273 is identical to pLN290 (Fig. 2D) except that the 290-bp sequence was inserted in an orientation opposite to transcription. (There were no AATAAA motifs in the antisense sequence.) JZ211 and JZ273 were introduced into step 3 cells as described

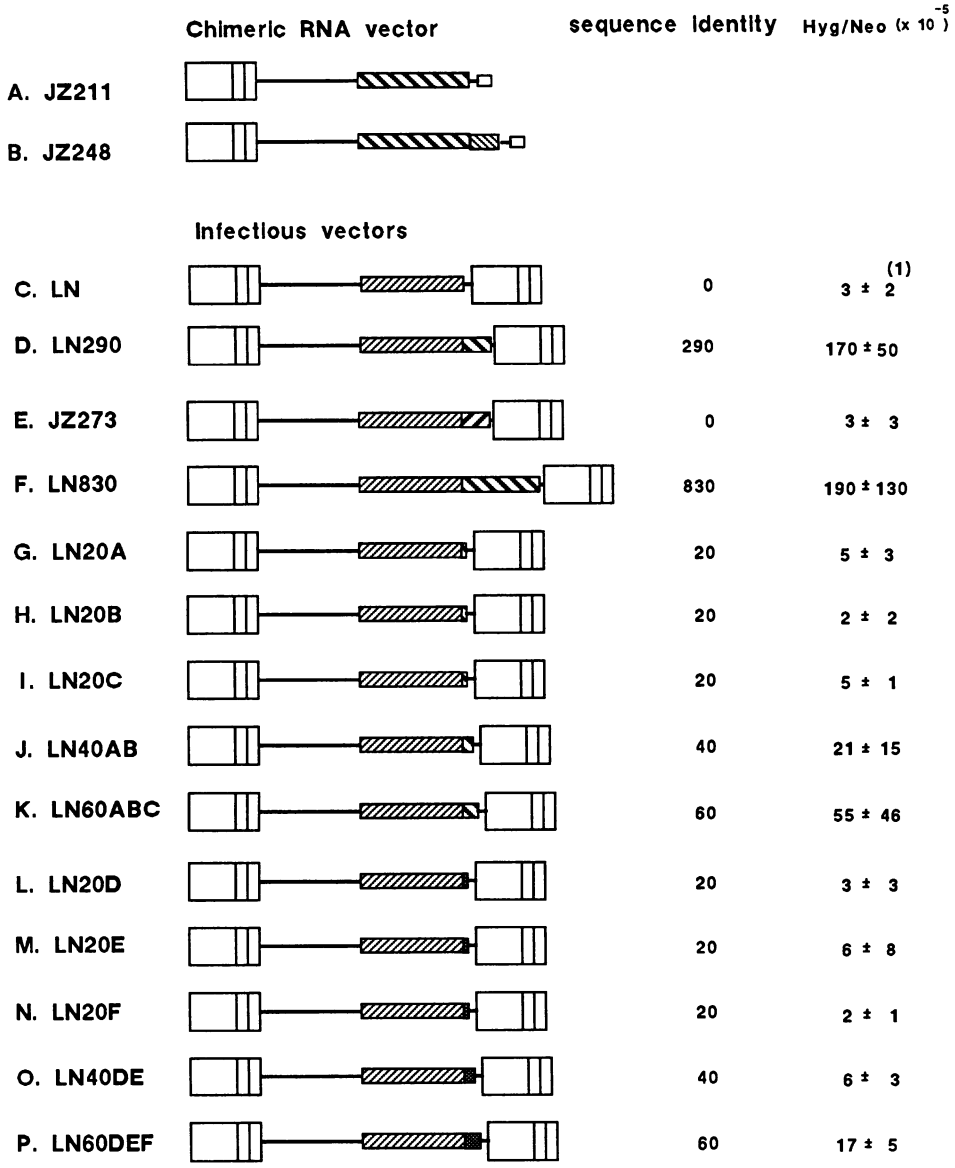

FIG. 2. Chimeric RNA vector (*hyg*), different infectious vectors (*neo*), and the ratio of Hyg^r to Neo^r CFU produced by infection with viruses from step 3 cells. (A and B) structures of the chimeric RNA vectors JZ211 (panel A) and JZ248 (panel B). The chimeric RNA vectors contain a *hyg* gene expressed by the MoMuLV 5′ LTR and a herpes simplex virus thymidine kinase termination sequence. The larger empty box represents the MLV 5′ LTR. The lines in the LTR separate U3, R, and U5 regions. The heavily striped box represents the *hyg* gene. The smaller empty box represents the thymidine kinase termination sequence. (C) Structure of infectious vector pLN and the ratio of Hyg^r to Neo^r CFU produced in the recombination experiments. pLN contains a *neo* gene between two MoMuLV LTRs. pLN does not contain extensive 3′ sequence identity with the chimeric RNA vector JZ211. (D to P) Structures of different infectious vectors and the ratio of Hyg^r to Neo^r CFU produced. The infectious vectors contained two MoMuLV LTRs and a *neo* gene between the two LTRs. They also contained sequences from the chimeric RNA vector JZ211. The lightly striped boxes represent the *neo* gene, and the stippled boxes represent sequences from the 3′ untranslated region of *hyg*. The length of sequence identity for each vector is shown under the column "sequence identity," and the ratio of Hyg^r to Neo^r CFU produced for each vector is shown under the column Hyg/Neo.

previously. The supernatant media from five different step 3 cell clones were used to infect D17 cells, and the ratio of Hyg^r to Neo^r CFU was determined. This ratio was $(2.8 \pm 3.2) \times 10^{-5}$, similar to that determined from recombination between JZ211 and LN (completely nonhomologous) and about 60 times lower than the rate of recombination between JZ211 and LN290, $(1.7 \pm 0.5) \times 10^{-3}$ (Fig. 2D), which contained the same 290-bp sequence of the *hyg* gene but in the same orientation as the *hyg* gene in JZ211.

We also constructed another chimeric *hyg* RNA vector, pJZ248 (Fig. 2B). pJZ248 was similar to pJZ211 except that it contained a 200-bp sequence from the 3′ end of *neo* inserted into the 3′ end of *hyg* in the opposite orientation. JZ248 and LN (Fig. 2C) were introduced into step 3 cells as described

TABLE 1. Rate of recombination between sequence identities per cycle

Vector	Length of insert (bp)	Rate[a]	Rate/bp[b]
LN	0		5×10^{-8}[c]
LN20B	20	1.9×10^{-5}	1×10^{-6}[d]
LN40AB	40	2.1×10^{-4}	ND[e]
LN60ABC	60	5.5×10^{-4}	9×10^{-6}[f]
LN290	290	1.7×10^{-3}	6×10^{-6}[g]
LN830	830	1.9×10^{-3}	2×10^{-6}[h]
WH13	NA[i]		2×10^{-5}[j]

[a] See Fig. 2.
[b] Rates of recombination for proviruses that switched in the sequence identity divided by the length of sequence identity.
[c] Rate of nonhomologous recombination per base pair per cycle (12).
[d] Of 18 recombinants of LN20B and JZ211, 7 were at the 20-bp insert and 11 were in the surrounding sequence. Therefore, the rate of recombination that switched in the 20-bp sequence was (7/11) × rate of nonhomologous recombination or (7/11) × 2.7×10^{-5} (12) = 1.7×10^{-5}. Therefore, the rate of recombination per base pair per cycle is 1×10^{-6} ($1.7 \times 10^{-5}/20$).
[e] ND, not determined.
[f] The rate of recombination that switched in the 60-bp sequence identity was 22/24 × the rate of recombination between LN60ABC and JZ211, i.e., 5.1×10^{-4} [(22/24) × 5.5×10^{-4} (Fig. 2I)]. Therefore, the rate of recombination per base pair per cycle is 9×10^{-6} ($5.1 \times 10^{-4}/60$).
[g] The rate of recombination per base pair per cycle for LN290 is $1.7 \times 10^{-3}/290$.
[h] The rate of recombination per base pair per cycle for LN830 is $1.9 \times 10^{-3}/830$.
[i] NA, not applicable.
[j] Rate of essentially homologous recombination from reference 4.

previously. The ratio of Hyg^r to Neo^r CFU, (3.4 ± 2.3) × 10^{-5}, was also similar to that of JZ211 with no extra sequence identity.

Therefore, the recombination rate between a sequence and its antisense sequence was not increased.

The efficiency of recombination depends on the length of sequence identity. To test the effect of different sizes of sequence identity, were constructed and tested 10 different infectious vectors as described above. Each vector contained a complete *neo* gene and a different-sized part of the *hyg* gene (Fig. 2G to P). pLN20A, pLN20B, and pLN20C each contained contiguous 20-bp regions from the 3′ end of *hyg*; pLN40AB contained two 20-bp contiguous regions from the 3′ end of *hyg* (the 20-bp sequences from both LN20A and LN20B); and pLN60ABC contained three 20-bp contiguous regions from the 3′ end of *hyg* (the 20-bp sequences from LN20A, LN20B, and LN20C) (Fig. 3). No significant increase in the rate of recombination occurred when only 20 bp of sequence identity was added (compare LN and LN20A, LN20B, or LN20C) (Fig. 2C, G, H, and I). However, the rate of recombination increased significantly when 40 or 60 bp was present (Fig. 2J and K) (compare LN and LN40AB or LN60ABC) (Wilcoxon rank sum test for LN40AB, $P < 0.001$; Wilcoxon rank sum test for LN40ABC, $P < 0.002$).

DNA from individual step 4 Hyg^r colonies was isolated. The DNA sequences at the *hyg*-MoMuLV junctions were amplified by PCR with two primers: one primer was located upstream from the 3′ end of the *hyg* gene, and the other primer was located in the MoMuLV U5 region (12). The sequences of these amplified fragments were determined. The sequences of 18 recombinant Hyg^r proviruses resulting from recombination between LN20B and JZ211 and of 24 recombinant Hyg^r proviruses resulting from recombination between LN60ABC and JZ211 were determined. Of the 18 LN20B recombinants, 7 resulted from the switch of the growing point of reverse transcription in the 20-bp sequence identity. Sequencing showed that 22 of 24 recombinants of LN60ABC and JZ211 resulted from the switch of the growing point of reverse transcription in the 60-bp sequence identity. The sequence analysis, therefore, was consistent with the results of the measurement of recombination rates.

The rate of completely nonhomologous recombination is 3×10^{-5} per cycle (Fig. 2C). The rate of completely nonhomologous recombination was approximately equal to the ratio of Hyg^r to Neo^r titer (12). The rate of recombination occurring in this 20-bp sequence was about 1.9×10^{-5} or 1×10^{-6} per bp (Table 1). The rate of recombination for LN60ABC is 55×10^{-5} (Fig. 2K) or 9×10^{-6} per bp per cycle (Table 1). The rate of recombination for LN290 is 6×10^{-6} per bp per cycle (Table 1), and the rate of recombination for LN830 is 2×10^{-6} per bp per cycle (Table 1). The rate of homologous recombination is about 2×10^{-5} per bp per cycle (4). The rate

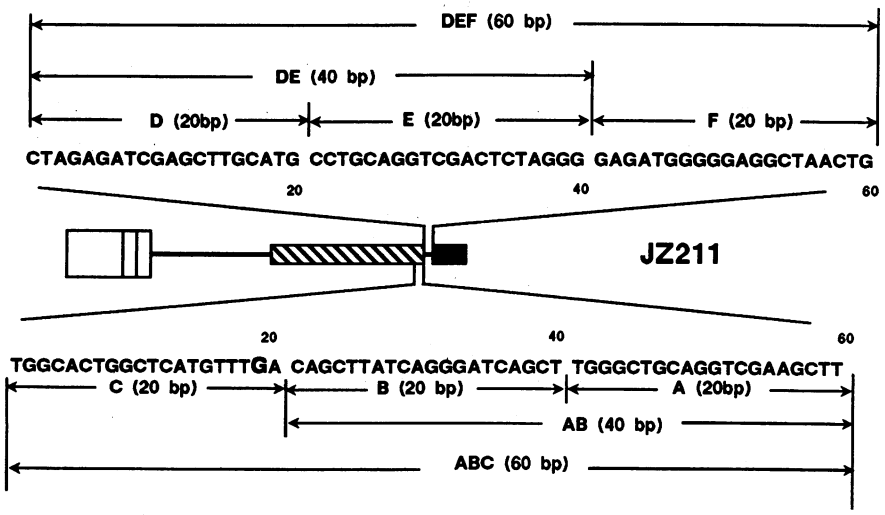

FIG. 3. Sequence identity from JZ211 in infectious vectors. Sequences A to F were the inserts 3′ of *neo* that are shown in the heavily striped and stippled boxes in Fig. 2G to P. LN60ABC contained a nucleotide that was not in JZ211, as a result of a mistake in the synthesis of the oligonucleotide. This mistake was shown in region C and is marked in boldface type. The nucleotide G was a substitution for C.

of recombination increased with the extent of sequence identity in the midst of nonidentical sequences (Table 1). However, the increase in rate of recombination per base pair per replication cycle was not linear. It increased when the sequence identity increased from 20 bp to 60 bp but decreased when the sequence identity increased from 60 bp to 830 bp. We have no complete explanation but hypothesize that the relative positions of sequence identity in the midst of nonidentity in the two RNA molecules in the virus may be important.

The products of crossover for LN20B and LN20C were different from the products for LN20A, LN40AB, and LN60ABC in that a few amino acids at the carboxyl end of *hyg* were deleted. Native *hyg* does not have these amino acids; the sequence at the 3′ end of *hyg* in JZ211 is from pBR322. Previous work indicated that these carboxyl-terminal amino acids are not important for formation of a Hyg^r colony (12). To test whether different sequences would give the same result, we also constructed two vectors containing two *hyg* genes with the same sequence as the recombinants between LN20B and JZ211 and between LN20C and JZ211. These two vectors had titers similar to that of JZ206 (data not shown).

To determine whether the requirement for sequence identity was sequence specific, we constructed LN20D, LN20E, LN20F, LN40DE, and LN60DEF (Fig. 2L to P). pLN20D, pLN20E, and pLN20F each contained contiguous 20-bp regions from the 3′ end of *hyg*; pLN40DE contained two 20-bp contiguous regions from the 3′ end of *hyg* (the 20-bp sequences from both LN20D and LN20E); and pLN60DEF contained three 20-bp contiguous regions from the 3′ end of *hyg* (the 20-bp sequences from LN20D, LN20E, and LN20F) (Fig. 3). Similar results were found with pLN20D, pLN20E, pLN20F, and pLN40DE. No significant increase in the rate of recombination occurred when only 20 or 40 bp of sequence identity was added. However, the rate increased markedly when 60 bp was present (Wilcoxon rank sum test for LN/LN60DEF, $P < 0.002$) (Fig. 2L to P). These results indicate that the requirement for sequence identity to increase the rate of recombination was not a result of a specific sequence. They also suggest that some sequences may be preferred for recombination, since in this case (LN40DE, Fig. 2O) a 40-bp sequence identity did not increase the rate of recombination significantly whereas it did in LN40AB (Fig. 2J).

Recombination is not error prone. Peliska and Benkovic (7) reported that cell-free HIV-1 reverse transcriptase incorporated additional bases beyond the 5′ end of the RNA template, resulting in a base misincorporation upon DNA strand transfer, and suggested that retrovirus recombination is error prone. To determine whether recombination is error-prone, we determined the sequences of the junctions between the chimeric RNA vector (JZ211) and infectious vectors in recombinant (Hyg^r) proviruses in step 4 cells. Since the rate of recombination increased markedly when the 60-bp sequence was present and 22 of 24 sequenced recombinants were at the 60-bp insert, we examined the 60-bp sequence in the recombinants for errors. If retroviral recombination is error prone, we should observe that the 60-bp sequence insert contained one base misincorporation in each recombinant in which the recombination occurred in this 60-bp sequence identity.

No mutations were detected in the 60-bp sequence in the 22 recombinants (data not shown). In addition, the 20-bp sequence identity of seven recombinants from LN20B resulting from the switch of the growing point of reverse transcription in this 20-bp sequence identity was also determined. Not a single mutation was detected in these 20-bp sequences in the seven recombinants resulting from recombination between LN20B and JZ211 (data not shown). Therefore, high-fidelity recombination occurred when the growing point of reverse transcription of MoMuLV switched its template in our system.

DISCUSSION

The rate of recombination between essentially homologous molecules is about 2×10^{-5} per bp per cycle (4), and the rate of completely nonhomologous recombination is about 5×10^{-8} per bp per cycle (12). Therefore, the rate of recombination between essentially homologous molecules is about 1,000 times higher than the rate of nonhomologous recombination (4, 12). When nonhomologous recombination occurs, a short region of sequence identity is frequently used for the strand switching (12, 13). The results presented here indicate that more frequent recombination events occur when a larger region of sequence identity exists between the chimeric and infectious viral RNAs. However, not all larger regions of sequence identity have the same effect on the rate of recombination.

The efficiency of recombination depends on the length of sequence identity. The rate of recombination increased with the extent of sequence identity in the midst of nonidentical sequence. However, the rate of recombination per base pair per replication cycle was not linear. It increased when the sequence identity increased from 20 to 60 bp but decreased when the sequence identity increased from 60 to 830 bp. It is interesting that the R region of MoMuLV, which is used to transfer the minus-strand primer, is about 60 bp.

Many perfect matches are found between two randomly chosen sequences of sufficient length, but not all the perfect matches are equally used when retrovirus recombination occurs (12). Different larger regions of sequence (40 bp) also have different effects on the rate of recombination (for example, 40AB and 40DE). Therefore, adding a very short sequence of identity between the chimeric and infectious viral RNAs will not necessarily increase the rate of recombination significantly. During retroviral replication, the reverse transcription growing point switches its template to synthesize the LTRs (1). The efficiency of transferring to the chimeric RNA (JZ211) depends on the length of sequence identity in our system. The NC protein in the retroviral virion may align the two identical single-stranded RNAs as RecA protein aligns a single-stranded DNA with identical double-stranded DNA (8). A large sequence identity may be required to allow formation of a stable duplex structure.

The rate of recombination between two partially identical RNA molecules never reached that of recombination between essentially homologous molecules even when there was extensive sequence identity, and the rate of recombination per base pair decreased when the sequence identity increased from 60 to 830 bp. These results suggest that sequence identity in the midst of an otherwise nonidentical sequence does not function as well as two almost completely identical sequences (homologous recombination).

The rate of recombination of a retrovirus is a function of the product of the rate of leaving of the original template and the rate of switching to the new template. In our system, the growing point of reverse transcription leaves the infectious vector RNA genome and switches to the chimeric RNA genome during minus-strand DNA synthesis (Fig. 1) (12). Recombination occurred more frequently as the sequence identity was increased between the chimeric and infectious viral RNAs. The infectious vectors were similar except for the size of the sequence identity. If a nonspecific sequence was inserted in the same position, the rate of recombination between the two vectors did not increase (Fig. 2B and E). These results indicate that the limiting step in nonhomologous

recombination is the switching. In other words, in most cases reverse transcription growing points leaving the infectious vector did not switch to the chimeric vector RNA molecule. The higher rate of recombination between essentially homologous molecules results from the presence of an ideal switching area for the reverse transcription growing points that have left the infectious vector template.

Peliska and Benkovic (7) reported that cell-free HIV-1 reverse transcriptase incorporates additional bases beyond the 5′ end of the RNA template, resulting in a base misincorporation upon DNA strand transfer. Such a process occurring in vivo during retroviral recombination could contribute to the hypermutability of the HIV-1 genome. We used our system to test whether base misincorporation occurred during MoMuLV reverse transcription in cell culture. We analyzed sequences of 29 recombinant Hyg^r proviruses resulting from the switch of the growing point of reverse transcription at an inserted region of sequence identity. No mutations were detected at the recombination site in these 29 recombinants. Therefore, high-fidelity recombination occurred when the growing point of reverse transcription of MoMuLV switched its template in cell culture. These two different results may reflect differences between HIV-1 and MoMuLV, between cell-free and cell culture experiments, or between forced copy choice and copy choice recombination.

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