Replication of the Retroviral Terminal Repeat Sequence during In Vivo Reverse Transcription

CATHERINE A. RAMSEY AND ANTONITO T. PANGANIBAN*

McArdle Laboratory for Cancer Research, University of Wisconsin at Madison, Madison, Wisconsin 53706

Received 31 December 1992/Accepted 31 March 1993

There is a copy of a short terminal repeat segment, *r*, at each end of the retroviral RNA genome. During reverse transcription, *r* is copied from the genomic RNA to form the R component of the long terminal repeat in viral DNA. Although our current model for reverse transcription suggests that the 5' *r* is copied, it is not known whether the 5' copy, the 3' copy, or part of each *r* in the genomic RNA serves as the template for the R region in the progeny viral DNA. To assess the relative contribution of the 5' and 3' *r* templates, we examined the effect of mutations located at the center of the 5' or 3' *r* of spleen necrosis virus and determined the sequence of the R region in the progeny proviruses after a single round of retroviral replication. In approximately 90% of the proviruses, the 5' *r* marker was copied, whereas 10% of the proviruses had derived the R marker from the 3' *r*.

Retroviruses are positive-stranded RNA viruses that replicate through a DNA intermediate and have evolved a complex strategy to replicate the ends of the genome. Reverse transcription is primed by a cellular tRNA molecule base paired to a sequence termed the primer-binding site, which is located near the 5' end of the viral genome (26). The nascent minus-strand DNA copy is transferred to the 3' end of the genomic RNA for reverse transcription to continue (9) (Fig. 1). After extension of the transferred minus-strand DNA, site-specific cleavage of the genomic RNA by the RNase H activity of reverse transcriptase at a sequence adjacent to u3 (16) yields a primer for initiation of plus-strand DNA synthesis. The plus-strand DNA is transferred to the 3' end of the nearly full-length minus-strand DNA in the second-strand transfer step. Subsequent elongation of the plus and minus strands results in the generation of a full-length double-stranded DNA copy.

The strand transfer steps and further synthesis generate long terminal repeats (LTRs) in the DNA, which were not present in the genomic RNA (Fig. 1). The LTRs consist of three regions, designated U3, R, and U5. R is derived from the short terminal repeat in the genomic RNA and makes up the central segment of the LTR. U3 is present only at the 3' end of the genomic RNA but is located at the 5' end of both LTRs in the viral DNA. Similarly, U5 is present only at the 5' end of the genomic RNA but forms the 3' segment of both LTRs. The minus-strand transfer allows juxtaposition of U5 and R with U3 to generate one LTR structure, and the plus-strand transfer carries the LTR to the other end of the genome. For clarity of presentation, the segments of the LTRs in the DNA and the RNA will be designated here by capital letters and lowercase letters, respectively.

A simple way to explain the minus-strand transfer step is that reverse transcription initiates at the tRNA primer adjacent to u5 (Fig. 1), elongates across the u5 of the RNA template, and proceeds all the way through the *r* region to the 5' end of the genome. Generation of such a full-length nascent minus-strand strong stop DNA is normally invoked in models of reverse transcription (Fig. 1) (13, 18, 22).

According to these models, the RNase H activity of reverse transcriptase removes the RNA in *r* and u5, leaving a minus-strand DNA copy containing a full-length R which is free to interact with the complementary *r* at the 3' end of the genome (9). Minus-strand strong stop DNA can transfer to either the 3' end of the same genomic RNA or the second copy of the genome present in the viral capsid (13, 18). In contrast, the plus-strand transfer is an intrastrand event in more than 95% of reverse transcription events; therefore, the two LTRs in a provirus are almost always identical (13, 18).

One consequence of the model presented in Fig. 1 is that the 5' *r* region is always copied into both LTRs of the progeny provirus (Fig. 2A). In vitro reverse transcription studies have detected an initial discrete product, originally called minus-strand strong stop DNA, consisting of U5 and R sequences (1, 22), which supports the hypothesis that *r* is completely reverse transcribed prior to transfer. In contrast, studies on replication-competent murine leukemia virus (MLV) with alterations in R suggested that reverse transcriptase can transfer prior to copying all of *r*, since mutations in the 5' *r* were always lost (15). However, the transfer site was not uniform or constant, since the same mutations introduced into the 3' end were also always lost (unless present in both LTRs of the parental virus) (15).

Studies focusing on strand transfer have demonstrated that reverse transcriptase is the only protein required in vitro for this process (17, 22). RNase H activity is not absolutely required for strand transfer in the in vitro reaction with MLV reverse transcriptase, although it does enhance the efficiency of transfer (17). However, in vitro studies with human immunodeficiency virus type 1 reverse transcriptase have shown no detectable strand transfer when reverse transcriptase with a mutation in the RNase H domain was used (22). In vivo, RNase H mutants of MLV are blocked in replication prior to strand transfer (25), which suggests that RNase H activity is required in vivo for strand transfer.

In other in vitro reverse transcription assays, it was observed that RNase H does not remove all of the ribonucleotides remaining after digestion of the genomic RNA but,

* Corresponding author.

FIG. 1. Reverse transcription of the retroviral genome. RNA sequences are indicated by thin lines, and DNA sequences are indicated by thick lines. Named regions of the genome are indicated by lowercase letters in the RNA and capital letters in the DNA. The cellular tRNA is shown interacting with the primer-binding site (pbs) near the 5' end of the genome, where it is used as a primer for DNA production. The polypurine tract where site-specific cleavage by RNase H generates a primer for plus-strand synthesis is designated ppt. The LTRs created in the viral DNA are indicated at the bottom. The initial steps of reverse transcription are discussed in the text.

rather, that a terminal 14- to 18-base oligoribonucleotide remains base paired with the nascent DNA (6, 17, 22). These latter observations support a model in which the RNA is not completely removed from the minus-strand strong-stop DNA prior to strand transfer.

Previous studies on the propagation of mutations in the 5' or 3' R region of replication-competent MLV demonstrated that mutations were lost in the course of replication and that they were maintained only when present in both LTRs of the starting provirus (15). However, in these studies, the analysis of r complementarity in strand switching may have been complicated by genetic selection. Multiple replication cycles were used in the analysis of the r mutations, and some of the mutations were deleterious to growth. Therefore, recombination could have taken place, resulting in the generation of wild-type virus that would replicate more efficiently than its defective counterpart.

To learn more about the minus-strand transfer step of reverse transcription, we made alterations in the sequence of the r region of spleen necrosis virus (SNV). We tested these mutations to determine whether they affected replication and to determine whether reverse transcriptase copies these mutations within r prior to the minus-strand transfer step of reverse transcription. By using a packaging cell line and vector system with a single, defined starting vector, it was possible to examine the results of a single round of replication and unambiguously determine the source of the R region of the LTRs. The results of this analysis indicate that strand transfer can occur prior to the completion of reverse transcription of the r segment. Approximately 10% of the progeny proviruses we examined had transferred prior to reverse transcription of a marker that is located in the center of r, 40 bases from u5. However strand transfer usually occurs after

FIG. 2. Alternative fates of mutations in the 5' r. Conventions are as in Fig. 1. (A) A mutation in the 5' r is copied into DNA and transferred to the 3' end and thus will be present in both LTRs of the provirus. (B) Reverse transcriptase transfers prior to copying a mutation in the 5' r; therefore the 3' r is copied in that region and will yield a provirus with the R region of the LTRs derived from the 3' r.

the reverse transcription of at least half (40 bases) of r in spleen necrosis virus.

MATERIALS AND METHODS

Plasmid constructions. All of the LTRs used for mutagenesis contain a deletion of 8 bp in U5 between the two *RsaI* sites and an inserted 8-bp *HindIII* linker at that site (APH-neo') (18). The parental plasmid was linearized with *BstBI*, which cleaves at the center of R, treated with Klenow polymerase fragment in the presence of deoxynucleotides to fill in the resulting recessed 3' end, and then ligated in the presence of an 8-bp *XhoI* linker (Pharmacia). A short deletion was created in the middle of R from the unique *XhoI* site by using the 3'-specific exonuclease activity of T4 DNA polymerase and the single-strand-specific DNA nuclease activity of S1 nuclease.

PCR. Polymerase chain reaction (PCR) amplification was performed by using the Gene Amp PCR Reagent Kit and DNA Thermal Cycler (Perkin Elmer Cetus). Primers were supplied by the University of Wisconsin Biotechnology Center and the McArdle Laboratory DNA synthesis facility. Primers for amplification of the 5' LTR were in U3 (CGCG CACCGAAGGCAAGCATCA) and either adjacent to the primer-binding site (CTGCCGATGGGGAGGGTATCC) or in the 5' end of the Neo^r gene (CGCTTCCTTTAGCAGC CCTTGC). For amplification of the 3' LTR, the primers

were in the 3' end of the Neo^r gene (GGATCTCATGCTG GAGTTCTTCG) and in U5 (GAACAAAATCTCCTCTC ACTGC). Genomic DNA for PCR analysis was prepared as a crude cell lysate which was subjected to proteolysis at 65°C for 1 to 12 h, after which the proteinase K was inactivated by a 10-min incubation at 95°C (10). The 100-μl PCR contained an estimated 0.5 μg of genomic DNA, 0.3 μg of each primer, and 100 μM each deoxynucleoside triphosphate. The reaction mixture was overlaid with mineral oil, incubated for 5 min at 95°C, and amplified through 30 cycles by using the following specifications: 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C per cycle. Finally, the reaction was incubated for 5 min at 72°C. PCR-amplified DNA samples were purified by using the Magic PCR Prep DNA Purification System (Promega) prior to sequencing.

DNA sequencing. Dideoxy DNA sequencing was performed by using the Sequenase Version 2.0 Kit (United States Biochemical Corp.). The sequencing primer, supplied by the University of Wisconsin Biotechnology Center, anneals to a region of U3, 45 bp upstream of R (GTCATG TAACACCCTG).

To denature double-stranded DNA for sequencing, 5 μg of DNA was incubated for 30 min at 37°C in 200 mM sodium hydroxide–2 μM EDTA. The DNA was then precipitated by addition of 300 mM sodium acetate (pH 5.2) and 3 volumes of ice-cold ethanol. The DNA pellet was dried and then resuspended in water.

FIG. 3. Sequence of mutations in the R region. (A) The primary sequences of two mutations in R are compared with that of wild-type (WT) R. The inverted repeat present in the wild-type sequence is indicated by arrows. In BX, the inverted repeat is extended by the introduction of an *Xho*I linker and the duplication of CG (in brackets) by the Klenow enzyme. Five bases are deleted in $\Delta 5$, and this would be expected to remove the putative stem-loop structure. Several differences between the wild-type sequence shown here and the published sequence (27) are noted in boldface and reflect our sequencing of the starting plasmid as well as all of the mutants. (B) Putative secondary structure of the inverted repeats in the wild type and the BX mutant.

Cell culture, transfection, and infection. The D17 cell line, a canine osteosarcoma cell line (American Type Culture Collection), was maintained in Dulbecco's modified Eagle's medium supplemented with 7% bovine calf serum (HyClone). DSO cells were provided by W.-S. Hu and H. Temin, McArdle Laboratory, and were maintained in Dulbecco's modified Eagle's medium supplemented with 64 nM ouabain and 7% bovine calf serum (HyClone). DSO cells were made by using the same SNV expression plasmids as the previously described cell line DSN (4), but cotransfection with the expression plasmids was performed with a plasmid that encodes a protein that confers resistance to ouabain.

Transfections were performed by the Polybrene-dimethyl sulfoxide method (14). G418 selection (400 μ g of G418 per ml) was applied 24 h after transfection. For infections, virus was harvested and centrifuged or filtered through a 0.2- μ m-pore-size Acrodisc (Gelman) to remove cells and cellular debris. Then 1 or 2 ml of virus-containing medium was placed on the DSO or D17 cells in a 100-mm dish, and Polybrene (Aldrich) was added to a concentration of 100 μ g/ml. Following a 40-min incubation at 37°C, 10 ml of medium was added, and G418 selection at 400 μ g/ml was applied 24 h later.

RESULTS

Construction of retroviral vectors with markers in r. If reverse transcriptase copies all of the 5' r prior to strand transfer, the 5' r will be the sole source of r sequence information for the progeny (Fig. 2A). If this occurs, alterations introduced into the 5' r will be maintained in both

copies of r in the progeny provirus and alterations in the 3' r will be lost after a single round of replication. However, if strand transfer occurs prior to complete reverse transcription of the 5' r, the 3' r will also be a source of sequence information for the progeny. In this case, mutations introduced into the 5' r might be lost, depending on the site of transfer (Fig. 2B), and mutations introduced into the 3' r might be maintained. If strand transfer occurs at different times during reverse transcription of r, the progeny could be a mixed population of proviruses, all of which contain identical LTRs either with or without the mutation.

To monitor the replication of r during retroviral reverse transcription, we introduced markers into the center of the R sequence of a SNV vector. If markers introduced into the 5' r region are consistently copied into both LTRs, reverse transcription must proceed through the site of the marker prior to strand transfer (Fig. 2A). If all progeny proviruses contain the markers of the 3' r, strand transfer must occur consistently early during reverse transcription of r (Fig. 2B). A mixed population of proviruses would indicate a flexible mechanism in which the amount of r copied prior to strand transfer was not fixed.

There is an inverted repeat in the center of R, which has no previously known function and which contains a *Bst*BI site (Fig. 3). This appeared to be a potentially useful site for the introduction of markers to monitor the genesis of R. It has been suggested that sites of secondary structure in the RNA might cause pauses during reverse transcription, at which time strand transfer could occur (3, 15). If this is the case, the site of a mutation with respect to regions of secondary structure, or changes in secondary structure, could affect the propagation of a mutation. In one of the r

FIG. 4. Efficiency of replication of mutants. The first column gives the vector name, the second shows a schematic diagram of the structure of the vector, and the third gives the relative titers. The constructs with altered 5' LTRs have a *HindIII* linker in the 5' U5 and are compared with the parental APHneo'. Those with altered 3' LTRs have wild-type 5' LTRs and are compared with JD214Neo. For APHneo' the titers ranged from 10 to 100 G418 resistance-transforming units/ml; for JD214Neo the range was 70 to 710 units/ml. The relative titers are the average results from four transfections. Restriction site abbreviations: β , *BstBI*; H, *HindIII*; X, *XhoI*; Δ , a 5-bp deletion obliterating the restriction site.

mutants (BX [Fig. 3]) the inverted repeat is extended from 14 to 24 bp through the introduction of an *XhoI* linker into the *BstBI* site. In the second mutant, a short deletion which removed 5 bp from one side of the original inverted repeat was made ($\Delta 5$ [Fig. 3]).

Effect of r mutations on replication. We wished to determine whether the mutations in r affected the efficiency of propagation of the vector through a single round of replication. If a mutation reduces the efficiency of replication, it might yield progeny of one type (e.g., wild type) more readily than another (e.g., mutant) and thus give a biased estimate of the tendency of reverse transcriptase to switch strands at a given point in R. We therefore tested the efficiency of replication of SNV vectors with each of our mutations in either the 5' or the 3' LTR or both. Each of the mutated LTRs was introduced into a SNV vector, JD214Neo (5), replacing either the 5' or 3' LTR or both. The vector JD214Neo carries no retroviral genes and expresses the neomycin phosphotransferase gene (*Neo^r*), conferring G418 resistance on mammalian cells. To determine the efficiency of replication of each of the mutated constructs, we transfected an SNV-packaging cell line (DSO) with each of the vectors. The DSO cell line expresses the SNV *gag*, *pol*, and *env* genes from heterologous promoters. After selection with G418, we pooled all the G418-resistant colonies, plated out an equal number of cells per line, and used these as a source of virus to infect D17 cells. The titer of G418 resistance-transforming virus was determined by counting the G418-resistant colonies that arose following selection of the D17 cells.

Figure 4 summarizes the results of the replication assay. The *HindIII* linker present in U5 of the mutated LTR reduces the efficiency of replication when present in the 5'

FIG. 5. Experimental method to determine the sequence of the R region of the progeny proviruses from a single defined mutant provirus. The vector DNAs were introduced into SNV packaging cells by transfection. After selection with G418, clonal populations were isolated and the number of proviruses was determined by nucleic acid hybridization. The sequence of the LTRs of the provirus was confirmed by amplification of each LTR and restriction analysis. Appropriate virus-producing cell lines (two for each vector) were chosen as a source of virus to infect D17 cells which were selected with G418, and clonal populations were grown out and analyzed by PCR as before. For LTRs that contained a deletion and were not cut by *BstBI*, DNA sequence analysis was performed to confirm that the progeny contained the same R sequence as the deleted LTR of the parental vector.

LTR; therefore, the 5' mutant LTR vectors and vectors with two mutant LTRs are compared with the APHneo' vector, which contains this alteration, and the vectors with 3' mutant LTRs are compared with JD214Neo. The titers of 5-BX and 2-BX indicate that the BX insertion does not strongly affect the efficiency of replication. The short deletion in $\Delta 5$ may have a slightly detrimental effect on replication, as shown by the slightly reduced titers of 5- $\Delta 5$ and 2- $\Delta 5$. Both of these mutations in the 3' LTR appear to be slightly detrimental to replication.

Generation of the LTR R from the genomic r. The experimental strategy that we used to analyze the progeny of these vectors is shown in Fig. 5. To propagate the starting vectors through a single round of replication, we again transfected the SNV-packaging cell line with each vector. G418 selection was applied to the transfected cells, and individual cell clones were then isolated and characterized by nucleic acid hybridization to determine the copy number of the introduced vectors. As virus-producing cells we chose only clones that contained no more than two copies of the introduced vector (data not shown).

To be sure that the markers in the LTRs had not under-

FIG. 6. Restriction analysis of PCR-amplified 5' LTRs of progeny proviruses. D17 cells were infected with the 5-BX vector, G418 selection was applied, the resulting colonies were isolated, and genomic DNA was prepared as described in Materials and Methods. The DNA of the 5' LTR was amplified by using primers in U3 and the primer-binding site. The product was purified, incubated with either *Bst*BI (A) or *Xho*I (B), and subjected to electrophoresis on a 1% SeaPlaque Agarose (FMC) gel. Sample 1 was cleaved by *Bst*BI but not by *Xho*I. Samples 2 to 6 were cleaved by *Xho*I but not *Bst*BI, indicating that they contain the sequence at that site in R from the 5' LTR of the parental vector and not the 3' LTR.

gone mutation or gene conversion during transfection as a result of homologous recombination between the two LTRs, we determined the sequence of the LTRs of the introduced vectors in virus-producing cell clones. The proximal and distal LTRs were individually analyzed by using PCR to amplify specifically the 5' or 3' SNV LTR (see Materials and Methods), and then the amplified DNA was either digested with diagnostic restriction enzymes or characterized by

DNA sequence analysis. We had previously determined that we could detect a mixed population of proviruses with two different LTR types, containing half of each type, by using primers that amplified both LTRs of our vector and showed that we could reliably detect both species of LTR. In positive controls for the PCR analysis with plasmid DNA, we did not detect products that would represent the results of recombinant PCR creating LTR fragments with restriction sites from the inappropriate LTR. We identified and isolated two clonal populations of virus-producing cells for each vector. We used these cell clones as a source of virus to infect target D17 cells. G418-resistant colonies were isolated, and the proviral LTRs were analyzed as described above. The plus-strand transfer is usually an intrastrand event, and the two LTRs in a provirus are almost always identical (13, 18). We analyzed both the 5' and 3' LTRs for each of the progeny proviruses derived from 5-BX and 3-BX, and in all cases they were identical. We determined the sequence of only the 5' LTR in each of the progeny proviruses of the $\Delta 5$ clones.

A representative example of restriction analysis is presented in Fig. 6, which shows the 5' LTR PCR products of six of the 5-BX progeny proviruses. The figure shows one provirus with an LTR derived from the 3' parental LTR and five with LTRs derived from the 5' parental LTR. Overall, we examined 13 progeny proviruses derived from 5-BX. Of these, 11 contained the *Xho*I linker derived from the mutated 5' LTR and 2 contained the wild-type *Bst*BI site from the 3' LTR (Fig. 7). When the *Xho*I linker was present in the 3' LTR instead (3-BX), 18 of the progeny examined contained the *Bst*BI site derived from the 5' LTR and 2 contained a *Xho*I site derived from the 3' LTR. We examined 16 progeny proviruses from 5- $\Delta 5$, in which a 5-bp deletion was introduced into the 5' LTR; 15 proviruses had LTRs that contain the deletion from the 5' LTR and one contained the wild-

FIG. 7. Origin of R in progeny proviruses. The structures of parental vectors are indicated on the left, and the structures of the two expected progeny types for each vector are indicated on the right, along with the number of proviruses of each observed type.

type *Bst*BI site derived from the 3' LTR. When this short deletion was present in the 3' end (3-Δ5), all 11 progeny proviruses examined contained the *Bst*BI site derived from the 5' LTR. Therefore, overall, only about 10% of the progeny derived their R sequences from the 3' LTRs, while the great majority copied the 5' LTR marker.

DISCUSSION

The r region of SNV is 82 nucleotides long. The results of our analysis indicate that the r region is usually reverse transcribed at least 40 bases into r (through the *Bst*BI site) before transfer to the 3' end occurs. However, transfer can also take place prior to complete reverse transcription of the r region, as shown by examples (approximately 10%) in which the 3' r markers were copied.

It had been proposed that reverse transcriptase pauses at sites of secondary structure (3, 15, 21). Strand transfer might occur during such a pause. Therefore, the position of a mutation with respect to a pause site would be expected to affect the way in which it was replicated. If a mutation were on the 5' side of a region of secondary structure in the genomic RNA and the reverse transcriptase paused at the structure, strand transfer could potentially occur prior to the replication of the mutation, and the 3' r region would serve as the template for that region (Fig. 2B). Our results indicate that there is apparently no strong preference for switching before the potential stem-loop structure at the *Bst*BI or *Xho*I site when compared with a vector that lacks obvious secondary structure at this site. Comparison of the progeny proviruses of 5-BX, which contains a predicted stem-loop structure (Fig. 3B), with 5-Δ5, in which there is no predicted secondary structure at this site, and 3-BX, which contains the shorter wild-type stem-loop (Fig. 3B), indicates that in all cases the 5' r was copied most of the time. The marginal increase in the usage of the 3' LTR in 5-BX compared with either 5-Δ5 or 3-BX is not statistically significant as determined by Fisher's exact test ($P > 0.3$).

The model of reverse transcription presented in Fig. 1 presumes that the minus-strand DNA contains at its 3' end the primer-binding site sequence but not R or U5, because the r and u5 segments at the 5' end of the RNA template were degraded by RNase H. This would arise when the minus-strand transfer is intrastrand (transfers to its own 3' end) or when minus-strand transfer is interstrand and reverse transcription has initiated in parallel on both viral genomes present in the capsid (the second is not specifically shown). However, it is possible that reverse transcription starts on only one of the genomic RNAs and the nascent DNA transfers to the other genome. In this case, R and U5 would be present in the minus-strand DNA and could provide greater sequence complementarity for the plus-strand transfer. Studies on the plus-strand transfer step of reverse transcription have suggested that transfer can proceed prior to the complete reverse transcription of the primer-binding site from the tRNA (23). This is similar to our results with the minus-strand transfer: reverse transcriptase does not always copy all the way to the 5' end of the genome prior to strand transfer. Early transfer of the plus-strand DNA could potentially lead to a provirus containing two LTRs of different types (such structures have been observed by other researchers [13]) or a population of proviruses derived from the same viral DNA in which there is a mixture of types at the 5' LTR as a result of a mismatch present in the plus-strand transfer that was not repaired. We examined both LTRs of our 5-BX and 3-BX progeny proviruses, and

all of them contained the same LTR at the 5' as the 3' end; therefore, we did not detect any early plus-strand transfer in the BX proviruses, although not all early transfers would be detectable in this system.

Reverse transcriptase has a well-documented tendency to switch templates during polymerization, which has been variously attributed to a need to alternate genomic templates when they are damaged or broken and to the necessity of translocation from the 5' end to the 3' end of the genome during normal replication. Transfer can occur either to a homologous sequence (11–13, 23) or to a region which has no apparent sequence similarity (19, 20, 23, 24). Strand transfer (or copy choice) by reverse transcriptase has been invoked to explain the relatively high rate of recombination between coinfecting retroviruses (2) and transduction of cellular genes, especially oncogenes, into retroviral genomes (7, 8, 24).

The recombination rate between two almost identical SNV vectors was determined to be 2% per kb in a single round of replication (12). In our study, approximately 10% of viruses switched templates within a 40-nucleotide region of the 3' half of r. This is a much higher rate than that observed between markers in the internal portion of the genome (12). It is intriguing to consider what causes this high rate of strand transfer prior to complete copying of the 5' r. It may be that the proximity of a free end influences the rate of strand switching. Since two potential reasons for reverse transcriptase to switch templates are to bypass a nicked site in the genome and to carry out the terminal transfer required for replication, and since both of these involve free 3' ends of RNA, perhaps reverse transcriptase has a tendency to switch to a homologous strand of RNA when it approaches any 3' end on a template.

Are there sequences within r which promote template jumping? If one were to make mutations along the length of r, specific regions required for strand transfer might be identified. Such a set of mutations could also be used to determine whether there is a hot spot for switching or whether there is a simple gradient of increased switching along r as reverse transcriptase nears the 5' end. There could be some minimum of overlapping sequence complementarity required for strand transfer to proceed, and during reverse transcription of r, the growing DNA strand has an increased region of complementarity with the 3' end. It is interesting in this context that the R regions of different retroviruses vary tremendously in length: in SNV, R is 82 nucleotides long, in human T-cell lymphotropic viruses I and II it is over 200 nucleotides (27), and in mouse mammary tumor virus it is only 15 nucleotides (27). The R region of MLV can be shortened from 68 to 39 bases, deleting most of its 5' half, without completely blocking replication (15). Clearly, retroviral reverse transcriptases can accommodate variability in the length of r complementarity for strand transfer.

Although reverse transcriptase is sufficient to promote strand transfer in vitro, perhaps other proteins are involved in vivo to juxtapose the two ends and align the complementary sequences efficiently. Our results indicate that strand transfer can occur prior to complete reverse transcription of r. However, much still remains to be learned about this unique step in retrovirus reverse transcription.

ACKNOWLEDGMENTS

We thank J. Jones and H. M. Temin for useful comments on the manuscript; D. Fiore, K. Talbot, and H. Hermanson for technical assistance; and R. Geraghty for generation of the BX mutant.

This work was supported by grant RO1 CA49741-03 from the National Institutes of Health.

REFERENCES

1. Boone, L. R., and A. M. Skalka. 1981. Viral DNA synthesized in vitro by avian retrovirus particles permeabilized with melittin. I. Kinetics of synthesis and size of minus- and plus-strand transcripts. *J. Virol.* **37**:109–116.
2. Coffin, J. 1979. Structure, replication and recombination of retrovirus genomes: some unifying hypotheses. *J. Gen. Virol.* **42**:1–26.
3. DeStefano, J., R. Buiser, L. Mallaber, P. Fay, and R. Bambara. 1992. Parameters that influence processive synthesis and site-specific termination by human immunodeficiency virus reverse transcriptase on RNA and DNA templates. *Biochim. Biophys. Acta* **1131**:270–280.
4. Dougherty, J., R. Wisniewski, S. Yang, B. Rhode, and H. Temin. 1989. New retrovirus helper cells with almost no nucleotide sequence homology to retrovirus vectors. *J. Virol.* **63**:3209–3212.
5. Dougherty, J. P., and H. M. Temin. 1986. High mutation rate of a spleen necrosis virus-based retrovirus vector. *Mol. Cell. Biol.* **6**:4387–4395.
6. Fu, T.-B., and J. Taylor. 1992. When retroviral reverse transcriptases reach the end of their RNA templates. *J. Virol.* **66**:4271–4278.
7. Goodrich, D., and P. Duesberg. 1988. Retroviral transduction of oncogenic sequences involves viral DNA instead of RNA. *Proc. Natl. Acad. Sci. USA* **85**:3733–3737.
8. Goodrich, D., and P. Duesberg. 1990. Retroviral recombination during reverse transcription. *Proc. Natl. Acad. Sci. USA* **87**:2052–2056.
9. Haseltine, W., J. Coffin, and T. Hageman. 1978. Structure of products of the Moloney murine leukemia endogenous DNA polymerase reaction. *J. Virol.* **30**:375–383.
10. Higuchi, R. 1989. Simple and rapid preparation of samples for PCR, p. 31–38. *In* H. Erlich (ed.), *PCR technology: principles and applications for DNA amplification*. Stockton Press, New York.
11. Hu, W.-S., and H. Temin. 1992. Effect of gamma radiation on retroviral recombination. *J. Virol.* **66**:4457–4463.
12. Hu, W.-S., and H. M. Temin. 1990. Genetic consequences of packaging two RNA genomes in one retroviral particle: pseudodiploidy and high rate of genetic recombination. *Proc. Natl. Acad. Sci. USA* **87**:1556–1560.
13. Hu, W.-S., and H. M. Temin. 1990. Retroviral recombination and reverse transcription. *Science* **250**:1227–1233.
14. Kawai, S., and H. Nishizawa. 1984. New procedure for DNA transfection with polycation and dimethyl sulfoxide. *Mol. Cell. Biol.* **4**:1172–1174.
15. Lobel, L. I., and S. P. Goff. 1985. Reverse transcription of retroviral genomes: mutations in the terminal repeat sequences. *J. Virol.* **53**:447–455.
16. Luo, G., L. Sharmeen, and J. Taylor. 1990. Specificities involved in the initiation of retroviral plus-strand DNA. *J. Virol.* **64**:592–597.
17. Luo, G., and J. Taylor. 1990. Template switching by reverse transcriptase during DNA synthesis. *J. Virol.* **64**:4321–4328.
18. Panganiban, A. T., and D. Fiore. 1988. Ordered interstrand and intrastrand DNA transfer during reverse transcription. *Science* **241**:1064–1069.
19. Pathak, V., and H. Temin. 1990. Broad spectrum of in vivo forward mutations, hypermutations, and mutational hotspots in a retroviral shuttle vector after a single replication cycle: substitutions, frameshifts, and hypermutations. *Proc. Natl. Acad. Sci. USA* **87**:6019–6023.
20. Pathak, V., and H. Temin. 1990. Broad spectrum of in vivo forward mutations, hypermutations, and mutational hotspots in a retroviral shuttle vector after a single replication cycle: deletions and deletions with insertions. *Proc. Natl. Acad. Sci. USA* **87**:6024–6028.
21. Pathak, V., and H. Temin. 1992. 5-Azacytidine and RNA secondary structure increase the retrovirus mutation rate. *J. Virol.* **66**:3093–3100.
22. Peliska, J., and S. Benkovic. 1992. Mechanism of DNA strand transfer reactions catalyzed by HIV-1 reverse transcriptase. *Science* **258**:1112–1118.
23. Pulsinelli, G., and H. Temin. 1991. Characterization of large deletions occurring during a single round of retrovirus vector replication: novel deletion mechanism involving errors in strand transfer. *J. Virol.* **65**:4786–4797.
24. Swain, A., and J. M. Coffin. 1992. Mechanism of transduction by retroviruses. *Science* **255**:841–845.
25. Tanese, N., A. Telesnitsky, and S. P. Goff. 1991. Abortive reverse transcription by mutants of Moloney murine leukemia virus deficient in the reverse transcriptase-associated RNase H function. *J. Virol.* **65**:4387–4397.
26. Taylor, J. M., and R. Illmensee. 1975. Site on the RNA of an avian sarcoma virus at which primer is bound. *J. Virol.* **16**:553–558.
27. Van Bevrn, C., J. Coffin, and S. Hughes. 1985. Appendix B. Nucleotide sequences complemented with functional and structural analysis, p. 567–1148. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), *RNA tumor viruses (supplements and appendixes)*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.