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Utilization of Nonhomologous Minus-Strand DNA Transfer To Generate Recombinant Retroviruses

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During reverse transcription, minus-strand DNA transfer connects the sequences located at the two ends of the viral RNA to generate a long terminal repeat. It is thought that the homology in the repeat (R) regions located at the two ends of the viral RNA sequences facilitate minus-strand DNA transfer. In this report, the effects of diminished R-region homology on DNA synthesis and virus titer were examined. A retrovirus vector, PY31, was constructed to contain the 5′ and 3′ *cis*-acting elements from Moloney murine sarcoma virus and spleen necrosis virus. These two viruses are genetically distinct, and the two R regions contain little homology. In one round of replication, the PY31 titer was approximately 3,000-fold lower than that of a control vector with highly homologous R regions. The molecular characteristics of the junctions of minus-strand DNA transfer were analyzed in both unintegrated DNA and integrated proviruses. Short stretches of homology were found at the transfer junctions and were likely to be used to facilitate minus-strand DNA transfer. Both minus-strand strong-stop DNA and weak-stop DNA were observed to mediate strand transfer. The ability of PY31 to complete reverse transcription indicates that minus-strand DNA transfer can be used to join sequences from two different viruses to form recombinant viruses. These results suggest the provocative possibility that genetically distinct viruses can interact through this mechanism.

Reverse transcription initiates near the 5′ end of the retrovirus RNA, using a tRNA primer that binds to the primer binding site (9, 17, 59, 61, 62). Reverse transcriptase (RT) copies the unique 5′ sequence (U5) and the 5′ repeated (R) region of the RNA to form minus-strand strong-stop DNA. This DNA is then transferred to the 3′ end of the viral RNA genome in a step known as minus-strand strong-stop DNA transfer (17, 21, 23, 40).

It is thought that RT-associated RNase H degrades the RNA of the RNA-DNA hybrid, to expose the R region in the minus-strand strong-stop DNA (6, 58). This allows the newly synthesized DNA to base pair with the R region in the 3′ end of the RNA and facilitate the DNA transfer. This view is supported by the fact that strand transfer events were reduced during DNA synthesis by RNase-H deficient RTs in both in vivo and in vitro systems (3, 12, 30, 44, 57).

The lengths of the R regions vary between different retroviruses, ranging from 249 nucleotides (nt) in human T-cell leukemia virus type 2 to 12 nt in mouse mammary tumor virus (5, 9, 26). This finding suggests that a relatively short region of homology (12 nt) is sufficient for the minus-strand DNA transfer and that only a portion of the longer R regions may be necessary for the transfer. Using markers located in the R regions, premature minus-strand DNA transfer events were observed in murine leukemia virus (MLV), spleen necrosis virus (SNV), and human immunodeficiency virus 1 (HIV-1) (26, 29, 47). The lengths of the R regions in MLV, SNV, and HIV-1 are 69, 82, and 97 nt, respectively. It was found that the minus-strand DNA transfer can occur with DNA containing only partially synthesized R regions (weak-stop DNA) (26, 29, 47). However, minus-strand strong-stop DNA containing complete R regions was predominantly used (47). The term minus-strand DNA transfer is used in this report to include both strong-stop DNA and weak-stop DNA transfer events.

Genetic recombination has been observed to occur between related viruses as well as between viruses and endogenous elements such as VL30 (28, 46, 54). In contrast to genetic recombination, minus-strand DNA transfer is an essential step during reverse transcription. Therefore, minus-strand DNA transfer between genetically distinct retroviruses may potentially be another mechanism for increasing viral variation.

We now report experiments to determine whether minus-strand DNA transfer can occur between different R regions and whether the DNA products generated from such transfers can integrate and express their genomes. Two retroviral vectors, pPY31 and pAR2, were constructed to contain different 5′ and 3′ long terminal repeat (LTR) sequences (Fig. 1A). Their viral titers were compared in one round of retroviral replication, and the characteristics of the minus-strand DNA transfers were studied. In this report, pPY31 and pAR2 refer to plasmids, while PY31 and AR2 refer to the viruses derived from these plasmids.

Retrovirus vector pPY31 was constructed to contain the 5′ LTR from Moloney murine sarcoma virus (Mo-MSV) and the 3′ LTR from SNV. Mo-MSV is derived from Moloney MLV (Mo-MLV); these two viruses contain very similar *cis*-acting sequences (27, 60). SNV is an avian type C virus that belongs to a different subgenus of the MLV-related viruses. Thus, SNV is genetically distinct from and yet distantly related to Mo-MLV and Mo-MSV (8). The R regions of SNV and Mo-MSV contains no significant relationship outside of the poly(A) signal found in all retroviruses (Fig. 1C).

Retrovirus vector pAR2 was derived from pLN series of vectors, which contain the 5′ LTR from Mo-MSV and the 3′ LTR from Mo-MLV (1, 35). The R regions of Mo-MSV and Mo-MLV contain 87% homology (Fig. 1D).

The structures of pPY31 and pAR2 are illustrated in Fig.

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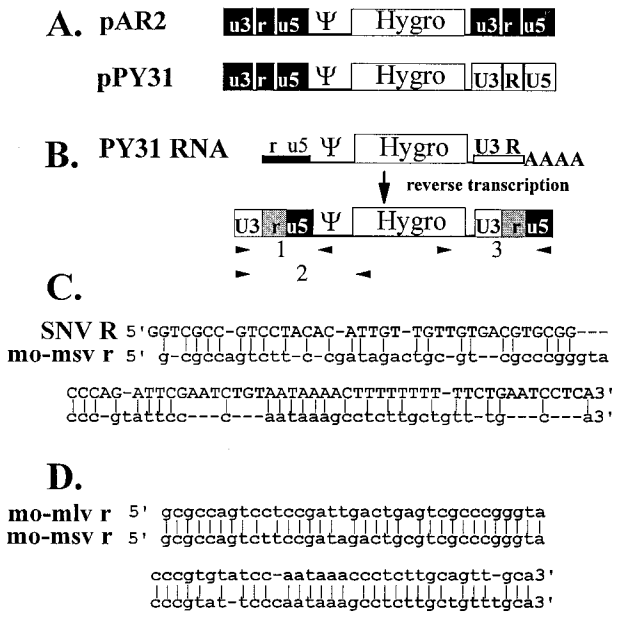

FIG. 1. MLV-based retrovirus vectors used to study minus-strand DNA transfer. (A) Structures of pAR2 and pPY31. Black boxes, Mo-MLV/Mo-MSV LTR sequences including U3, R, and U5; white boxes, SNV LTR sequences; Ψ, Mo-MSV/Mo-MLV packaging signal; Hygro, hygromycin phosphotransferase B gene. All viral *cis*-acting sequences of pAR2 are derived from either Mo-MLV or Mo-MSV. The viral sequences of pPY31 are derived from Mo-MSV (all sequences upstream of *hygro*) and SNV (all sequences downstream of *hygro*). Standard molecular cloning techniques were used to construct these vectors (51). (B) Reverse transcription of PY31 RNA into viral DNA. AAAA, poly(A) tract; shaded boxes, sequences generated after minus-strand DNA transfer which may originate from either Mo-MSV or SNV. Black arrows below the proviral structure indicate the locations and the senses of the primers used in PCR. Primer sets 1 and 2 were used to amplify the 5′ end of the proviral genome, whereas primer set 3 was used to amplify the 3′ end of the unintegrated DNA. (C) Sequence comparison of the 3′ (SNV) and 5′ (Mo-MSV) R regions of PY31 generated by the computer program DNASIS (Hitachi). The SNV R region is 82 nt, and the Mo-MSV R region is 69 nt. Short vertical lines connecting the bases indicate identical nucleotides. Mo-MSV sequences are in lowercase; SNV sequences are in uppercase. (D) Sequence comparison of the 3′ (Mo-MLV) and 5′ (Mo-MSV) R regions of AR2. The Mo-MLV R region is 68 nt.

1A. Both vectors contain an Mo-MSV 5′ LTR, packaging signal, and a hygromycin phosphotransferase B gene (*hygro*) (19). However, these two vectors contain different sequences at the 3′ end of the viral genome. Vector pAR2 contains the Mo-MLV polypurine tract (PPT) and 3′ LTR, whereas pPY31 contains the SNV PPT and 3′ LTR. The RNA structure of PY31 is shown in Fig. 1B.

An Mo-MSV U3 promoter drives the transcription of both vector plasmids. AR2 RNA and PY31 RNA contain the same packaging signal. In both viral vector RNAs, reverse transcription initiates from an Mo-MSV primer binding site and copies the Mo-MSV U5 and R sequences to form minus-strand strong-stop DNA. AR2 RNA contains two highly homologous R regions to facilitate the minus-strand DNA transfer events. In contrast, PY31 RNA contains little homology in the R regions, which may inhibit the minus-strand DNA transfer events. Alternatively, minus-strand DNA transfer of PY31 may occur with reduced regions of complementarity between the newly synthesized DNA and the viral RNA. The nature and efficiency of the minus-strand DNA transfer were examined in the experiments described below.

In these transfer events, the 5′ end of the RNA genome containing the Mo-MSV R and U5 regions is referred to as a donor template. The 3′ ends of the viral RNA genome containing the SNV U3 and R regions (PY31) or the Mo-MLV U3 and R regions (AR2) are referred to as the acceptor template.

Experimental protocol to study minus-strand DNA transfer. The two viral constructs pPY31 and pAR2 were transfected separately into PG13 helper cells by the dimethyl sulfoxide-Polybrene method (25). PG13 is a murine helper cell line that expresses MLV Gag-Pol proteins and Env proteins of gibbon ape leukemia virus (33). The transfected cells were selected for hygromycin resistance, and the resulting colonies were pooled. The average pool size was 200 colonies. The pooled cells were trypsinized, and 10^6 cells expressing either PY31 or AR2 were plated on 60-mm-diameter dishes. After 24 to 48 h, viruses were harvested from PY31- and AR2-producing PG13 cells and used to infect D17 target cells. D17 is a dog osteosarcoma cell line permissive to infection by both SNV and MLV (48). The infected D17 target cells were selected with hygromycin. Virus titers of PY31 and AR2 were determined by counting the number of hygromycin-resistant colonies. Hygromycin-resistant D17 colonies containing PY31 proviruses were isolated, and genomic DNA lysates were generated from these clones. Proviral DNA structures were amplified by PCR (50); the nature of the minus-strand DNA transfer in these clones was analyzed by DNA sequencing (52).

Murine cells do not contain receptors for gibbon ape leukemia virus; thus, PG13 cells cannot be reinfected by the virus that they produce. This eliminates superinfection during the propagation of helper cells and possible amplification of the observed differences. Therefore, this system measures events occurring in a single cycle of retroviral replication.

Cellular RNA and viral RNA were isolated from the transfected PG13 cell pools. RNA hybridization analysis demonstrated that AR2- and PY31-containing PG13 cells had similar levels of vector RNA expression and produced equivalent amounts of cell-free viral RNA (data not shown). This allowed us to directly compare the efficiencies of minus-strand DNA transfer and the replication of AR2 and PY31.

PY31 can replicate with nonhomologous LTRs. Five separate experiments were performed, and the viral titers generated by PY31 and AR2 were compared (Table 1). The titers of PY31 ranged from 0.3×10^2 to 3.6×10^2 CFU/ml; the average was 1.7×10^2 CFU/ml. The titers of AR2 ranged from 1×10^5 to 5.7×10^5 CFU/ml; the average was 4×10^5 CFU/ml. The AR2 titer/PY31 titer ratios ranged from 861 to 18,667 in different experiment sets. Because the differences in viral titers between AR2 and PY31 were in the range of 10^3 to 10^4, the log of the ratio of the two virus titers was used to measure the differences in viral titers to avoid excessive influence of individual measurements. On average, replication of PY31 was reduced 3,162-fold relative to that of AR2 ($P = 0.00016$). These results indicate that AR2 can be propagated in PG13 cells efficiently. These viral titers are comparable to those of

TABLE 1. Comparison of viral titers obtained with PY31 and AR2[a]

Expt.	Titer (CFU/ml)	
	PY31 (10^2)	AR2 (10^5)
1	3.6	3.1
2	0.6	1.0
3	0.3	5.6
4	0.7	5.7
5	3.4	5.0

[a] On average, PY31 replication was 3,162-fold less efficient than AR2 replication.

other MLV-based vectors with two Mo-MLV-derived R regions, which agrees with previously published observations (35). These data also demonstrate that PY31 can complete reverse transcription, integrate into the host chromosome, and express *hygro*. However, PY31 titers were significantly lower than the AR2 titers in all experiments, indicating that PY31 replicated with reduced efficiency.

Analysis of minus-strand DNA transfer junctions. The nature of the minus-strand DNA transfer in PY31 was studied by isolating target cell clones containing PY31 proviruses and analyzing the junctions of minus-strand DNA transfer. The multiplicities of infection used to generate these PY31-containing colonies were low (approximately 0.001). Thus, each colony should contain a single provirus. Genomic DNA lysates from each colony were isolated, and the 5′ LTRs of the integrated proviruses were amplified by PCR (primer sets 1 and 2 [Fig. 1B]). To determine the transfer junctions, the amplified PCR products were either sequenced directly or sequenced after cloning of the PCR product into pUC19.

Results obtained from analysis of 13 individual proviruses are shown in Fig. 2. To ensure that the examined proviruses were generated through independent events, hygromycin-resistant colonies were isolated from five independent infection experiments and from separate cell culture dishes. Among these 13 proviruses, three different minus-strand DNA transfer junctions (MST#1, MST#2, and MST#3) were identified. The predominant minus-strand DNA transfer junction observed was MST#1 (10 of 13 proviruses) (Fig. 2A). In proviruses containing the MST#1 junction, the viral LTRs contained the complete Mo-MSV R and U5 sequences and most of the SNV U3 sequence except for a 147-bp deletion at the 3′ end of SNV U3. Sequence comparison indicates that a 6-nt overlap, GCG CCA, bridges the SNV U3 and Mo-MSV R sequences (Fig. 2A). This 6-nt sequence is present at the 5′ end of the Mo-MSV R sequence as well as in SNV U3 at 147 nt from its 3′ end (Fig. 2B and C). Judging from the content of the proviral LTR structures, viruses with MST#1 junctions probably generated minus-strand strong-stop DNA and subsequently used the 6-nt complementarity between the Mo-MSV R DNA and SNV U3 RNA to mediate the minus-strand DNA transfer. As a result, all SNV U3 RNA sequences 3′ from this strand transfer junction were lost in the proviral LTR sequence.

Only one provirus with the MST#2 junction was observed; this provirus contained the complete Mo-MSV R region, Mo-MSV U5, and most of SNV U3. However, only 31 bp, rather than 147 bp, of the 3′ end of SNV U3 was deleted from this provirus. Sequence comparison indicated a single nucleotide overlap (G) between the donor template (Mo-MSV R region) and the acceptor template (SNV U3) (Fig. 2A). Thus, this virus probably generated the minus-strand strong-stop DNA and used the 1-nt sequence complementarity to transfer to the SNV U3 region.

MST#3 was observed in 2 of the 13 proviruses examined. The LTRs of proviruses with MST#3 contained a complete Mo-MSV U5, a portion of the Mo-MSV R region, and all of SNV U3 except for a 13-nt deletion at the 3′ end. These LTRs contained only 41 nt from the 3′ end of the Mo-MSV R region; the 28 nt from the 5′ end were deleted. Sequence comparison indicates that the junction sequences between the Mo-MSV R region and SNV U3 contain an overlap of 8 nt (CCGGGTAC) (Fig. 2A). During reverse transcription of the virus, minus-strand DNA transfer probably occurred before completion of strong-stop DNA synthesis, using the 8-nt complementarity between the newly synthesized DNA and SNV U3 RNA. As a result, the 5′ end of the Mo-MSV R region and a portion of SNV U3 were deleted from these proviruses. The presence of these two proviruses indicated that weak-stop DNA was used in a premature minus-strand DNA transfer.

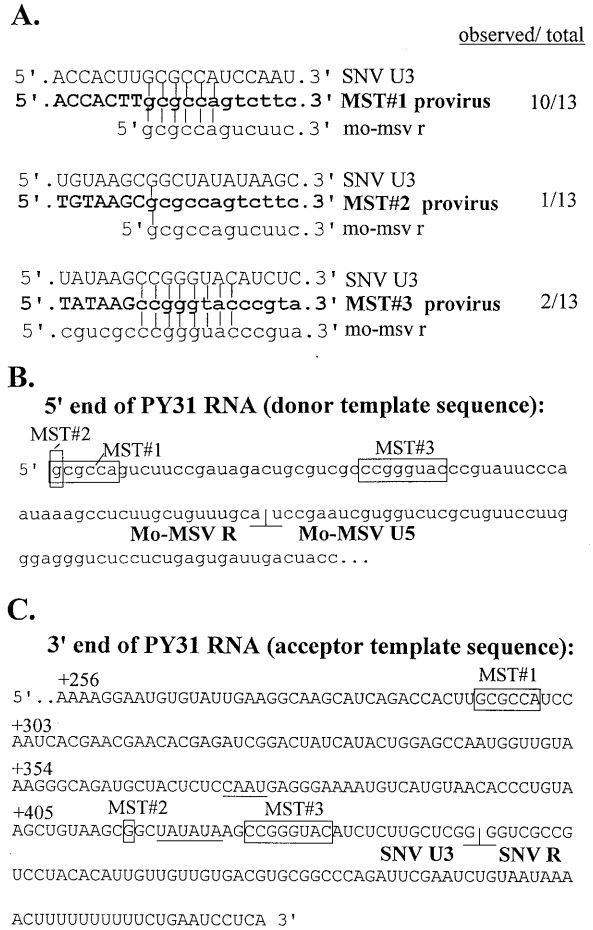

FIG. 2. Sequences of the minus-strand DNA transfer junctions. (A) Three observed minus-strand DNA transfer junctions in PY31. These junctions are designated MST#1, MST#2, and MST#3. In all junctions, acceptor template sequences (SNV U3) are shown on the top (uppercase letters), the observed proviral sequences are shown in the middle (bold-face letters), and the donor template sequences (Mo-MSV R region) are shown on the bottom (lowercase letters). The acceptor templates and donor templates are shown as RNA sequences, whereas the proviruses generated are shown as DNA sequences. Homologies are indicated by vertical lines. The frequencies of the observed events versus the total events are shown on the right. (B) Locations of the minus-strand DNA transfer junctions in the donor templates. (C) Locations of the minus-strand DNA transfer junctions in the acceptor templates. Not all of the SNV U3 is shown; the beginning of the U3 is designated +1. TATA box and CAAT promoter elements are underlined.

Strand transfer substrates and homology at the transfer junctions. Of the 13 proviruses analyzed, 11 (85%) contain the complete Mo-MSV R (MST#1 and MST#2) and 2 (15%) contain only a portion of the Mo-MSV R (MST#3), indicating that minus-strand strong-stop DNA as well as weak-stop DNA were generated and used for the minus-strand DNA transfer events in these viruses. This observation is consistent with previously published studies (26, 29, 47).

In all 13 PY31 proviruses, minus-strand DNA transfer occurred, using short stretches of sequence homology varying from 8 to 1 nt. This finding suggests that in vivo, the transfer is homology driven and that it can be carried out with as little as 1 nt of homology. All three stretches of homology are G/C-rich sequences. Five of 6 nt are G/C in the homology used in

MST# 1, and 6 of 8 nt are G/C in the homology used in MST#3. The 1 nt used in MST#2 is a G.

In all 13 proviruses examined, the acceptor template sequences used for minus-strand DNA transfer were located in the 3′ end of SNV U3 rather than in the SNV R region (Fig. 2C). This observation indicates that the acceptor template used in minus-strand DNA transfer is not limited to the very 3′ end of the viral sequences. We did not observe any proviral structures in which the donor DNA transferred to acceptor sequences located 5′ of the SNV U3. Although it is possible for such transfers to occur, the product of these transfers will lack the U3 promoter, the 5′ *att* sites, PPT, and perhaps a portion of *hygro*. Thus, the products of such transfers are unlikely to complete reverse transcription, integration, and expression of *hygro* and will not be observed in these experiments.

Rescue of PY31 proviruses by replication-competent MLV. Although the PY31 proviruses isolated were selected for the ability to confer hygromycin resistance, all of the PY31 proviruses examined lost a portion of SNV U3. Therefore, it is not clear whether these PY31 proviruses can be efficiently expressed and further transferred to other cells when viral proteins are provided in *trans*. To examine the abilities of these PY31 viruses to be propagated, a PY31-infected cell pool as well as three cell clones containing PY31 proviruses with the MST#1, MST#2, or MST#3 junction were transfected with pAMS (34), a plasmid containing the replication-competent MLV genome. As a positive control, a cell clone containing an AR2 provirus was also transfected with pAMS. Five days after the transfection, viruses were harvested, serially diluted, and used to infect D17 cells. The infected D17 cells were placed on hygromycin selection, and the hygromycin-resistant cells were counted to determine viral titers.

Hygromycin-resistant colonies were observed in D17 cells infected with viruses generated from the AR2-containing cells, PY31 cell pools, and all three different cell clones containing PY31 with the MST#1, MST#2, or MST#3 junction. This finding indicates that PY31 proviruses can be expressed and transferred although portions of the U3 sequence were deleted. Cell clones containing PY31 proviruses with the MST#2 or MST#3 junction, and the cell pool containing PY31, generated hygromycin resistance titers similar to those of the cell clone containing AR2. In contrast, the cell clone containing PY31 with MST#1 generated a titer approximately 100-fold lower than those of the other cells. Because a replication-competent virus was used in this system and multiple cycles of viral replication were involved, these viral titers were not evaluated in a highly quantitative manner. However, the result suggested that PY31 proviruses which contained the largest 147-bp deletion in the U3 promoter may have reduce viral expression and thus propagated less efficiently. In contrast, PY31 viruses with MST#2 or MST#3 junctions were propagated relatively efficiently.

Analysis of minus-strand DNA transfer junctions in unintegrated viral DNA. To examine minus-strand DNA transfer events without selecting for the completion of reverse transcription or integration, unintegrated viral DNAs were analyzed. The experimental protocol is illustrated in Fig. 3A. Viruses were harvested from the pAR2- and pPY31-transfected PG13 cells and used to infect D17 cells. Low-molecular-weight DNAs were isolated from infected D17 cells at 6 h and 12 h postinfection and then analyzed by PCR amplification (20).

The 3′ ends of the unintegrated DNAs that had completed minus-strand DNA transfer were amplified by PCR using a set of primers located in *hygro* and in Mo-MSV U5 (primer set 3 [Fig. 1B]). PCR amplifications were performed, and the resulting products were analyzed by agarose gel electrophoresis and ethidium bromide staining to visualize the DNA (Fig. 3B). When low-molecular-weight DNAs from AR2-infected cells were used as templates for PCR amplification, a discrete DNA product approximately 800 bp in length was observed (Fig. 3B). This is the size expected if strand transfer occurred by using homology between the 5′ and 3′ R regions. Direct DNA sequencing analysis of the PCR product indicated that all se-

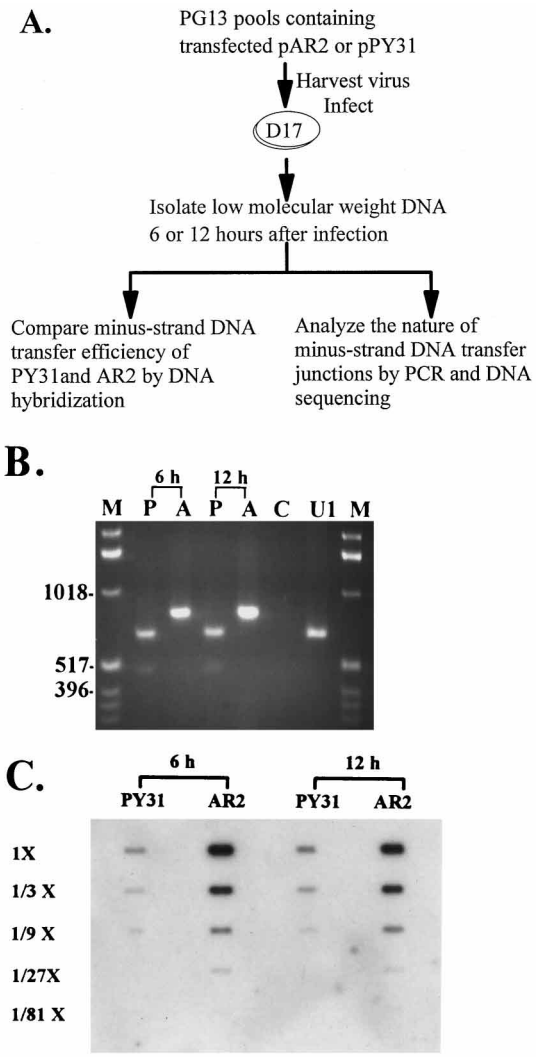

FIG. 3. (A) Protocol to study minus-strand DNA transfer in unintegrated viral DNA. Viruses were harvested from equal numbers of transfected PG13 cells and used to infect D17 cells. Low-molecular-weight DNAs were isolated 6 and 12 h after virus infection. Viral DNAs were amplified by PCR, and the nature of the minus-strand DNA transfers was determined by direct DNA sequencing of the PCR product. The efficiency of minus-strand DNA transfer in PY31 was compared to that of AR2 by DNA hybridization. (B) Analysis of unintegrated viral DNA amplified by PCR using electrophoresis. Lanes marked 6 h and 12 h represent DNAs harvested 6 and 12 h postinfection and used as templates for PCR amplification. DNA samples containing PY31 (lanes P) or AR2 (lanes A) unintegrated viral DNA were used as templates for amplification. Lane C, negative control for the amplification reaction (no template DNA was added to the reaction). Lane U1, DNA lysate from cell clone U1, used as template for the amplification reaction. U1 contains a PY31 provirus with MST#1. Lane M, molecular weight markers with sizes (in base pairs) indicated on the left. (C) Slot blot hybridization of unintegrated viral DNA isolated from PY31- and AR2-infected D17 cells. Slot blots were hybridized with a probe generated by labeling a 800-bp DNA fragment containing the 3′ end of *hygro*. DNA dilutions are shown on the left.

quences from the R region were derived from Mo-MSV, and the junction between the R and U3 regions is as expected. This result indicates that the majority of the minus-strand DNA transfers in AR2 were precise and homology driven; furthermore, most of the transfers utilized minus-strand strong-stop DNA. In contrast, when low-molecular-weight DNAs from PY31-infected cells were used as templates for PCR amplification, two DNA products approximately 690 and 500 bp in length were observed (Fig. 3B). The size of the 690-bp DNA fragment corresponded to minus-strand DNA transfer using the MST#1 junction (Fig. 2). This 690-bp DNA fragment also migrated at the same position as the PCR product from cell clone U1 (Fig. 3B, lanes P and U1), which contains a PY31 provirus with the MST#1 junction. Direct DNA sequencing of the PCR product confirmed that the 690-bp fragment amplified from unintegrated DNA contained an LTR with the MST#1 junction. The strong intensity of the 690-bp band indicates that DNA with the MST#1 junction is the most abundant product of minus-strand DNA transfer in PY31 unintegrated viral DNA. This inference is consistent with the PY31 provirus analysis in which 10 of 13 proviruses contained the MST#1 minus-strand DNA transfer junction. Together, these data indicate that the MST#1 junction is predominantly used in minus-strand DNA transfer during the replication of PY31. The 500-bp band is much weaker in intensity; its presence indicates that other minor strand transfer events also occurred. The exact nature of the transfer junction was not determined. The absence of PCR-amplified DNA that corresponded to the sizes of MST#2 and MST#3 (804 and 794 bp, respectively) provided further evidence that strand transfers using these two junctions were low-frequency events.

The efficiency of minus-strand DNA transfer using diminished R-region homology. The relative amounts of unintegrated viral DNA generated by PY31 or AR2 after infection of target D17 cells were compared to estimate the efficiency of minus-strand DNA transfer using nonhomologous R regions. Viruses were harvested from the same number of AR2- or PY31-containing PG13 cells and used to infect target D17 cells (Fig. 3A). Cellular vector RNA expression and cell-free viral RNA were produced at similar levels in PY31- and AR2-containing PG13 cells (data not shown). Therefore, the efficiencies of minus-strand DNA transfer of PY31 and AR2 can be directly compared. Low-molecular-weight DNAs were isolated from the same number of cells infected with either PY31 or AR2. Serial dilutions of the DNA were performed, and the samples were loaded onto a slot blot. These slot blots were hybridized with a probe generated from an 800-bp DNA fragment containing the 3′ portion of *hygro* by random priming reactions (14). As a result, only the viral DNA that completed minus-strand DNA transfer and continued to copy the *hygro* portion of the viral RNA can be detected. An example of the slot blot is shown in Fig. 3C. Two separate sets of experiments were performed to compare the efficiencies of minus-strand DNA transfer in PY31 and AR2. The intensities of the bands in the slot blots were quantified by phosphorimager analysis. The data indicated that the intensities of the signals from PY31 were 3 to 7% of the intensities of the signals from AR2 at the 6-h time point and were 12 to 17% of the intensities of the signals from AR2 at the 12-h time point. This finding indicates that in PY31, the efficiency of minus-strand DNA transfer is at least 3% of that of AR2. The differences observed here reflect the minimum efficiency of minus-strand DNA transfer in PY31. Because the probe contains the 3′ *hygro* sequences, any minus-strand DNA transfer that deleted a portion of the 3′ *hygro* will have a reduced signal. In addition, the probe also hybridizes to both plus-strand and minus-strand DNA. Any decrease in synthesis of either DNA strand will also reduce the signal.

DNA hybridization experiments indicate that the estimated minus-strand DNA transfer efficiency is much higher than the observed PY31 titer (Fig. 3C; Table 1), which suggests that some other factor(s) may also play a role in the reduction of the PY31 titer. All of the PY31 proviruses contain deletions of the U3 region, which may weaken the strength of the promoter in these viruses. When hygromycin selection is used to determine the viral titers, some of the integrated proviruses with the weaker promoters may not express *hygro* sufficiently to survive the drug selection. This can lead to a reduction in the observed viral titer. This possibility is supported by the rescue experiment described earlier, which showed that PY31 with the largest deletion in the U3 (MST#1) was propagated with the lowest efficiency. It is also possible that PY31 viral DNA is not an efficient substrate for viral integrase. Viral integrase recognizes the sequences at the ends of the viral DNA. Most retroviruses have the AATG/CATT inverted repeat at the terminal sequences of the viral DNA that is known to be important for integration (9). An additional 3 and 8 bp at the 5′ and 3′ ends of the viral DNA, respectively, are important for efficient integration of MLV (37). Mo-MLV and Mo-MSV have the same terminal nucleotide sequences. Similarly, an additional 8 and 4 bp at the 5′ and 3′ ends of the viral DNA, respectively, may be important for integration of SNV (41). The only apparent conserved sequences between MLV and SNV terminal nucleotides are the minimal 4-bp inverted repeat. After reverse transcription, PY31 viral DNA contains the 5′-terminal sequences from SNV and 3′-terminal sequences from MLV. It has been demonstrated that correct terminal sequences at both ends of the DNA are essential for efficient integration in MLV (36). Thus, PY31 DNA may not be efficiently integrated. Last, it has not been shown that MLV RT can use the SNV PPT with the same efficiency as the MLV PPT for the priming of plus-strand DNA synthesis. Therefore, it is possible that the efficiency of plus-strand DNA synthesis of PY31 is an additional factor that can contribute to the reduction of the viral titers.

Role of short stretches of homology between the donor and acceptor templates during minus-strand DNA transfer. Minus-strand DNA transfer occurred efficiently in AR2, which contains highly homologous R regions. Direct sequencing of the unintegrated DNA confirmed that the minus-strand DNA transfer occurs precisely in AR2. Minus-strand DNA transfer is drastically reduced in PY31, which contains two R regions with little homology. This finding indicates that minus-strand DNA transfer is a homology-driven event and that the length of the R-region homology increases the efficiency of minus-strand DNA transfer.

In our study, 1, 6, and 8 nt overlapped the donor and acceptor templates in the PY31 proviruses examined. Using purified MLV RT and in vitro-synthesized DNA and RNA templates, it was found that strand transfer can be observed with a 20-nt homology, but not 8- or 3-nt homology, between the donor and acceptor templates (30). Similar results were observed in studies using HIV-1 RT (44). The major differences between our results and those from in vitro studies may reside in the systems used. In in vitro systems, purified RT, templates, and primers are used, with the possible inclusion of other purified viral proteins such as NC (2, 4, 12, 15, 22, 30, 38, 44, 45, 49). In contrast, the microenvironment where reverse transcription is carried out in vivo involves multiple viral proteins and physiological conditions. In addition, less frequent strand transfer events can be selected in vivo to increase the sensitivity of the assay.

Although this is the first documentation of minus-strand DNA transfer using short stretches of homology in vivo, mechanistically similar events have been observed to occur elsewhere in the viral genome. Short stretches of homology have been observed to mediate plus-strand DNA transfer (4 nt) (13), deletion (31, 32, 42, 43), nonhomologous recombination (65), and transduction of nonviral sequences (56). The rate of deletion is 2×10^{-6}/nt/cycle in SNV (43), whereas the rate of nonhomologous recombination is 5×10^{-8}/nt/cycle in MLV (65). The efficiency of the PY31 minus-strand DNA transfer is much higher than the efficiency of the other observed events. The efficiency of PY31 minus-strand DNA transfer is at least 3 to 7% of the efficiency of transfer generated by a vector with highly homologous R regions. A possible explanation for the difference in frequencies may be the structure of the RNA genome. Deletion and nonhomologous recombination may not require breaks in the genome (24, 64); in minus-strand strong-stop DNA transfer, RT has to switch to a different location of the RNA genome because it reaches the end of the template. This is conceptually similar to the forced copy-choice model of recombination (7), in which the breaks in the viral RNA force RT to switch templates in order to complete DNA synthesis.

The 3′ end of minus-strand DNA can mediate strand transfer. One model of minus-strand DNA transfer (44) suggests that the 3′ end of the minus-strand DNA remains annealed to a fragment of RNA that escapes RNase H degradation (6, 11, 16, 18, 39, 53, 58). An RNA:DNA:RNA three-strand intermediate is formed, then the undegraded RNA is displaced with the new template, and DNA synthesis is reinitiated (44). An implication of the model is that the internal region of the newly synthesized DNA, not the terminal sequence, is used to initiate the base pairing to the acceptor template.

In this report, short stretches of homology were observed to bridge the donor and acceptor templates. The regions flanking the transfer junctions in both the donor and acceptor templates were analyzed. No additional sequence homologies were identified, which indicates that the terminal sequences of the newly synthesized DNA, rather than the internal region, are used to base pair with the acceptor template. If the 3′ ends of the newly synthesized DNAs are used to search for homology, then the undegraded RNA is probably no longer annealed to the 3′ end of the DNA to mask terminal sequences. Two mechanisms can release the RNA without the involvement of the acceptor template: the proposed helicase activity associated with the RT (10) and kinetic dissociation of the short, undegraded RNA. Alternatively, the RNA may be dissociated from the DNA by competition not from the acceptor RNA template but from the newly synthesized DNA itself. In the newly synthesized minus-strand DNA containing the Mo-MSV R region, a stem structure can be formed by pairing 9 of 10 nt of the 3′-terminal sequences with 9 of 10 nt of the internal sequences, located 17 to 27 nt from the 3′ end of the R region. This stem structure may play a role in dissociating the undegraded RNA. Any of the above-mentioned mechanisms can result in a newly synthesized DNA with a single-stranded terminal region free to pair with complementary regions in the acceptor templates. This finding, however, does not suggest that the internal sequences are not used to enhance the efficiency of transfer in wild-type viruses.

Minus-strand DNA transfer uses both strong-stop DNA and weak-stop DNA. It has been demonstrated that both minus-strand strong-stop DNA transfer and weak-stop DNA transfer can occur in MLV, SNV, and HIV-1 (26, 29, 47). Both types of transfer were also observed in this study. It is not known whether the RT stops randomly in the viral RNA to generate weak-stop DNA with various ends, or whether weak-stop DNA with specific ends are generated more frequently. The two weak-stop DNA transfer events observed in this report were generated from two separate experiments and used the same 8-nt homology. It is likely that the observed weak-stop DNA is generated more frequently than random events. Pausing during DNA synthesis has been postulated to increase the frequency of strand transfer (4). It is possible that a pause site is located near the end of this weak-stop DNA.

Short stretches of homology exist between the SNV and Mo-MSV R region, such as the AATAAA sequences near the central portions of the two R regions (Fig. 1C). However, minus-strand DNA transfer using these sequences was not observed in integrated proviruses or unintegrated DNAs. It is possible that minus-strand DNA synthesis does not stop near the AATAAA sequence to generate weak-stop DNA. Alternatively, the A/T-rich sequence may not be an ideal substrate to facilitate the minus-strand DNA transfer. All three of the transfer junctions observed were G/C rich. Although it is difficult to draw conclusions based on only three transfer junctions, it is possible that the base composition of the homology, as well as the length of the homology, plays a role in the efficiency of minus-strand DNA transfer.

Short homology not located in the R region can serve as acceptor templates for minus-strand DNA transfer. In all PY31 proviruses examined, the homologies in the acceptor templates were located in the U3 region rather than the R region, indicating that minus-strand DNA can transfer to regions other than the R region in the acceptor templates. In a wild-type virus, although the R region marks the end of the viral sequences, a poly(A) tail is located at the 3′ end of the downstream R region. Thus, the minus-strand DNA transfer does not occur between the two ends of the viral RNA. It has been demonstrated that a Rous-associated virus-1 (RAV-1) mutant that allows readthrough transcription can replicate efficiently (55). This finding indicates that the minus-strand DNA transfer of RAV-1 can occur despite the additional sequences downstream from the 3′ R region. Our data together with those from the RAV-1 study indicate that homology, rather than location of the sequences, dictates the transfer.

Whether strand transfer events lead to mutations has been a debatable point in the literature (45, 63, 66). In two in vitro systems, mutations near the transfer junctions have been observed (45, 63). However, no mutations near the strand transfer junctions were observed in an in vivo study on nonhomologous recombination (66). In this study, the transfer junctions and the regions flanking the junctions were sequenced in proviruses and unintegrated DNA. No mutations flanking the transfer junctions were observed.

Potential genetic interactions between different retroviruses during minus-strand DNA transfer. In this report, we demonstrated that a retrovirus vector containing *cis*-acting sequences from two different retroviruses can replicate. Despite the lack of homology in the R regions, minus-strand DNA transfer occurred. Furthermore, the resulting viral DNAs were able to integrate with only the AATG/CATT terminal inverted repeat sequences. Mobilization experiments demonstrated that these hybrid viruses can be further propagated in the presence of helper viruses. This finding suggests the possibility that once copackaged, genetically distinct viruses can interact and use minus-strand DNA transfer as a mechanism to generate hybrid viruses. The hybrid viruses may contain different combinations of regulatory sequences in the LTR derived from the parental viruses. This potential for genetic interaction may have important implications for retrovirus replication, pathogenesis, and evolution.

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