

Requirements for Strand Transfer between Internal Regions of Heteropolymer Templates by Human Immunodeficiency Virus Reverse Transcriptase

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We have examined the ability of the reverse transcriptase (RT) from human immunodeficiency virus (HIV) to carry out strand transfer synthesis (i.e., switching of the primer to a new template) from internal regions of natural-sequence RNA. A 142-nucleotide RNA template (donor) primed with a specific 20-nucleotide DNA oligonucleotide was used to initiate synthesis. DNA oligonucleotides with homology to internal regions of the donor were used as acceptors. In this system, HIV RT produced strand transfer products. An HIV RT having RNase H depleted to 3% of normal (HIV RT_{RD}) catalyzed the transfer reaction inefficiently. An RNase H-minus deletion mutant of murine leukemia virus RT was unable to catalyze strand transfer. HIV RT_{RD}, however, efficiently catalyzed transfer when *Escherichia coli* RNase H was included in the reactions, while the mutant murine leukemia virus RT was not efficiently complemented by the *E. coli* enzyme. Evidently, RNase H activity enhances, or is required for, internal strand transfer. Two acceptors homologous to 27-nucleotide regions of the donor, one offset from the other by 6 nucleotides, were tested. The offset eliminated a sequence homologous to a prevalent DNA synthesis pause site in the donor. Strand transfer to this acceptor was about 25% less efficient, suggesting that RT pausing can enhance strand transfer. When the deoxynucleoside triphosphates in the reactions were reduced from 50 to 0.2 μ M, increasing RT pausing, the efficiency of strand transfer also increased. A model for RT-catalyzed strand transfer consistent with our results is presented.

Strand transfer (also referred to as template switching or strand jumping), an event whereby a DNA primer synthesized over a particular region of a template is translocated to another region on the same or a different template, occurs during retrovirus replication (4, 10, 26-28). The single-stranded RNA viral genome is primed near its 5' end by a cellular tRNA (19, 25, 31), from which synthesis of the first (minus)-strand DNA is initiated by the viral reverse transcriptase (RT). Primer extension to the 5' terminus of the RNA template produces the minus-strand strong-stop DNA. The 3'-end region of this DNA is transferred to a complementary segment of template at the 3' end of the RNA genome, allowing subsequent reverse transcription of the majority of the genomic RNA. Degradation of the viral RNA by the RT RNase H is carried out on the RNA-DNA hybrid product. Similarly, a second (plus)-strand strong-stop DNA is initiated from a unique, purine-rich segment of the viral RNA left from RNase H action near the 5' end of the minus-strand DNA (2, 9, 15, 17, 20, 23, 24). The 3' end of the plus-strand strong-stop DNA then transfers to a complementary region at the 3' end of the minus-strand DNA for continued elongation.

Since infectious particles each contain two copies of the viral genome, it is reasonable to expect that the transfers could occur either intra- or intermolecularly. Experiments in which cells were infected with heterodimeric retroviral particles (containing two nonhomologous RNA molecules) suggested that transfer events involving the minus-strand strong-stop DNA occur intermolecularly, while those involving the plus-strand strong-stop DNA are intramolecular (18). Results obtained by others confirm the intramolecular

nature of transfers with plus-strand DNA but suggest that those involving minus-strand DNA can occur either intra- or intermolecularly (12).

Strand transfers involving either the plus- or minus-strand strong-stop DNA have in common that they occur near the terminal regions of the template from which synthesis of the DNAs is initiated. This type of strand transfer reaction was performed in vitro, with the viral RT as the only required protein (20). The polynucleotide requirement in vitro was that there be a greater than 10-nucleotide sequence homology between the donor and acceptor templates. Strand transfer was more efficient when the donor template was RNA but also occurred with a DNA donor. When the donor template was RNA, RNase H activity substantially increased the efficiency of transfer but was not an absolute requirement.

Strand transfer has also been demonstrated to occur from within internal regions of viral RNA during infection (3, 11, 13, 29). In one set of experiments (11), the investigators generated heterodimeric virus particles with one RNA derived from a genetically altered Harvey sarcoma virus genome and the other from Moloney murine leukemia virus (Mu-LV). The RNAs shared an internal region of homology flanked by nonhomologous regions. The polymerase chain reaction was used to amplify specific recombinant viral RNAs that were generated either in vivo by infection with heterodimeric virus or in vitro from disrupted virus particles. Whereas recombination was detected following infection in vivo or with use of disrupted viral particles in vitro, no recombination was detected in a system using the RNA extracted from the heterodimeric virus and purified RT. This result led the experimenters to suggest that "undefined viral structural proteins together with the proximity of the two

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parental genomes within the viral particle (11) are essential for crossing-over during reverse transcription *in vitro* (11).

In this report, we demonstrate that strand transfer between internal regions of RNA templates occurs *in vitro* in a system containing only relatively (in comparison with viral genomic RNA) short donor and acceptor templates and viral RT. No other viral or host proteins are required. A form of human immunodeficiency virus (HIV) RT nearly depleted of RNase H activity (RT_{RD}) catalyzed strand transfer relatively poorly but could be complemented by *Escherichia coli* RNase H. Sequences that promote pausing of synthesis by the RT in the region of homology between the donor and acceptor templates have an effect on strand transfer. The presence of one particular sequence feature on the template used in these experiments substantially increased the efficiency of strand transfer. Possible explanations for this observation are discussed.

MATERIALS AND METHODS

Materials. Recombinant HIV RT with native primary structure was graciously provided by Genetics Institute (Cambridge, Mass.). This enzyme had a specific activity of approximately 21,000 U/mg [1 U of RT is defined as the amount required to incorporate 1 nmol of dTTP into nucleic acid product in 10 min at 37°C, using oligo(dT)-poly(rA) as the template primer]. HIV RT_{RD} (specific activity of approximately 10,000 U/mg) was produced from a form of HIV RT produced in *E. coli* such that p51 was generated through the action of bacterial proteases. The carboxyl terminus of this enzyme is not unique. This enzyme has a 30-fold-reduced RNase H-to-polymerase ratio (1). Subjecting HIV RT to cycles of freezing in dry ice-ethanol and then thawing it at 30°C for 20 min reduces the RNase H-to-polymerase ratio. Application of at least eight cycles is required to reduce the RNase H-to-polymerase ratio by 30-fold. The properties of HIV RT_{RD} (before freeze-thawing) are as described by Huber et al. (14). Aliquots of HIV RT and RT_{RD} were stored frozen at -70°C, and a fresh aliquot was used for each experiment. RNase H-minus MuLV RT, purified to a specific activity of approximately 103,000 U/mg, was obtained from Bethesda Research Laboratories. *Hind*III and *Eco*RI were also from Bethesda Research Laboratories; *Bst*NI was from New England Biolabs. T4 DNA ligase, T4 polynucleotide kinase, T7 RNA polymerase, *E. coli* DNA polymerase I (Klenow fragment), and *E. coli* RNase H were obtained from United States Biochemical Corp. Bovine pancreatic DNase (RNase free), placental RNase inhibitor, RNase A (DNase free), and ribonucleoside triphosphates (rNTPs) were obtained from Boehringer Mannheim Biochemicals; dNTPs were obtained from Pharmacia. Deoxyoligonucleotide acceptor templates A and B (Fig. 1) and the 20-nucleotide-long DNA primer were synthesized by Genosys Inc. (formerly Genetic Designs), Houston, Tex. All other chemicals were from Sigma Chemical Co. Radiolabeled compounds were from New England Nuclear.

Preparation of pBSM13+(Δ). A purified preparation of pBSM13+ was digested with *Hind*III and then with *Eco*RI. The resulting restriction fragments (51 and 3,153 nucleotides in length) were blunt ended by using the Klenow fragment of *E. coli* DNA polymerase I, and ligation was performed as described previously (21) in a volume of 10 μl at a DNA concentration of 0.1 μg/μl (the low concentration was used to promote circularization of the 3,153-bp cleavage product). The ligated products were transformed into competent *E. coli* XL1-Blue cells as described previously (21). Plasmid DNA was isolated from cells by using a Qiagen plasmid kit

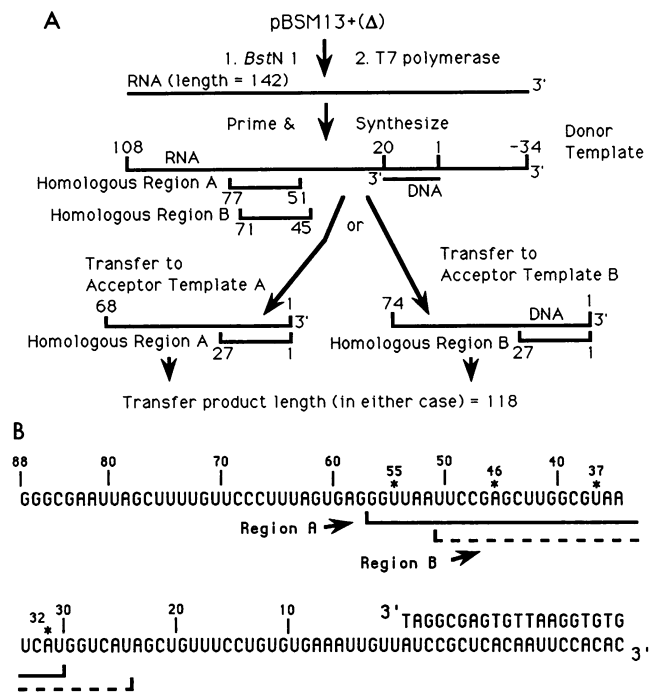


FIG. 1. Construction of the strand transfer template. (A) Steps involved in the production of the RNA donor template from pBSM13+(Δ) (see Materials and Methods for a detailed description). Also shown is the position where the DNA primer hybridizes to the donor template (position 1 denotes the base on the donor template that is complementary to the 5'-terminal base of the DNA primer) and the regions of the donor which are homologous to acceptors A and B. Calculations for the lengths (bases) of strand transfer products resulting from homologous transfer to acceptors A and B are shown. DNA synthesis products resulting from transfer and subsequent extension to the end of the acceptor templates would be 118 bases long, while those resulting from full-length donor template-directed synthesis would be 108 bases long. (B) Nucleotide sequence of the portion of the RNA template on which DNA synthesis was performed. Also shown is the sequence and binding position of the 20-nucleotide 5'-³²P-labeled DNA primer used in this template (positioned over the appropriate region of the RNA donor). The 27-nucleotide-long regions of the donor where acceptor A (filled line) or B (dashed line) has homology are indicated. The nucleotide sequences of acceptors A and B were 3'-(homologous region A)-(AGTC)₁₀-A-5' (total length, 68 nucleotides) and 3'-(homologous region B)-CC-(AGTC)₁₁-A-5' (total length, 74 nucleotides), respectively. The template is numbered starting with the first nucleotide after the 3'-terminal nucleotide of the DNA primer. Positions where prominent DNA synthesis pause sites occur on the template (see Fig. 5) were determined by direct sequencing of the template and are indicated (*).

as described by the manufacturer. Deletion of a 47-bp fragment (as expected after filling in recessed ends; see above) from the multiple cloning site region of the plasmid was verified by restriction enzyme analysis and DNA sequence analysis.

Standard strand transfer reactions and those with *E. coli* RNase H. In the standard reaction, various amounts of RT were preincubated with 2 nM primer-donor template and 200 nM acceptor (A or B) template for 3 min in 20 μl of 50 mM Tris-HCl (pH 8.0)-80 mM KCl-1 mM dithiothreitol-0.1 mM EDTA-2% (vol/vol) glycerol (buffer A). Assays with HIV RT and RT_{RD} were initiated by the addition of MgCl₂ and dNTPs in 5 μl of buffer A to give a final concentration of 6 mM MgCl₂ and 1 μM each dATP, dTTP, dGTP, and dCTP.

RNase H-minus MuLV RT reactions were initiated as described above except that the final $MgCl_2$ concentration was 1 mM and $MnCl_2$ was included at 1 mM. In some reactions, the concentrations of KCl or dNTPs or the amount of acceptor template was varied. In assays that included *E. coli* RNase H, this enzyme was added at the start of the reaction in various amounts. Samples were incubated for 1 h at 37°C unless otherwise indicated, and reactions were terminated by addition of 20 μ l of gel loading buffer (90% formamide, 10 mM EDTA [pH 8.0], 0.1% xylene cyanol, 0.1% bromophenol blue).

Hybridizations. A specific 20-nucleotide-long deoxyoligonucleotide was labeled with ^{32}P at the 5' end, using T4 polynucleotide kinase. The labeled primer was hybridized such that its 3'-terminal nucleotide was 89 nucleotides from the 5' end of the RNA transcript (donor template; Fig. 1). The hybrids were prepared by mixing primer and transcript at a 2:1 (primer/transcript) ratio of 3' termini in 10 mM Tris-HCl (pH 7.5)–1 mM EDTA–50 mM KCl. The mixture was heated to 65°C for 10 min and then slow cooled to room temperature.

Gel electrophoresis. Denaturing 8% polyacrylamide sequencing gels (19:1, acrylamide-bisacrylamide) containing 7 M urea were prepared and subjected to electrophoresis as described previously (21).

Quantitation of RNA donor transcript and acceptor templates. The concentration of the RNA transcript was estimated by titration of a known amount (0.025 pmol) of 5'- ^{32}P -labeled 20-nucleotide-long DNA primer (Fig. 1) with RNA transcript, using the conditions of the standard strand transfer assay as described for RNase H-minus MuLV RT. Reactions were performed and the samples were processed as described for the standard strand transfer assay except that the incubation time was 10 min. The concentration of RNA was estimated from the level of RNA required to extend 50% of the primers, assuming 100% hybridization efficiency. The concentrations of DNA primer and of the DNA acceptor templates were estimated by spectrophotometry.

Runoff transcript. Runoff transcription was done as described in the *Promega Protocols and Applications Guide*. Plasmid pBSM13+(Δ) prepared and purified as described above was cleaved with *Bst*NI. T7 RNA polymerase was used to produce runoff RNA transcripts 142 nucleotides in length. The DNA template was digested with bovine pancreatic RNase-free DNase I (1 U/mg of DNA). The RNA transcript was then extracted with 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1) and ethanol precipitated twice, using 3 volumes of ethanol and a final concentration of 1.25 M ammonium acetate. This RNA was quantified and used directly for hybridizations as described above.

Quantitation of primer extension products. RT extension products were quantitated by scanning of the autoradiogram with a densitometer. Film exposure times were chosen such that the densitometer readings were approximately proportional to the quantity of radioactivity in each band.

RESULTS

Construction of substrates for detection of strand transfer. Retroviral recombination in vivo potentially involves strand transfers from an RNA donor to an RNA acceptor template or a DNA donor to a DNA acceptor template. In the experiments presented in this report, we have modeled RNA-to-RNA transfer, using an RNA donor template and DNA acceptor templates. Although this type of transfer

probably does not occur in vivo, it has been shown that DNA or RNA can act as an efficient acceptor when DNA is used as the donor template in strand transfer reactions in vitro (16). We have used DNA as the acceptor template in all of our experiments because of the relative ease by which long oligomers of DNA versus RNA can be chemically synthesized. Use of these readily made oligomers allowed us to manipulate the size and position of the homologous acceptor sequence. In so doing, we were also able to test the effect of a specific synthesis pause site within the donor template on the efficiency of strand transfer. The system used in these experiments, although not directly representing RNA-to-RNA transfer in vivo, emphasizes events occurring on the donor template that can influence strand transfer. Examples include degradation (by RT RNase H) or displacement of the donor RNA to allow the acceptor to hybridize to the nascently synthesized DNA or pausing by the RT to allow sufficient time for events required for transfer to occur.

The polynucleotide substrates were designed to allow the DNA product of the strand transfer reaction to be longer than the DNA product resulting from maximum extension on the donor template RNA. This approach allows the strand transfer product to be distinguished readily from products generated by premature termination of synthesis on the donor template. The RNA transcript used as the donor template was generated from pBSM13+(Δ), which has a deletion that limits the transcript length to 142 nucleotides and the maximum donor DNA length to 108 nucleotides (Fig. 1). We used chemically synthesized DNA oligonucleotides 68 (acceptor A) and 74 (acceptor B) nucleotides in length, with a homologous sequence located such that products of homologous strand transfer are 118 nucleotides long.

We have previously examined DNA synthesis by HIV RT on a large RNA template, derived from pBSM13+, that overlapped the same sequence present in the donor RNA template used here (7). In doing so, we had mapped the positions of sequences that promote pausing of DNA synthesis on the large template. A particularly effective pause sequence appears within the smaller donor RNA. The acceptor templates used in the current experiments were designed with the intention of examining the effect of this sequence on the efficiency of strand transfer.

Integrity of the RNA transcript used in the transfer reactions. To ascertain that strand transfer that we observed occurred from the internal portion of the RNA donor template, it was important to determine the condition of the RNA template. If the RNA transcript was truncated as a result of breakdown or incomplete synthesis, then strand transfer from the ends of the truncated templates could potentially occur. This would be possible if there were a population of transcripts that terminated on truncated templates within the region of homology between the donor and acceptor templates. Such transcripts could generate strand transfer products by a forced-copy-choice mechanism (4, 30). (In the copy-choice model, it is proposed that when the RT encounters a break in the genomic RNA, it can transfer to the second copy of genomic RNA [present in the dimeric virion] and use it as a template for continued synthesis.)

We examined ^{32}P -labeled RNA transcript prepared identically (except for the inclusion of [α - ^{32}P]CTP in the transcription reactions) to that used in the transfer reactions (Fig. 2). Incubations (for 1 h except for the reaction in lane 1, which was not incubated) were performed by using the conditions for the transfer assay in the absence of enzyme (lanes 1 and 2) or with HIV RT, HIV RT_{RD}, RNase H-minus MuLV RT, or *E. coli* RNase H (lanes 3 to 6, respectively). No truncated transcripts were observed under any condi-

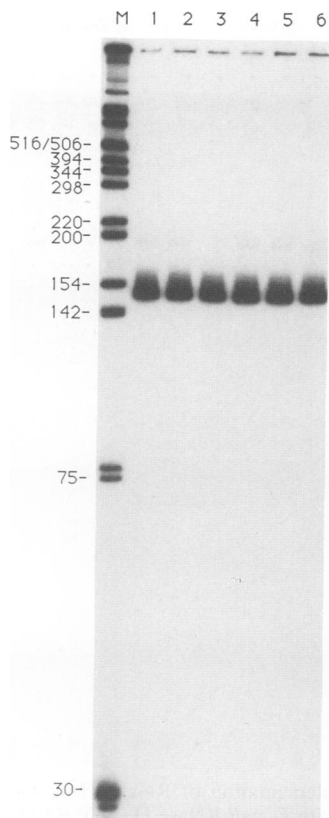


FIG. 2. Test for single-strand-specific nuclease activity in RTs and *E. coli* RNase H. RNA transcript was prepared as described in Materials and Methods except that [α - 32 P]CTP (approximately 2.5 Ci/mmol) was included in the transcription reactions. Transcript (2 nM) was incubated in the absence of enzyme (lanes 1 and 2), with 3 U of HIV RT (lane 3) or HIV RT_{RD} (lane 4), with 4 U of RNase H-minus MuLV RT (lane 5), or with 1 U of *E. coli* RNase H (lane 6). Incubations were at 37°C for 0 h (lane 1) or 1 h (lanes 2 to 6). Reactions were terminated, and samples were processed as described for the strand transfer assay. Reaction conditions were also as described for the strand transfer assay except that dNTPs were not included. Radiolabeled DNA size markers of the indicated lengths (bases) were run in lane M.

tions at the level of exposure shown in Fig. 2. This result indicated that the enzymes used in these experiments did not contain significant levels of contaminating single-strand-specific nuclease activity that could potentially cleave the RNA transcript. Further analysis of the intense transcript band located between the 142- and 154-base DNA markers on Fig. 2 showed that it did not consist of a single RNA species. Rather, there were RNAs of various quantities ranging from approximately 139 to 144 nucleotides (data not shown). Since the region of homology between the donor and acceptor templates began 32 bases upstream of the 142-nucleotide RNA, the presence of low levels of slightly smaller or larger RNAs would not be expected to interfere with the assessment of strand transfer.

An autoradiogram of the gel shown in Fig. 2 that was exposed for five times as long as the autoradiogram shown in Fig. 2 did show some faint truncated RNAs under all the conditions shown in Fig. 2 (data not shown). We calculated, by densitometry scanning of autoradiograms exposed for various time periods, that the truncated transcripts comprise less than 1% of the total RNA transcript. Only those donor

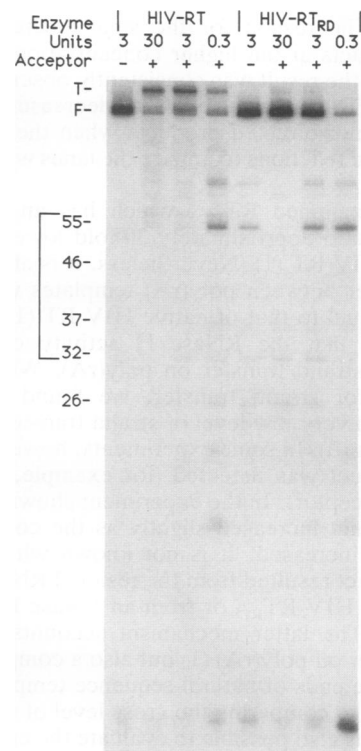


FIG. 3. Internal strand transfer with HIV and RT_{RD}. HIV RT and RT_{RD} at the indicated amounts were incubated in standard strand transfer reactions in the presence or absence of acceptor template A (as indicated) and processed thereafter as described in Materials and Methods. The positions of full-length donor template-directed DNA synthesis products (F) and products produced by strand transfer events (T) are indicated. The approximate region (bases) where the donor and acceptor templates were homologous is bracketed. The positions of prominent DNA synthesis pause sites (see Fig. 5) are also indicated.

transcripts that terminate within the 27-base region of homology between the donor and acceptor could generate specific transfer products derived from transfers at the end of truncated donor. Thus, in these experiments, the level of transfer products potentially generated from truncated donors should have been much less than 1%. In the strand transfer experiment shown in Fig. 3, when 3 U of HIV RT was used in the presence of an acceptor template, approximately 32% of the total primer extension products were transfer products (determined by densitometry scanning). This result indicates that the vast majority of transfer products result from internal transfer events occurring on intact donor templates.

Strand transfer by HIV RT and RT_{RD}. We tested the abilities of HIV RT and RT_{RD} to catalyze strand transfer synthesis (Fig. 3). HIV RT catalyzed strand transfer, as indicated by the appearance, in the presence of acceptor template, of a DNA extension product of a length corresponding to that of the predicted transfer product. HIV RT produced the greatest amount of strand transfer products when 3 U of enzyme was used. Increasing or decreasing the concentration 10-fold resulted in a decrease in the amount of transfer product. At least part of the decrease with a low enzyme concentration probably resulted from a shortening of the average length of extension products. Consequently, there was a decrease in the fraction of products that reached the area of the template where transfer occurs (as indicated

on Fig. 3). The reason for the small decrease in strand transfer products at the higher concentration of enzyme is not clear, but the result was consistently observed in several experiments. There was also a large decrease in the level of full-length donor-directed products when the acceptor was included in the reactions (compare the lanes with 3 U of HIV RT).

We also examined RT_{RD}, which has an RNase H-to-polymerase ratio approximately 30-fold lower than that of the normal HIV RT (1). Nevertheless, it is able to catalyze strand transfer between poly(rA) templates with efficiency essentially equal to that of native HIV RT (1). This finding demonstrates that the RNase H activity of RTs is not required for strand transfer on poly(rA). When we tested HIV RT_{RD} for strand transfer, we found that in most experiments a very low level of strand transfer product was generated (Fig. 3). In some experiments, however, no strand transfer product was detected (for example, Fig. 4, HIV-RT_{RD} plus acceptor). In the experiment shown in Fig. 3, the level of product increased slightly as the concentration of enzyme was increased. It is not known whether this low level of product resulted from the residual RNase H activity possessed by HIV RT_{RD} or from an RNase H-independent mechanism. The latter mechanism accounts for not only strand transfer on poly(rA) (1) but also a component of total transfer at the ends of natural-sequence templates (16).

In addition to comparing the gross level of strand transfer product, it was also possible to evaluate the efficiency of the transfer reaction. We define the efficiency of transfer as the quantity of DNA primers extended to the end of the acceptor template (labeled T [transfer product] on the figures) divided by the sum of the quantity extended to the end of the donor template (labeled F [full-length donor-directed product] on the figures) plus those extended to the end of the acceptor. The greater the T/(T + F) ratio, the greater the efficiency.

The efficiency of transfer for HIV RT was considerably greater than that for HIV RT_{RD} (Fig. 3). For example, when 3 U of each enzyme was used in the transfer assay, the T/(T + F) ratio was approximately 0.55 with HIV RT, whereas it was 0.07 with HIV RT_{RD}. This experiment was repeated several times, and the efficiency of transfer was always severalfold greater with HIV RT than with HIV RT_{RD}.

In reactions in which strand transfer occurred, a single discrete band corresponding to the predicted transfer product (Fig. 1) appeared. This result indicated that the observed primer transfers occurred only in the regions of the templates where the donor and acceptor have identical sequences. Strand transfer in nonhomologous regions would have generated products longer or shorter than the observed product. Products shorter than the maximum-length extension on the donor template did appear and technically could have derived from strand transfer in nonhomologous areas. However, these products were relatively faint and probably resulted from incomplete extension of some of the primers on the acceptor template, since they are most evident when the acceptor is present. We also note that strand transfer at the ends of natural-sequence templates requires substantial homology (16).

Complementation of HIV RT_{RD} for strand transfer with *E. coli* RNase H. If the inability of HIV RT_{RD} to catalyze efficient strand transfer resulted from a deficiency in RNase H, we postulated that strand transfer would be stimulated by addition of an exogenous RNase H. To accomplish this, various levels of *E. coli* RNase H were added to standard strand transfer reactions (Fig. 4). Addition of *E. coli* RNase H to reactions with the normal HIV RT had little effect on the level of strand transfer products made in the reactions,

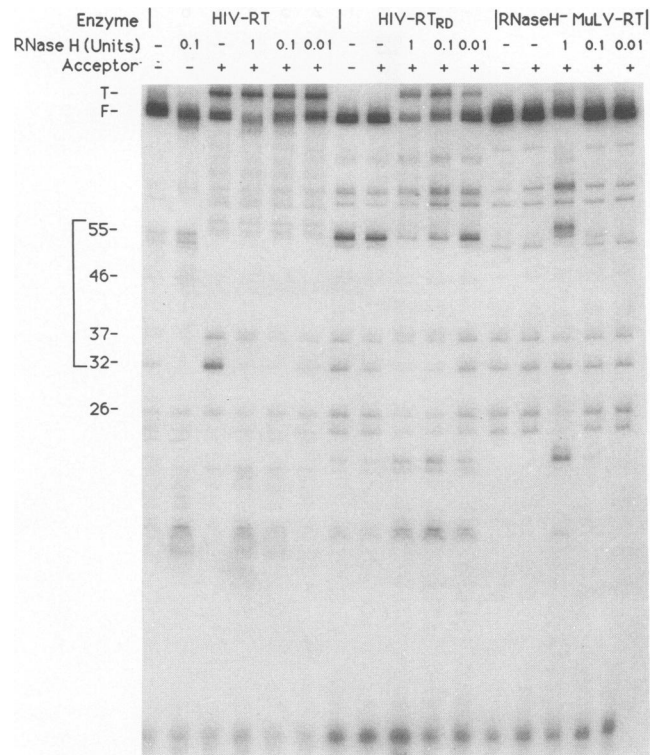


FIG. 4. Complementation of RNase H-deficient forms of HIV and MuLV RT with *E. coli* RNase H. HIV RT (3 U), HIV RT_{RD} (3 U), and RNase H-minus MuLV RT (4 U) were incubated in the presence or absence (as indicated) of acceptor template A (see Fig. 1) and processed thereafter as described in Materials and Methods. *E. coli* RNase H at the indicated amounts was added at the start of the reactions. The positions of full-length donor template-directed DNA synthesis products (F) and products produced by strand transfer events (T) are indicated. The region (bases) where the donor and acceptor templates were homologous is bracketed. The positions of prominent DNA synthesis pause sites (see Fig. 5) are also indicated.

although the efficiency of transfer increased at the highest concentration of RNase H. In contrast, when RNase H was added to reactions with HIV RT_{RD}, the level of strand transfer product was increased to near the quantity made by the normal enzyme. The level of strand transfer product was highest when 0.1 U of *E. coli* RNase H was added. Increasing or decreasing the concentration of RNase H 10-fold from the optimal amount decreased the level of product. The decrease observed at 10-fold-higher concentration of RNase H (1 U) could have been caused, at least in part, by a decrease in the level of total primer extension products. This decrease may have resulted from the destruction of some primer-template complexes (by *E. coli* RNase H) prior to the initiation of DNA synthesis. The results with *E. coli* RNase H suggest that the inefficiency in strand transfer by HIV RT_{RD} was caused by low RNase H activity in the RT and not by some other defect present in this modified enzyme.

Unlike the RNase H-deficient HIV enzyme, RNase H-minus MuLV RT was not effectively complemented by exogenous RNase H. This enzyme produced no detectable transfer product in the presence of acceptor without added RNase H (Fig. 4). At the highest concentration of added *E. coli* RNase H, a very low level of strand transfer product was observed. The very low efficiency of the reaction may be a

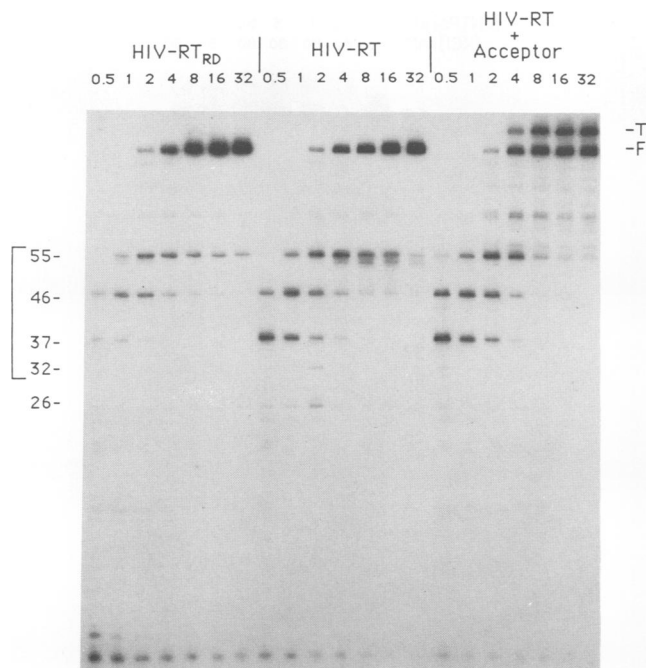


FIG. 5. Time course of primer extension and strand transfer with HIV RT and RT_{RD}. HIV-RT_{RD} (3 U) and HIV RT (3 U) were incubated in standard strand transfer reactions in the presence or absence (as indicated) of acceptor template A (see Fig. 1) as described in Materials and Methods. The reactions were terminated at the time (minutes) indicated above each lane and processed thereafter as described in Materials and Methods. The positions (bases) of full-length donor template-directed DNA synthesis products (F) and products produced by strand transfer events (T) are indicated. The region where the donor and acceptor templates were homologous is bracketed. Also indicated are positions (see Fig. 1) of prominent pause sites within the regions of homology for acceptors A and B.

consequence of the deletion mutation in the RNase H domain of this enzyme, which also affects parameters of DNA synthesis (1). It is possible that this deletion directly influences the strand transfer activity of the enzyme independently of RNase H activity. In contrast to RNase H-minus MuLV RT, parameters of DNA synthesis of HIV RT_{RD} are similar to those of HIV RT (1) (Fig. 5).

Time course of primer extension and strand transfer with HIV RT. To examine the time frame of primer extension and the appearance of full-length donor-directed versus transfer products, we performed reactions for various times with HIV RT_{RD} in the absence, or HIV-RT in the absence and presence, of acceptor template (Fig. 5). The patterns of primer extension products produced by HIV RT and RT_{RD} at a given time point were essentially identical. This result indicates that both enzymes pause during synthesis at the same positions on the template. There were three sites on the template, within the region in which strand transfer could occur, that induced prominent pausing. These sites were located 37, 46, and 55 nucleotides downstream of the primer 3' terminus.

Full-length donor template-directed products appear at about 2 min into the reaction, while strand transfer products appear at about 4 min (HIV RT with acceptor). The levels of both products increase up to approximately 16 min.

Effects of acceptor template concentration and of pausing during synthesis on the level of strand transfer. The presence

of pause sites within the region of homology between the donor and acceptor templates suggested the possibility that pausing promotes strand transfer. To test this possibility, we synthesized a second acceptor template (acceptor B; Fig. 1) in which the region of homology with the donor was shifted 6 nucleotides closer to the primer. That is, the new acceptor had homology to nucleotides 25 to 51 of the donor, while the original (acceptor A) was homologous to nucleotides 31 to 57. The number of homologous nucleotides was held at 27 (Fig. 1). Hence, acceptor B could not hybridize to the end region of a primer that had extended to donor nucleotides 52 to 57 (3'-AATTGG-5'), and so transfer presumably would not occur from that region. Conversely, a new region of potential transfer was available at donor nucleotides 25 to 30 (3'-TACTGG-5'). This change would not be expected to significantly affect the nucleotide composition of the complementary region and consequently the strength of a hybrid formed between the DNA synthesis product of the donor template and the acceptor. One characteristic of acceptor B is that it can no longer act as an acceptor template for DNA synthesis products that terminate at position 55, a prevalent synthesis termination site on the donor template (Fig. 5).

We performed experiments in which the concentrations of acceptors A and B were varied relative to the concentration of the donor template (Fig. 6). Acceptor/donor ratios of 1:1, 10:1, 100:1, and 1,000:1 were used with A or B as the acceptor. The results indicated that the level of strand transfer products and the efficiency of transfer increased as the relative level of the acceptor was increased up to about a 100:1 ratio. Strand transfer products were detected even when the ratio was 1:1.

The level of strand transfer products, and the efficiency of the transfer reaction at a given ratio of acceptor to donor, varied depending on the acceptor used in the reactions. It is apparent from Fig. 6 that the amount of transfer product at all ratios of acceptor to donor was greater when acceptor A was used. Densitometry revealed a 25 to 50% increase in the amount of transfer product when acceptor A rather than acceptor B was used. The ratio of transfer to the sum of full-length donor-directed plus-strand transfer products (efficiency of transfer) was also evaluated at 100:1 and 1,000:1 ratios. There was an approximately 25% decrease in this ratio when transfer assays were performed with acceptor B. In the experiment shown in Fig. 6, the T/(T + F) ratios for acceptors A and B were 0.55 and 0.44, respectively, when assays were performed with a 100:1 acceptor-to-donor ratio. At 1,000:1, the ratios were 0.62 and 0.47, respectively. These experiments were repeated two additional times, with similar results.

Optimal conditions for strand transfer. We tested the effect of varying the KCl and dNTP concentrations on the level of strand transfer. The greatest amount of strand transfer product was observed with 1 μ M dNTPs and 80 mM KCl (Fig. 7). Decreasing the dNTP concentration to 0.2 μ M decreased the level of transfer product about twofold. There was also a decrease in the amount of primers extended into the strand transfer region of the template, an effect that accounts, at least in part, for the lower level of transfer products. Increasing the dNTP concentration to 5 and 50 μ M had no effect on the level of extension products reaching the strand transfer region but lowered both the amount of strand transfer product and the efficiency of the transfer reaction. In the experiment shown in Fig. 7, the T/(T + F) ratio was about 2.5-fold lower with 50 μ M dNTPs than with 0.2 μ M dNTPs. Lowering the KCl concentration to 5 mM did not have a large effect on the level of transfer product or the efficiency of transfer, while both decreased when 150 mM

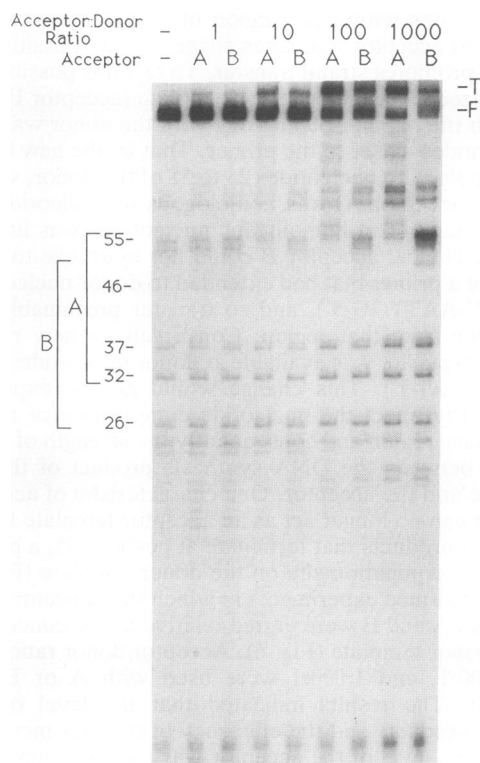


FIG. 6. Strand transfer with various amounts of acceptor templates A and B. HIV RT (3 U) was incubated in a standard strand transfer reaction in the presence of acceptor template A or template B or without acceptor (as indicated) and processed thereafter as described in Materials and Methods. The ratios of acceptor to donor templates in the reactions are indicated above the lanes. The positions (bases) of full-length donor template-directed DNA synthesis products (F) and products produced by strand transfer events (T) are indicated. The region where the donor and acceptor templates (A and B for the corresponding acceptor template) were homologous is bracketed. The positions of prominent DNA synthesis pause sites (see Fig. 5) are also indicated.

KCl was used. The amount of primer extended into the transfer region was also decreased when 150 mM KCl was used.

DISCUSSION

We have investigated the ability of HIV RT to catalyze strand transfer from an internal region of a heteropolymer RNA template to a complementary internal region of a DNA template. The reaction was qualitatively similar to strand transfer that initiates at terminal regions of template RNA (16). Both require an area of homology between the donor and acceptor templates. The amount of strand transfer product generated in the reactions increased as the concentration of acceptor template was increased (Fig. 6), and both types of reactions were stimulated by RNase H activity (Fig. 3 and 4) (16).

We were unable to detect internal strand transfer with use of DNA donor and DNA acceptor templates (data not shown). The templates used were different in sequence from the RNA donor and DNA acceptor templates used in these experiments, but the lengths of the regions of homology were about the same. This result contrasts with strand transfer at terminal regions of the template, where DNA-to-DNA jumps can occur (16). The results suggest that there

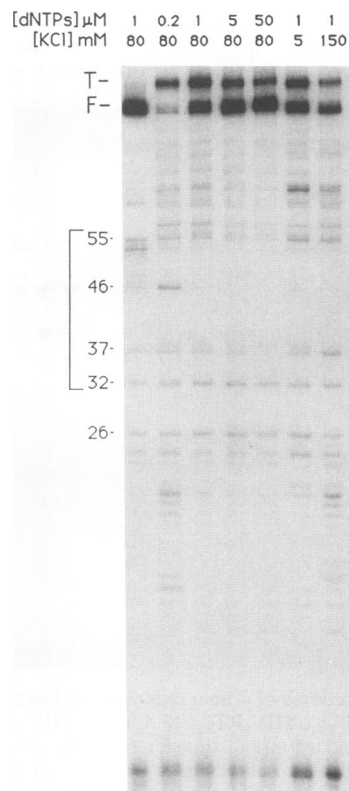


FIG. 7. Effects of varying the concentration of dNTPs and KCl on the level of strand transfer. HIV RT (3 U) was incubated in the presence (all lanes except the leftmost lane) or absence (leftmost lane) of acceptor template A (200 nM, 100:1 acceptor/donor ratio) and processed thereafter as described in Materials and Methods. The concentrations of KCl and the four dNTPs used in the reactions are indicated above each lane. The positions (bases) of full-length donor template-directed DNA synthesis products (F) and products produced by strand transfer events (T) are indicated. The region where the donor and acceptor templates were homologous is bracketed. The positions of prominent DNA synthesis pause sites (see Fig. 5) are also indicated.

may be additional constraints on strand transfer within internal versus terminal regions of template polymers.

It was clear from the results obtained in complementation experiments with *E. coli* RNase H and HIV RT_{RD} that RNase H activity is highly stimulatory to strand transfer on an RNA template (Fig. 4). The stimulatory effect of RNase H also indicates that the low level of strand transfer (or in some experiments the absence of strand transfer) by HIV RT_{RD} was probably caused solely by the RNase H deficiency of the enzyme. That is, the procedure used to deplete the enzyme of RNase H activity did not affect parameters of the polymerization reaction that may be important to strand transfer. This conclusion was also supported by results of the time course experiment (Fig. 5), which showed that the distribution of primer extension products made by HIV RT_{RD} was essentially identical to that made by normal HIV RT. Furthermore, complementation of the lost RNase H activity of HIV RT_{RD} by an exogenous activity shows that the physical association of the two activities on the RT is not required for strand transfer.

The influence of the region of homology shared by the donor and acceptor templates on the efficiency with which HIV RT catalyzed strand transfer was evaluated (Fig. 6). In

experiments in which the length of this region was held constant (27 nucleotides) but was shifted 6 nucleotides toward the primer on the donor RNA, through use of a different acceptor DNA (acceptor B), there was an approximately 25% decrease in the efficiency of the transfer reaction. This 6-base shift removed from the complementary region a portion of the template containing a prominent DNA synthesis pause site (position 55; Fig. 1 and 5). This result suggests that pausing during synthesis promotes strand transfer. Obviously, we cannot rule out the possibility that the decrease in strand transfer with acceptor template B resulted from some other specific sequence change in the region of homology. However, the strength of potential hybrids between donor-directed DNA synthetic products and the acceptor templates would not be expected to be altered by the shift. Furthermore, the homology was shifted toward the primer, favoring efficient synthesis on the donor template into the homologous region.

Consistent with a role for pausing in strand transfer was the fact that high concentrations of dNTPs decreased the level of strand transfer products and the efficiency of the transfer reaction (Fig. 7). We have found that during processive synthesis on RNA or DNA templates, the frequency of DNA synthesis termination at specific sites is increased when reactions are performed with 1 μ M rather than 50 μ M dNTPs (7).

A mechanism by which pausing of synthesis could promote transfer is considered in the following model. Results in our laboratory (6) have indicated that the synthetic and RNase H activities of RTs are not strictly coupled. An enzyme reverse transcribing the RNA does not appear to extensively degrade the RNA template during this process. If the RT pauses during synthesis and its rate of RNase H function remains the same, RNA cleavages will be favored to occur near the pause site. In fact, it has been suggested that DNA synthesis pause sites may facilitate the conversion of the enzyme-hybrid polymerase complex into an RNase H mode (8). RNA degradation would then clear a region on the nascent DNA to hybridize to an acceptor template with appropriate homology.

It has been suggested that the rate-limiting step in strand transfer involves interaction of the elongated DNA product with the acceptor template (16). The fact that the level of strand transfer increased as the concentration of acceptor template was increased in our experiments is consistent with this suggestion. Perhaps there is not sufficient time for this step to occur efficiently unless the RT pauses in DNA synthesis.

In Fig. 8, three possible modes for internal strand transfer are presented. Mode A illustrates transfer occurring in an RNase H-independent manner. Such a mechanism has been demonstrated for strand transfer on a poly(rA) template (1) and as a component of transfer at the ends of natural-sequence templates (16). Our results with HIV RT_{RD} indicate that RNase H-independent internal transfer occurs rarely if at all. Since HIV RT_{RD} retains approximately 3% of the RNase H activity of the normal enzyme, it was not possible to unequivocally determine whether internal transfer could occur in the complete absence of RNase H.

Modes B and C illustrate two RNase H-dependent transfer mechanisms. In mode B, the RNase H activity of the RT (this could be the RT that was performing DNA synthesis and/or other RTs that bind to the RNA-DNA hybrid region upstream of the DNA 3' terminus) degrades a portion of the RNA donor upstream of the 3' terminus of the nascent DNA. However, a stretch of RNA remains hybridized to the 3'-terminal region of the DNA. Strand transfer occurs when

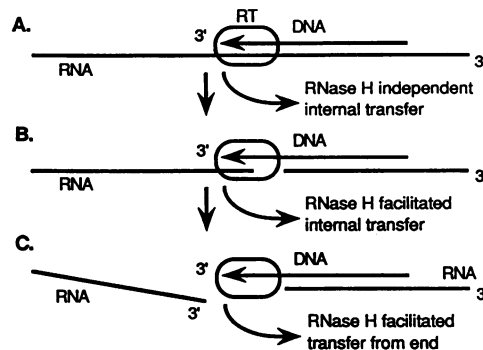


FIG. 8. Three possible modes (A, B, and C) for strand transfer within an internal region of an RNA. The region where the RNA donor is homologous to the acceptor template is circled. Regions of the RNA template where RNase H-mediated degradation is proposed to have occurred are indicated as gaps.

the acceptor template first binds to the region of the DNA where the RNA template was removed and then displaces that portion of the RNA bound to the 3'-terminal region of the DNA. Alternatively, DNA extension may continue on the RNA donor template if an acceptor does not bind.

Mode C is similar to mode B except the RNase H activity of the RT degrades the RNA template such that it no longer forms a stable hybrid with the nascent DNA. Transfer could then occur when the acceptor template binds to the single-stranded DNA terminal region. This mode predicts the formation of incomplete DNA extension products that can no longer be extended to full length on the donor. We did observe some less than full-length DNA extension products in the absence of acceptor. Many of these DNAs terminated at positions corresponding to the pause sites indicated in Fig. 5. The level of these DNAs varied somewhat from experiment to experiment but was in general very low in comparison with the level of fully extended product. In addition, most of those DNAs having 3' termini in the transfer zone in the absence of acceptor were not chased into greater lengths when acceptor was added. If a substantial level of the transfer products that we observed had resulted from a mode C type of mechanism, then the addition of acceptor to the reactions should have allowed transfer of some of the DNAs in the transfer zone to the acceptor. This would result in a decrease in the level of DNA extension products that terminated in the transfer zone in the presence of acceptor. Consequently, although a mechanism like that illustrated in mode C may have accounted for a small portion of the total transfer that we observed, most of the transfer probably resulted from mode B.

In addition, mode B strand transfer is supported by previous results from our laboratory (5) and those reported by others (22). These results indicate that if a long piece of RNA is internally primed with a short piece of DNA, the RNase H activity of HIV RT is unable to cleave the RNA portion of the RNA-DNA hybrid when the length of the hybrid becomes shorter than about 7 nucleotides. This finding suggests that there are always 7 or more bases of the RNA template that could potentially hybridize to the terminal portion of the template-directed DNA. Thus, in the strand transfer assay, continued synthesis on the RNA donor template or strand transfer synthesis (if the RNA donor is displaced by a homologous acceptor) would be possible.

It is important to note that the demonstration of internal

strand transfer, with the RT as the only required protein, does not reduce the likelihood that retroviral recombination requires other factors or proteins. In the system used in these experiments, the donor and acceptor templates were very short in comparison with the length of a retrovirus genome. Also, we were able to increase the concentration of the acceptor template severalfold above that of the donor. This allowed us to easily detect strand transfer (although admittedly a low level of strand transfer was detected even when the molar ratio of acceptor to donor was 1:1). The considerably greater length of a normal retrovirus genome may place additional constraints on recombination between genomes. For example, the large size would probably make it more difficult for regions of homology within the genomes to line up appropriately for recombination to occur. This may explain why internal recombination between viral RNAs was demonstrated in disrupted virus particles, but the same RNAs did not recombine after they were extracted from the virion and incubated with purified RT (11). As these authors point out, viral structural proteins and the proximity of the two viral genomes in the virus particle appear to be required for strand transfer in the system that they used.

The results presented here indicate that all of the fundamental enzymatic activities required to catalyze an internal strand transfer event are possessed by the RT. Additional activities or structural constraints may also be utilized in vivo to align or bring into proximity the appropriate regions of the RNA genomes involved in an internal strand transfer.

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