Strand Displacement Synthesis in the Central Polypurine Tract Region of HIV-1 Promotes DNA to DNA Strand Transfer Recombination*

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Two distinct plus strand initiation sites have been identified in human immunodeficiency virus (HIV), the central polypurine tract (cPPT) and the polypurine tract located just upstream of the U3 region (U3-PPT). When synthesis from the U3-PPT reaches the cPPT and the elongating primer causes limited strand displacement of the product created from the cPPT, we examined whether reverse transcriptase (RT) catalyzed strand transfer recombination is promoted by this process. Using a substrate having the viral sequence of the displaced region, we measured transfer of an elongating DNA primer from a donor DNA to an acceptor DNA. Strand transfer synthesis was only efficient when RT was performing strand displacement synthesis. Transfer efficiency was directly related to acceptor concentration but independent of the reaction time. Transfer could occur to acceptors containing 80, 40, or 20 nucleotides of homology with the template DNA. Using different acceptors, we found that DNA to DNA transfer occurred at positions throughout the donor template, except near the 5’ end. This shows that a number of the sequences downstream of the cPPT region can promote transfer, but once synthesis has progressed to the point where the downstream segment is completely displaced transfer is not allowed. When the DNA to DNA transfer reactions were performed using a template containing nonviral sequences, the transfer efficiency dropped significantly. This indicates that transfer efficiency is determined by the sequences of the templates used. HIV-RT RNase H-dependent strand transfer between RNA templates is well documented. We propose a quite different mechanism for DNA to DNA transfer, consistent with the ability of RNase H minus RT to perform this reaction. If these DNA to DNA transfer events occur in vivo, they will result in plus strand recombination.

Genetic variability is a characteristic of the retrovirus family. Variability of HIV1 results mainly from recombination events and from misincorporation by the error prone viral reverse transcriptase (RT). Recombination during reverse transcription is possible because each HIV virion contains two genomic RNA molecules that are often different. Two models of retroviral recombination have been proposed, the forced copy choice (1) and the strand displacement/assimilation model (2, 3). The forced copy choice model proposes that recombination occurs during synthesis of the first strand of DNA by a process that involves the transfer of the newly synthesized DNA from one viral RNA to the second viral RNA. The strand displacement/assimilation model proposes that recombination occurs during synthesis of the second strand of DNA. According to this latter model, when a piece of downstream DNA is displaced by synthesis from an upstream primer, this unannealed fragment of DNA can bind a second copy of minus strand DNA and there serves as a primer (Fig. 1A).

The strand displacement/assimilation model assumes that each copy of the viral RNA is converted into DNA and that plus strand DNA is initiated at multiple sites. Two distinct plus strand initiation sites have been identified, the central polypurine tract (cPPT) and the polypurine tract located just upstream of the U3 region (U3-PPT) (4). When synthesis from the U3-PPT reaches synthesis from the cPPT, elongation of the U3-PPT causes limited displacement of the product created from the cPPT (5). Strand displacement is limited to approximately 100 nucleotides by the presence of very strong pause sites within this region, which are referred to as the central termination sequences (5). The final plus strand DNA product consists of two segments containing an approximately 100-base pair DNA overlap (5). Presumably the two DNA segments are repaired by cellular enzymes after the proviral DNA has been transported into the nucleus.

This paper deals with the ability of HIV-RT to transfer an elongating DNA primer from its DNA template to a homologous DNA. DNA to DNA transfer has been observed previously by us and others (6, 7). In vivo, DNA to DNA transfer events can lead to the formation of plus strand recombination products by strand displacement and assimilation (2, 3). Our proposed mechanism is different from the strand displacement/assimilation model in that it is the upstream primer rather than the displaced downstream strand that transfers and serves as a primer on the new template (Fig. 1B).

The ability of HIV-RT to perform strand transfer reactions between RNA templates is well documented. During reverse transcription an RNA to RNA strand transfer reaction is an obligatory part of each replication cycle (for a review see Ref. 8). Additionally, RNA to RNA transfer can occur in other areas of the genome, and this process can lead to minus strand recombination products via a mechanism similar to that proposed in the forced copy choice model (9, 10).

Here we analyzed the conditions that promote DNA to DNA transfer, and the extent of homology necessary for this event to occur. The template used was 101 nucleotides long and con-
DNA to DNA Strand Transfer by HIV-RT

**FIG. 1. Strand displacement/assimilation models for plus strand DNA recombination.** Panel A shows the strand displacement assimilation model as proposed by Junghans et al. (2). Panel B shows a model for the strand displacement/assimilation event as it occurs in our system. The latter model differs from the former in that it is the upstream primer rather than the downstream primer that transfers synthesis from DNA template 1, and continues synthesis on the new template. The latter model differs from the former in that it is the upstream primer rather than the downstream primer that transfers synthesis from DNA template 1, and continues synthesis on the new template.

The sequences of substrates used in this study are as follows. The nucleotide sequence of donor template 1, 3'-CCATGTCAGTCCCCTTTCTTTATCTCTGCTGTTAATGTTTGAATTTAATCTAATGTTTTAAAAAGCCCTAATTAGTTTGGCACCAAT

The substrates used in this study are shown in Fig. 2. The substrate sequences are found in the middle of the HIV genome, downstream of the central polyuracil tract. Using this model template, DNA to DNA transfer was only efficient when the enzyme was performing strand displacement synthesis. Transfer efficiency was directly related to acceptor concentration, but it was independent of the reaction time.

**EXPERIMENTAL PROCEDURES**

**Materials**

HIV-RT with native primary structure was provided by the Genetics Institute (Cambridge, MA). The enzyme had a specific activity of 40,000 units/mg. One unit is defined as the amount required to incorporate 1 nmol of dGTP into nucleic acid product in 10 min at 37 °C using poly(A)-oligo(dT) as template primer. The enzyme was divided into aliquots and stored at −70 °C, and a fresh aliquot was used for each experiment. An HIV-RT lacking Mg2+-dependent RNase H, (p66E), was obtained through the AIDS Research and Reference Reagent Program from Dr. Stuart Le Grice (11,12). T4 polynucleotide kinase, T4 DNA polymerase, dNTPs, and Quick Spin columns (G-25 Sephadex) were purchased from Boehringer Mannheim. The Sequenase version 2.0 DNA sequencing kit was obtained from U.S. Biochemical Corp.

**Preparation of Substrates**—The DNA oligonucleotides used in the experiments were synthesized by Genosys, Inc. The upstream primer was 5’-end-labeled with 32P using T4 polynucleotide kinase according to the manufacturer’s instructions and purified through G-25 Sephadex. Quick Spin columns (G-25 Sephadex) were purchased from Boehminger Mannheim. The Sequenase version 2.0 DNA sequencing kit was obtained from U.S. Biochemical Corp.

**Methods**

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donor template (Fig. 3A, lanes 9–13). A faint transfer product was occasionally observed in the absence of a downstream primer after long exposure times (data not shown). We calculated transfer efficiency using a PhosphorImager. Transfer efficiency is defined as the amount of full-length transfer product divided by the sum of the amounts of full-length donor template-directed synthesis and the amount of full-length transfer product (16). In general, the DNA to DNA transfer efficiency increased with increasing concentrations of acceptor template. This phenomenon has been previously observed for RNA to RNA transfer (7). In the absence of a downstream primer, the highest DNA to DNA strand transfer efficiency observed was 2.1%. In the presence of a downstream primer, this value increased to 18.6% (Fig. 3B). At the highest acceptor concentration used, DNA synthesis and strand transfer were inhibited. This could result from trapping of the enzyme by the large excess of DNA in the reaction. The DNA to DNA transfer efficiency increased with increasing concentrations of acceptor template. This phenomenon has been previously observed for RNA to RNA transfer (7). In the absence of a downstream primer, the highest DNA to DNA strand transfer efficiency observed was 2.1%. In the presence of a downstream primer, this value increased to 18.6% (Fig. 3B). At the highest acceptor concentration used, DNA synthesis and strand transfer were inhibited. This could result from trapping of the enzyme by the large excess of DNA in the reaction. The DNA to DNA transfer efficiency increased with increasing concentrations of acceptor template. This phenomenon has been previously observed for RNA to RNA transfer (7). In the absence of a downstream primer, the highest DNA to DNA strand transfer efficiency observed was 2.1%. In the presence of a downstream primer, this value increased to 18.6% (Fig. 3B). At the highest acceptor concentration used, DNA synthesis and strand transfer were inhibited. This could result from trapping of the enzyme by the large excess of DNA in the reaction. The DNA to DNA transfer efficiency increased with increasing concentrations of acceptor template. This phenomenon has been previously observed for RNA to RNA transfer (7).
DNA to DNA Strand Transfer by HIV-RT

Fig. 3. DNA to DNA strand transfer to acceptor 1A. A, primer-donor template 1 was incubated with 4 units of HIV-RT in the presence of increasing concentrations of acceptor. The first two lanes contain the extension products of primer-donor template 1 with T4 DNA polymerase in the absence and the presence of a downstream primer. T4 DNA polymerase cannot perform strand displacement, and synthesis stops when the elongating primer reaches the downstream primer (lane 2). The third lane shows a labeled DNA ladder, used to estimate the size of the extension and transfer products. The rest of the lanes correspond to reactions carried out with increasing concentrations of acceptor 1A in the absence (lanes 4–8) or the presence of a downstream primer (lanes 9–13). The acceptor was present at a 2.5-, 5-, 10-, or 20-fold excess over the template. B, the effect of acceptor concentration on transfer efficiency is shown. Strand transfer efficiency was assessed by quantitating the products of synthesis over the donor and acceptor templates using a PhosphorImager. Strand transfer efficiency was calculated as described under “Results.” C, primer-donor template 1 in the absence (lanes 1–6) or the presence (lanes 7–12) of a downstream primer was incubated with 4 units of HIV-RT. A 10:1 molar ratio of acceptor to donor template was used. The reactions in lanes 1–6 and 7–12 were stopped after 2.5, 5, 10, 20, 30, or 60 min. Full-length donor-directed products and transfer products are indicated by the letters F and T, respectively.
DNA to DNA Strand Transfer by HIV-RT

The Efficiency of DNA to DNA Strand Transfer Depends on the Overall Sequence of the Templates Used—To determine the positions of transfer, we tested whether DNA transfer could take place using substrate 2. The donor template was 104 nucleotides long, and the downstream primer was 79 nucleotides long (see Fig. 2). Thus, the strand displacement region in substrate 2 was approximately the same length as the one in substrate 1. The acceptor was 99 nucleotides long, and the first 79 bases of the acceptor were homologous to the last 79 bases of the donor template. Although the sequences of substrate 2 are not encountered in the virus, they also contain strong pause sites. Fig. 7 shows a reaction in which the primer-donor template 2, having or lacking a downstream primer, was incubated with increasing concentrations of HIV-RT in the presence of acceptor 2. Transfer products were detected only in the presence of a downstream primer, and the transfer efficiency ranged from 6–7.8%. This result indicates that the efficiency of DNA to DNA transfer is influenced by the overall sequences of the templates used in the reaction.

DISCUSSION

Two distinct plus strand initiation sites have been identified in HIV, the cPPT and the U3-PPT (4). When synthesis from the U5-PPT reaches the cPPT, the elongating primer causes limited strand displacement of the product created from the cPPT (5). Because this region is involved in strand displacement in vivo, we considered that it could be a site for efficient recombination via the strand displacement/assimilation model or a similar mechanism. HIV-RT catalyzed DNA strand transfer was found to occur efficiently in vitro, when measured on substrates having the viral sequences involved in displacement.

We cannot interpret moderate differences in transfer efficiency measured with different acceptor templates, because this process involves synthesis through different sequences. For example, primer elongation after transfer to acceptors A and C involves synthesis through the same strong pause sites, encountered at the end of the donor template DNA (indicated by an arrow on Fig. 3A). However, elongation on acceptors B and E may not involve synthesis through any strong pause sites, because these templates have large 5' regions that are not homologous to the donor template. This situation would cause an increase in transfer efficiency in experiments with acceptor B and E (Fig. 5, B and E). Interestingly, transfer efficiency did not drop when the homology in the acceptor template was reduced from 80 to 40 nucleotides.

At What Positions on the Sequence Does Transfer Take Place?—In order to determine the positions of transfer, we created an acceptor having a 2-nucleotide deletion every 18 nucleotides (acceptor F, Fig. 2). Using acceptor F, the length of the transfer product could be 113, 115, 117, or 119 nucleotides depending on the point of primer transfer. Transfer experiments were performed using acceptors A and F. The reaction products were separated on a 5% polyacrylamide gel (Fig. 6). To determine the precise size of the products, sequencing reactions were run in the same gel. Full-length donor-directed products were 99 and 100 nucleotides long. The 99-nucleotide-long product corresponds to the full-length product, and the 100-nucleotide-long product is generated by the addition of a nontemplate-directed nucleotide by HIV-RT before or after transfer. In the presence of acceptor F, we observed products 113–119 nucleotides long. These products correspond to the expected 113-, 115-, and 117-nucleotide-long transfer products, plus the corresponding set of products one nucleotide longer caused by nontemplate-directed nucleotide addition. Using acceptor F, none of the transfer products were 119 nucleotides long. The strongest transfer bands using acceptor F were at positions 115 and 117 rather than at position 113. This indicates that transfer is not uniquely enhanced at the position where the upstream primer initiates strand displacement synthesis. Furthermore, these results confirm that DNA to DNA transfer can occur anywhere throughout the template, except near the end.

Fig. 4. DNA to DNA strand transfer to acceptor 1A by RNase H minus HIV-RT. Primer-donor template 1, containing a downstream primer, was incubated with decreasing concentrations of enzyme and a 1:10 molar ratio of acceptor to donor template. Lanes 1–4 contained 4, 2, 1, and 0.5 units of RNase H minus HIV-RT. Full-length donor-directed products and transfer products are indicated by the letters F and T, respectively. The transfer efficiencies in lanes 1–4 are 21, 23, 26, and 29%, respectively.

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synthesis in vivo.

HIV-RT can catalyze the transfer of a DNA primer from a donor template DNA to an acceptor DNA. We show that this reaction requires strand displacement synthesis and is influenced by the acceptor concentration. The length, position, and sequence of the homology between the acceptor DNA and the template DNA are also important influences. Likewise, the overall sequences of the templates used to measure DNA to DNA transfer have a major impact on the efficiency of the process.

The experiments shown here were carried out with a 101-nucleotide-long template containing viral sequences downstream of the central polypurine tract. Using these sequences and other sequences of similar length, efficient DNA to DNA transfer was only observed when the enzyme was performing strand displacement synthesis. We have shown previously, us-

Fig. 5. DNA to DNA transfer from primer-donor template 1 to acceptors 1A-1E. Panels A–E show transfer to acceptors A–E, and lanes 1–4 contain 4, 2, 1, and 0.5 units of HIV-RT, respectively. In all cases the donor substrate contained a downstream primer, and the acceptor was present at a 10:1 molar ratio over the donor template. Full-length donor-directed products and transfer products are indicated by the letters F and T, respectively. For purposes of comparison, transfer efficiencies in lane 1 with acceptors A–E are 18, 31, 20, <2, and 36%, respectively.

Fig. 6. DNA to DNA transfer from primer-donor template 1 to acceptors 1A and 1F. The donor substrate contained a downstream primer. The reactions were carried out using 4 units of HIV-RT and a 10:1 molar ratio of acceptor to donor template. Sequencing reactions with single stranded M13mp18 were carried out using the Sequenase version 2.0 DNA sequencing kit according to the manufacturer's directions. The products were separated using a 5% denaturing sequencing gel containing 7 M urea. The sizes of the full-length donor-directed and transfer products are indicated in the figure.

Fig. 7. DNA to DNA transfer from substrate 2. Primer-donor template 2 in the absence (lanes 1–6) or presence (lanes 7–12) of a fully annealed downstream primer was incubated with decreasing concentrations of HIV-RT. Lanes 1–6 and 7–12 contained 16, 8, 4, 2, 1, or 0.5 units of HIV-RT. The reactions contained a 10-fold excess of acceptor template over the donor. Full-length donor-directed products and transfer products are indicated by the letters F and T, respectively.
DNA to DNA Strand Transfer by HIV-RT

ing a 667-nucleotide-long DNA template containing the sequences of the HIV long terminal repeats, that HIV-RT can catalyze strand transfer synthesis independent of the downstream primer (6). We proposed that with the long terminal repeat substrate, DNA to DNA transfer could be independent of the downstream primer because the template can form hairpin structures that resemble the strand displacement substrate. Displacement would then have to occur periodically as the primer was elongated. This interpretation was supported by results showing that the reaction was further stimulated when the presence of a downstream primer necessitated continuous strand displacement synthesis.

Strand transfer from an RNA template requires the action of the RNase H of the RT (7, 16, 17). It is thought to occur because RNase H-directed degradation of the RNA template frees the primer to bind to a new template. In this case, sequences and structures of the donor template that cause pausing of synthesis promote transfer. They do so by allowing the RNase H a longer opportunity to degrade the RNA template while the RT is paused. We hypothesize that strand displacement synthesis promotes primer transfer from a DNA template by an entirely different mechanism. The displaced downstream primer can compete with the extending upstream primer for binding to the template. The downstream primer could transiently bind the template beneath the upstream primer, dislodging a region of the upstream primer near its 3’ end. This region would then be available to bind to the acceptor template. The ability of the displaced downstream primer to compete with the upstream primer may be similar at all points throughout the reaction, because both primers are always equivalently complementary to the template at any position. This should tend to make the probability of transfer similar throughout the template. However, other factors, discussed below, could alter transfer efficiency at each position. The DNA to DNA transfer mechanism is not unique to HIV-RT, because Sequenase, which is capable of strand displacement synthesis, can also perform this reaction (data not shown).

The probability of transfer is not likely to be absolutely uniform along the donor template. Some sequences may allow either more rapid equilibration between the two competing primers or a longer region of displacement. Also it is possible that sequences that promote dissociation of the RT allow more primer equilibration. In this way, pause site sequences may still influence transfer but for a completely different reason than for transfer from RNA templates. In support of a lack of uniformity, we have shown that transfer from DNA templates is influenced by the overall sequence of the templates used in the reaction. The long terminal repeat and the sequences downstream of the central polyuridine tract are two areas of the viral genome known to be involved in strand displacement in vivo. Higher transfer efficiencies were observed using both of these viral sequences (Ref. 6; Fig. 3) than with the nonviral substrate 2 sequence (Fig. 7). This observation suggests that particular areas of the viral genome involved in strand displacement synthesis could form structures that promote DNA to DNA transfer.

In our experiments, transfer efficiency was directly related to acceptor concentration, but independent of the reaction time. As explained above, this observation suggests that transfer takes place actively during strand displacement synthesis and not after a full-length product has been synthesized. Furthermore, our results using acceptor templates with homologies to different regions of the donor template indicate that transfer can occur at more than one position in the homologous region. In particular, use of an acceptor template with periodic two nucleotide deletions allowed determination of the positions of transfer based on the final length distribution of transfer products. This experiment further showed that transfer occurred throughout the homologous region, except near the 5’ end of the donor template. Overall, these results show that once strand displacement is initiated, strand transfer can occur with high efficiency. The point at which the upstream primer on the donor template encounters the displaceable downstream primer is not a particularly hot spot for transfer. Similarly, it is not clear whether prominent pause sites for synthesis over the donor template are exceptionally effective at promoting transfer. Transfer does not occur near the 5’ end of the donor template, presumably because once synthesis has progressed to the point where the downstream primer is completely displaced transfer is not allowed. All of these observations support the hypothesis that competition for binding the template between the extending upstream primer and the partly displaced downstream primer forces transient dissociation of the 3’ end of the upstream primer, driving the transfer reaction.

In vivo, DNA to DNA transfer events could lead to the formation of plus strand recombination products via a mechanism similar to the strand displacement/assimilation model (Fig. 1B). The only direct evidence supporting the displacement/assimilation model of plus strand recombination is the observation of branched structures during replication using electron microscopy (2). The reactions discussed in this paper can give rise to the same phenotype. We are currently sequencing transfer products to investigate whether the DNA to DNA transfer process promotes mutagenesis.

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