Avian Retrovirus U3 and U5 DNA Inverted Repeats

ROLE OF NONSYMMETRICAL NUCLEOTIDES IN PROMOTING FULL-SITE INTEGRATION BY PURIFIED VIRION AND BACTERIAL RECOMBINANT INTEGRASES*

 AJaykumar C. Vora‡, Roger Chiu‡, Mark McCord‡, Goodarz Goodarzi‡, Stephen J. Stahl§, Timothy C. Mueser‡, C. Craig Hyde§, and Duane P. Grandgenett‡‡

From the ‡St. Louis University Health Sciences Center, Institute for Molecular Virology, St. Louis, Missouri 63110 and the §NIAMS, National Institutes of Health, Bethesda, Maryland 20892

The U3 and U5 termini of linear retrovirus DNA contain imperfect inverted repeats that are necessary for the concerted insertion of the termini into the host chromosome by viral integrase. Avian myeloblastosis virus integrase can efficiently insert the termini of retrovirus-like DNA donor substrates (480 base pairs) by a concerted mechanism (full-site reaction) into circular target DNA in vitro. The specific activities of virion-derived avian myeloblastosis virus integrase and bacterial recombinant Rous sarcoma virus (Prague A strain) integrase (~50 nM or less) appear similar upon catalyzing the full-site reaction with 3'-OH recessed wild type or mutant donor substrates. We examined the role of the three nonsymmetrical nucleotides located at the 5th, 8th, and 12th positions in the U3 and U5 15-base pair inverted repeats for their ability to modify the full-site and simultaneously, the half-site strand transfer reactions. Our data suggest that the nucleotide at the 5th position appears to be responsible for the 3-5-fold preference for wild type U3 ends over wild type U5 ends by integrase for concerted integration. Additional mutations at the 5th or 6th position, or both, of U3 or U5 termini significantly increased (~3 fold) the full-site reactions of mutant donors over wild type donors.

Upon retrovirus infection, the viral RNA genome is reverse transcribed into a linear blunt ended DNA genome. The retrovirus U3 and U5 DNA termini contain LTR1 sequences with short imperfect inverted repeats located at the very end of the blunt ended LTRs (1). In vivo, the inverted repeats are necessary for virally encoded integrase to catalyze the removal of a dinucleotide from the 3'-OH termini and the subsequent full-site integration reaction (2). The full-site reaction involves the concerted insertion of the two recessed LTR DNA termini into the host genome. This reaction also results in the formation of a small size host duplication at the site of insertion whose size is virus-specific (1).

In vitro, the mechanisms involved in the recognition of the blunt ended LTR termini by integrase for the 3'-OH processing reaction and for half-site strand transfer of the recessed LTR termini into target DNA have been investigated (2–10). The full-site reaction involves the insertion of only one LTR terminus into the DNA target. These in vitro analyses using purified integrase from several retrovirus species have established that the imperfect inverted repeat sequences located at the LTR termini are also necessary for catalysis (1, 11, 12). Besides the essential CA dinucleotide located 2 nucleotides downstream from the blunt ended viral termini, approximately 5–10 nucleotides internally also play varying roles in the 3'-OH processing and half-site strand transfer reactions (2, 13).

The full-site integration reaction can be catalyzed efficiently using retrovirus-like donor substrates with integrase purified from virions (14, 15). Study of the specific interactions of integrase with the U3 and U5 LTR termini for the full-site integration reaction in vitro would provide insights into the in vivo integration reaction. The bimolecular donor full-site reaction (see Fig. 1, bottom) catalyzed by AMV integrase produces the avian 6-bp host site duplication as demonstrated by DNA sequence analysis (14, 15, this report). The unimolecular donor full-site reaction, where two ends of one molecule are used, is less than 5% as efficient as the bimolecular reaction (15–18). The ability of bacterial recombinant RSV integrase (16) and AMV integrase (17, 18) to produce the 6-bp host duplication with the unimolecular donor reaction is also high.

We have reported previously that the avian retrovirus U3 LTR terminus is preferred over the U5 LTR terminus for both the 3'-OH processing (19) and the half-site and full-site strand transfer reactions (14, 15, 18–20). Three nonsymmetrical nucleotides are at the 5th, 8th, and 12th positions of the 15-bp inverted repeats located at the RSV LTR termini. It is unknown what role the three nonsymmetrical nucleotides have in modifying the recognition of integrase for either LTR terminus or their influence on the full-site integration reaction.

In this report we examined the role of the three nonsymmetrical nucleotides located in the avian U3 and U5 15-bp inverted repeat and several other LTR mutations in the formation of donor-target recombinants that are produced by the full-site integration reaction. The full-site reactions were catalyzed by either AMV integrase derived from virions or by recombinant RSV PrA integrase purified from bacteria. Of the seven LTR mutations examined, some had either a significant gain or a loss of function for the full-site reaction, whereas others had no affect. The interactions of integrase with wt and mutant LTR DNA termini for full-site strand transfer were evaluated by BglII digestion of labeled donor-target recombinants resolved by agarose gel electrophoresis and DNA sequence analysis of donor-target junctions. The specific activities of both virion and recombinant integrase appear equivalent when catalyzing the half-site and full-site strand transfer reactions with either wt or mutant LTR DNA sub-

* This research was supported by Grant CA16312 from the NCI, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: St. Louis University Health Sciences Center, Institute for Molecular Virology, 3681 Park Ave., St. Louis, MO 63110. E-mail: Grandgdp@sluvca.slu.edu

† The abbreviations used are: LTR, long terminal repeat; AMV, avian myeloblastosis virus; bp, base pair(s); RSV, Rous sarcoma virus; PrA, Prague A strain; wt, wild type; kbp, kilobase pair(s).

This paper is available on line at http://www.jbc.org.
Inverted Repeat Mutations Affecting Full-site Integration

**RSV LTR Mutant Series**

| Wt U3 | HindIII | Wt U5 |
|-------|---------|-------|
|       |         |       |
| 5'-TATGAGCTTTGCAATCTTTGAagcttgCAACCTGCACTGAAAGAAGCTCCTCA |         | U5P5-5/T/A |
|       |         |       |
| Mutants | U3P5-A/T | U5P8-G/A |
|         | U3P8-A/G | U5P12-G/T |
|         | U3P6-T/A | U5P5, 6-TT/AA |

**Bimolecular Full-Site Reaction**

![Diagram of Bimolecular Full-Site Reaction]

**Fig. 1. LTR mutations and the bimolecular full-site integration reaction.** The top line identifies the wt U3 and U5 RSV LTR terminal sequences. The three nonsymmetrical nucleotides are marked by dark circles, and the inverted repeat sequence is in bold. The HindIII site was used for insertion of the supF gene. The U3 mutations are identified on the left and the U5 mutations on the right. The positions of the mutations on the 3'-OH processed strands are numbered from the blunt end. The first nucleotide(s) identified with each mutation was changed to T at the 5th position of U3 in U3P5-AT. At the bottom is a diagram of two individual donor molecules (each 480 bp) held together for concerted insertion of the donors into a circular target (2,867 bp). The full-site reaction produces a linear 3.8-kbp donor-target recombinant. The U3 and U5 LTR termini are illustrated with the BglII site always located near (48 bp) the U3 terminus in all of the mutant and wt donor constructs. Restriction digestion of the linear 3.8-kbp DNA produces three different fragments whose sizes are dictated by various combinations of LTR termini that are used for full-site reactions.

**Strategies for Strand Transfer and 3'-OH Processing**—Standard reaction mixtures (20 μl) for full-site strand transfer contained 20 m M HEPES buffer (pH 7.5), 1 mM dithiothreitol, 8% polyethylene glycol 6000, 15% dioxane, and 330 mM NaCl (14). Briefly, AMV or RSV integrase (50 nM) was first preincubated on ice for 10 min in the above assay mixture with 15 ng of donor DNA. The strand transfer reactions were initiated by the addition of target (100 ng) and immediate incubation at 37 °C for 10 min. The molar ratio of dimeric integrase to donor ends was 12:1, respectively. The reactions were stopped, and the DNA products were separated on agarose gels (15). The amount of products produced was determined by a Molecular Dynamics PhosphorImager. The standard 3'-OH processing reaction conditions were 10 mM HEPES (pH 7.5), 2 mM dithiothreitol, 140 mM NaCl, and 20 mM MgCl₂ (19). AMV and RSV integrase was at either 10 or 20 nM. Integrase/blunt ended donor substrates ratios used for the 3'-OH processing reaction were similar to those described for strand transfer.

**Integrate from AMV Virions and Recombinant RSV Integrase from Bacteria—AMV (21) and RSV integrase (data not shown) were purified to near homogeneity. RSV integrase was cloned from an infectious PrA viral DNA clone (22) and expressed in bacteria using a pET11 vector.**

**RESULTS**

**Mutagenesis Strategy Used to Investigate the Full-site Integration Reaction in Vitro**—In this report we examined the contribution of the three nonsymmetrical nucleotides that map to the 5th, 8th, and 12th positions of the RSV U3 and U5 LTR DNA termini for the full-site integration reaction (Fig. 1). The mutations were introduced onto the 3'-OH processing strand and are numbered with respect to the blunt ended terminus. The U3 nonsymmetrical nucleotides were modified singly to U5 sequences, and the U5 nonsymmetrical nucleotides were modified singly to U3 sequences; in each case, the other two nonsymmetrical nucleotides were unchanged. Several other gain or
Fig. 2. Full-site and half-site integration reactions using wt and mutant U3 and U5 LTR donor substrates. Panel A, a standard strand transfer reaction with AMV integrase was performed, and the DNA products were resolved by 1% agarose gel electrophoresis. Standard amounts of each reaction followed by radioactive counting were analyzed, allowing for a direct comparison among the different input donor substrates having similar specific activities. The gel was dried and exposed to x-ray film and was also subjected to PhosphorImager analysis. The donor substrates are identified as five sets of two reactions each, one without (-) and one with (+) integrase. The vertical lettering on the top of the gel above each set identifies the donor substrates. The various DNA recombinant products and input donors are marked on the left. The half-site reaction involved the insertion of one donor molecule into pGEM, whereas the full-site reaction involved the concerted insertion of two donor molecules into pGEM producing linear 3.8-kbp DNA (Fig. 1). The donor-donor recombinants are caused by integrase inserting donor DNA into other donor molecules. The donor-donor recombinants are caused by integrase inserting donor DNA into other donor molecules. Panel B, the same DNA substrates and reaction conditions as described in panel A were used by RSV integrase to produce donor-target recombinants. Analysis of the reaction products was the same as in panel A.

loose of function mutations in the LTR termini were also examined. In all cases, a wt LTR terminus was present on the opposite end of each LTR mutant donor substrate.

Measuring Catalytic Rates of Wild Type and Mutant LTR Donor Substrates—The full-site and half-site strand transfer reactions using the 480-bp wt U3/U5, wt U3/U3, and three mutant LTR donor substrates (Fig. 1) with AMV integrase at 50 nM are shown in Fig. 2A. The wt U3/U5 and wt U3/U3 donors served as control substrates for measuring catalytic rates and for determining how the different wt LTR termini affect the full-site reaction. In a 10-min reaction at 37 °C, the total incorporation of each input donor into the pGEM target was 10.8, 15, 3.7, 10.8, and 16.3% for wt U3/U5, wt U3/U3, U3P5-A/T, U3P8-A/G, and U5P5,6-TT/AA donors (Fig. 2A) are shown on the bottom line as a percent of donor substrate inserted into pGEM. The vertical columns in the box were identified with each of the above donors. Schematics for the BgIII digestion products are shown in the left margin. The BgIII digestion of one donor molecule inserted into pGEM produces either “A” or “B” type structures depending on whether the U5 or U3 end was integrated, respectively. BgIII digestion of the linear 3.8-kbp donor-target recombinant produces four linear restriction fragments with the two 3.3-kbp U3/U5 fragments comigrating. Using PhosphorImager data, the percentage of each digested product produced was determined relative to the total donor-target products produced by that donor (Fig. 4A). The BgIII 3.7-kbp U5/U5 product (middle of first vertical column of numbers) obtained using wt U3/U5 donor was set to equal 1. The other numbers were derived by dividing the percentage of homologous U5/U5 full-site product of the wt donor into the percentage of each product obtained with a specific donor. For example, the numbers in the first column (wt U3/U5 donor) are very similar to the numbers in the fourth column (U3P8-A/G donor) because this mutation did not modify significantly the half-site and full-site reactions catalyzed by integrase. For visualization of the above comparison, compare lanes 2 and 8 of Fig. 4A.

![Fig. 3.](http://www.jbc.org/content/early/1988/263/11/23940/F2.large.jpg)

**Fig. 3.** Quantitative analysis of BgII restriction products of donor-target recombinants produced by AMV integrase. For easier visualization and understanding of the tabulated data in this figure, please see the BgII digestion data in Fig. 4A. The total amounts of donor-target recombinants produced by AMV integrase with wt U3/U5, wt U3/U3, U3P5-A/T, U3P8-A/G, and U5P5,6-TT/AA donors (Fig. 2A) are shown on the bottom line as a percent of donor substrate inserted into pGEM. The vertical columns in the box were identified with each of the above donors. Schematics for the BgII digestion products are shown in the left margin. The BgII digestion of one donor molecule inserted into pGEM produces either “A” or “B” type structures depending on whether the U5 or U3 end was integrated, respectively. BgII digestion of the linear 3.8-kbp donor-target recombinant produces four linear restriction fragments with the two 3.3-kbp U3/U5 fragments comigrating. Using PhosphorImager data, the percentage of each digested product produced was determined relative to the total donor-target products produced by that donor (Fig. 4A). The BgII 3.7-kbp U5/U5 product (middle of first vertical column of numbers) obtained using wt U3/U5 donor was set to equal 1. The other numbers were derived by dividing the percentage of homologous U5/U5 full-site product of the wt donor into the percentage of each product obtained with a specific donor. For example, the numbers in the first column (wt U3/U5 donor) are very similar to the numbers in the fourth column (U3P8-A/G donor) because this mutation did not modify significantly the half-site and full-site reactions catalyzed by integrase. For visualization of the above comparison, compare lanes 2 and 8 of Fig. 4A.
was established previously that BgIII digestion of half-site and full-site donor-target recombinants gives specific cleavage patterns (14, 15), as illustrated in Figs. 1 and 3. The quantity of each uncleaved and cleaved donor-target recombinant was determined by PhosphorImager analysis. For comparison among all donor-target recombinants, the full-site 3.7-kbp homologous U5/U5 recombinant obtained by BgIII digestion of the wt U3/U5 donor (Fig. 4A, lane 2) for AMV integrase was arbitrarily set to 1 (Fig. 3), in relationship to the other digested donor-target recombinants obtained with the other donor substrates. The full-site U5/U5 recombinants were produced at the lowest level for any of the wt full-site recombinants. Similar quantitative data (data not shown) were obtained as shown in Fig. 3 with the RSV integrase reactions with the same donor substrates (Fig. 4B).

A control strand transfer reaction was analyzed first with the wt U3/U5 donor substrate (Fig. 4, A and B, lanes 1 and 2; Fig. 3). With this donor, the recessed U3 LTR terminus was approximately 3-fold more effective than the recessed U5 LTR terminus for the half-site reaction (see column of wt U3/U5 donor in Fig. 3 and the column of BgII-digested products in Fig. 4, A or B, lane 2). The BgII-digested half-site donor-target recombinants were classified as “A” and “B” type structures that were produced by the use of either the U5 or U3 LTR terminus, respectively (Fig. 3). For the full-site reactions, the 2.9-kbp homologous U3/U3 recombinants were ~5-fold higher than the 3.7-kbp homologous U5/U5 recombinants whose quantity was set to 1. The full-site 3.3-kbp U3/U5 recombinants were ~3.6-fold higher than the U5/U5 recombinants. The data suggest that integrase recognizes and subsequently uses the U3 terminus at a significantly higher level than the U5 LTR terminus when the LTRs are presented at an equal molar ratio in the reaction mixture.

The BgII digestion of control wt U3/U3 donor reactions (Fig. 3, second column; Fig. 4, A and B, lane 4) demonstrated that the “A” and “B” half-site reactions were nearly equal, suggesting that sequences outside the 25-bp LTR region appeared not to influence strand transfer significantly. All full-site reactions with this donor would be homologous U3/U3 reactions only. The full-site 3.7-kbp and 2.9-kbp U3/U3 recombinants were near equal but were half the amount of the full-site 3.3-kbp U5/U5 recombinants. The individual wt U3/U3 donors can be inserted into pGEM in two orientations giving rise to the same size 3.3-kbp BgII restriction fragment. We cannot rule out the possibility of a minor negative effect exerted by the proximity of supF sequences located at the BgII side of the wt U3/U3 donor. The effect is seen when comparing the “B” with “A” half-site products or the full-site 2.9 kbp with 3.7 U3/U3 recombinants (Fig. 3, second column).

The two control reactions above demonstrate that BgII restriction analysis of the donor-target recombinants provides a quantitative and convenient method of simultaneously analyzing half-site and full-site integration reactions.

Effects of U3 Mutations on Full-site Strand Transfer—Because the U3 terminus is identical to the U5 terminus in sequence except for the nonsymmetrical nucleotides, the ability of integrase to recognize and to use recessed U3 over U5 LTR ends would be related to these nucleotides. We next examined how the individual nucleotide changes in the U3 LTR terminus modified the ability of integrase to catalyze both half-site reactions and full-site reactions with its own mutated U3 terminus and with wt U5 LTR ends. With the mutant U3P5-A/T donor and AMV integrase at 50 nm (Fig. 4A, lanes 5 and 6), the overall strand transfer products were decreased to one-third the level observed with the wt U3/U5 donor (Fig. 3). Increasing the concentration of AMV integrase to 88 or 120 nm did not

---

**Fig. 4. BgIII digestion of wt and mutant LTR donor-target recombinants.** The AMV and RSV reaction sets are shown in panels A and B, respectively. The DNA products produced by AMV and RSV integrase (Fig. 2) were subjected to phenol extraction and ethanol precipitation. Each reaction mixture was suspended in a constant volume of buffer. Approximately 20,000 cpm of each sample was (+) or was not (−) subjected to BgIII digestion. It should be noted that the 20,000 cpm of each sample included unused donor DNA as well as the donor-target recombinants, and therefore the observed donor-target recombinants on the gel reflect the catalytic rate of each reaction accurately. The samples were resolved on a 1.5% agarose gel. The donors are listed vertically above each set of two lanes. The half-site and full-site recombinants are indicated on the left, and the DNA products produced by BgIII digestion are indicated on the right.
increase the efficiency of catalysis of donor U3P5-A/T nor change its BgII restriction pattern relative to the wt U3/U5 donor (data not shown).

The BgII digestion pattern observed with the mutant donor U3P5-A/T was changed significantly relative to the wt U3/U5 donor (Fig. 4A, lanes 2 and 6). The most striking observation is that the U3P5-A/T LTR end appears to have activity similar to the wt U5 LTR end located on the same donor molecule. Almost all of the catalytic reactions with the U3P5-A/T donor more closely parallel wt U5 LTR than wt U3 LTR activities (Fig. 3, first and third columns). In addition, the half-site B U3P5-A/T LTR reaction of donor U3P5-A/T was inhibited 60% compared with the U3 LTR of wt donor U3/U5. The full-site 3.3-kbp U3/U5 and the 2.9-kbp homologous U3/U3 recombinant reactions of donor U3P5-A/T were decreased 50% and 85%, respectively, compared with the same wt U3/U5 donor reactions.

Similar results were obtained with the BgII digestion patterns using donor U3P5-A/T with RSV integrase at 50 nM (Fig. 4B, lane 6). With either AMV or RSV integrase (Fig. 4, A and B, lanes 2 and 8), changing the 8th nucleotide of the U3 LTR from A to G (donor U3P8-A/G) (Fig. 3, fourth column) had no apparent effect on the strand transfer reactions compared with the wt U3/U5 donor. The 12th nonsymmetrical nucleotide in the U3 inverted repeat was not examined, although a single nucleotide deletion 2 bp downstream of the 8th nucleotide decreases half-site and full-site catalytic rates (see below). The data suggest that the nonsymmetrical nucleotide at the 5th position has a significant effect on the preferential recognition of U3 ends over U5 ends by integrase as well as on subsequent catalysis.

The wt U5 end of donor U3P5-A/T was able to relieve some of the inhibitory effects of the single nucleotide change in the U3P5-A/T terminus. This conclusion was reached if one compares the ratio of the full-site U3/U5 (3.3 kbp) to homologous U3/U3 (2.9 kbp) reactions observed (0.66) with the wt U3/U5 donor (Fig. 3, first column) to the ratio observed (2.2) with the full-site 3.3-kbp to 2.9-kbp reactions of donor U3P5-A/T (Fig. 3, third column).

Fig. 5. BgII digestion of wt and mutant U5 LTR donor-target recombinants. The AMV and RSV reactions are shown in panels A and B, respectively. The same procedures used in Figs. 2 and 4 were also used in this analysis of the mutant U5 LTR donor-target recombinants. The wt U3/U5 and different U5 LTR mutant donor substrates were labeled to similar specific activities. Standard integration reactions were performed, and the amount of each donor incorporated into pGEM was determined by 1% agarose gel electrophoresis. The remaining samples were subjected to PhosphorImager analysis as shown previously.

Effects of U5 Mutations on Full-site Strand Transfer—We next examined the effects of three U5 mutations individually directed against the nonsymmetrical nucleotides on the strand transfer reactions. We compared the wt U3/U5 donor with donors containing single mutations in U5 at the 5th, 8th, and 12th positions (U5P5-T/A, U5P8-G/A, U5P12-G/T, respectively) using both AMV integrase (Fig. 5A) and RSV integrase (Fig. 5B). To determine if sequences downstream of the 8th nonsymmetrical nucleotide of U3 were important, we also investigated the inhibitory effects of the single nucleotide change in the U3 LTR from A to G (donor U3P8-A/G) (Fig. 3, fourth column) had no apparent effect on the half-site and full-site recombinants. As expected, the modification of the U5 LTR sequence to a U3 LTR sequence (T to A at the 5th position; donor U5P5-T/A) up-regulated the ability of both integrase proteins to use the mutated U5 terminus, which is now similar to the wt U3 terminus. For example, the half-site ("A") product and the full-site 3.7-kbp recombinants using the mutated U5 terminus of donor U5P5-T/A (Fig. 5, A or B, lane 4) were significantly higher (2–3-fold) than the same size products using the U5 end of the wt U3/U5 donor, shown in lane 2 of panels A and B. Similarly, the half-site wt U3 product ("B") of donor U5P5-T/A is nearly the same quantity as the half-site product ("A") with the mutated U5 terminus of the same donor (Fig. 5, A and B, lane 4). In lane 4 of both integrase sets, the mutated U5 end interactions with the wt U3 end to produce the full-site 3.3-kbp recombinants were similar in quantity to the full-site 2.9-kbp homologous U3/U3 product obtained with the wt U3/U5 donor shown in lane 2. The changing of the 8th (donor U5P8-G/A) or 12th (donor U5P12-G/T) position of the U5 LTR to U3 sequences (Fig. 5, A and B, lanes 6 and 8, respectively) had little effect on the mutated U5 LTR strand transfer reactions compared with reactions observed with the wt U5 LTR of the wt U3/U5 donor (lane 2). The modification of the U3 LTR by a single nucleotide deletion at the 10th position of donor U5P8-G/A had a modest effect on the overall catalytic rates (compare lanes 5 and 6 of 5A and 5B with the wt U3/U5 donor reactions in lanes 1 and 2). The catalytic rate was decreased with donor U5P8-G/A, but the BgII restriction pattern was not modified
significantly. The results suggest that the 5th nucleotide of the U5 LTR end also plays a significant role in regulating integrase recognition and catalysis.

**Effects of Double Mutations at the 5th and 6th Positions of U5 and U3**—As shown previously, modification of the 5th and 6th nucleotides of the U5 LTR from TT to AA (donor U5P5,6-TT/AA) increased strand transfer for both the half-site and full-site reactions relative to that observed with the wt U3/U5 donor (Figs. 2 and 3). There was a 1.5- to 2.3-fold increase in the catalytic rates observed with AMV and RSV integrase, respectively, when comparing donor U5P5,6-TT/AA with the wt U3/U5 donor. The double mutation at the 5th and 6th nucleotide of U5 results in a U3 LTR sequence up to the 5th position with an additional T to A change at the 6th position. The U5P5,6-TT/AA mutation enhanced markedly (3–5-fold) both the mutated U5 LTR half-site (“A”) and the full-site 3.7-kbp homologous U5/U5 reactions (Fig. 4A, lanes 9 and 10; Fig. 3, last column) compared with same size U5 LTR products obtained with the wt U3/U5 donor (Fig. 4A, lanes 1 and 2; Fig. 3, first column). In fact, the full-site 2.9-kbp homologous U3/U3 recombinant observed using donor U5P5,6-TT/AA was the poorest reaction, suggesting that integrase now has a much higher affinity for the modified U5 end than the U3 terminus in the same reaction mixture. The full-site 3.3-kbp U5/U5 product observed with donor U5P5,6-TT/AA was similar in quantity to the same size product obtained using the wt U3/U5 donor. Similar conclusions can be reached about the above strand transfer reactions using donor U5P5,6-TT/AA with RSV integrase (Fig. 4B, lanes 9 and 10). The changing of the two T pyrimidines to two A purines at the 5th and 6th positions of U5 enhanced markedly the ability of either AMV or RSV integrase to recognize and to use this modified LTR terminus compared with the wt U5 and U3 ends.

To establish further that the 5th and 6th positions of either U5 or U3 play critical roles in controlling full-site catalysis, we modified the sixth position of the U3 LTR from T to A (donor U3P6-T/A). The sequence of donor U3P6-T/A on the processed strand is 5′-GACAACA_{OH} which is similar to the above donor U5P5,6-TT/AA (5′-GGACAACA_{OH}). AMV integrase and RSV integrase at 25 or 50 nM were able to use the donor U3P6-T/A 3–4-fold better for full-site and half-site strand transfer reactions than the wt U3/U5 donor (data not shown). For example, in a 10-min reaction with AMV integrase at 50 nM, 40% and 24% of the input donor was incorporated into full-site and half-site products, respectively. BglII digestion of the half-site reaction products demonstrated that the U3P6-T/A LTR end was used 5-fold better than the wt U5 end. The homologous mutated U3/U3 and homologous wt U5/U5 full-site reactions accounted for 68% and 2% of the full-site products, respectively, whereas the wt U5/mutated U3 end reaction was 30%. The data support the results that were obtained with the U5P5,6-TT/AA donor and that the affinity of integrase for wt U3 is most likely controlled by both the 5th and 6th positions for full-site catalysis.

**Sequence Analysis of Wild Type and Mutant LTR Donor-Target Junctions**—AMV integrase produces the 6-bp avian host site duplication using a wt donor termed M-2 (14, 15), which is very similar to the wt U3/U5 donor in this study. The ability of RSV PrA integrase to produce the avian 6-bp host site duplication with wt U3/U5 and mutant U5P5,6-TT/AA as donor substrates was investigated. Standard reactions were performed with RSV and AMV integrase, and the donor-target recombinants were subjected to BglII digestion. The linear 3.3-kbp restriction fragments were isolated from each donor set, ligated, and transformed into CA244 cells. Recombinants from each donor set were sequenced at the donor-target junctions (Table I). The results show that both integrase proteins were capable of producing the avian 6-bp host site duplication with some 5-bp and 7-bp duplications. All of the donor LTR ends that were inserted into the target ended with the conserved CA dinucleotide. Several small size deletions were also observed with both donor reactions. The results show that purified RSV and AMV integrase can produce faithfully the avian 6-bp host site duplication in *vivo*.

3′-OH processing of blunt ended wt and mutant LTR DNA termini by AMV and RSV Integrase—Different assay conditions are needed for AMV integrase to process effectively a dinucleotide from blunt ended wt LTR donors compared with using the recessed LTR donors for strand transfer (14, 19). The aprotic solvents dimethyl sulfoxide or dioxane, or high NaCl concentrations (330 mM) severely inhibited the 3′-OH processing reaction. The *NdeI* sites of wt U3/U5 and mutant donors U5P5,6-T/A and U5P5,6-TT/AA were filled in at their *NdeI* sites using labeled TTP. The blunt ended donor substrates were digested with *Hinfl* and the appropriate fragments were isolated on agarose gels. Standard 3′-OH processing reactions were performed as described under “Experimental Procedures.” The concentration of integrase is shown for each set of reactions. Aliquots of each reaction were taken at the indicated times, and soluble trichloroacetic acid counts were determined. The percentages of donor substrate cleaved by integrase were determined (left).

**Table I.**

| Deletionsa | wt U3/U5 | U5P5,6-TT/AA |
|------------|----------|--------------|
| 5 bp       | 6 bp     | 7 bp         |
| AMV        | 5        | 41           | 2            |
| RSV        | 5        | 26           | 5            |
| Total      | 10       | 67           | 7            |

a The small size deletions varied in size from 27 to 98 bp.

b 45 of the AMV sequenced wt recombinants were produced using a wt donor termed M-2 (14, 15).
23% polyacrylamide DNA sequencing gels showed that both AMV and RSV integrase produced the same dinucleotide (data not shown; 19). The data suggest that integrase does not show a marked preference for blunt ended LTR donor substrates for processing to the same degree as was shown with their recessed LTR donor substrates for strand transfer.

DISCUSSION

Single or double nucleotide changes close to the recessed termini of U3 and U5 LTR substrates modify the ability of AMV and RSV integrase to catalyze the full-site integration reaction. The 5th nucleotide was the only nonsymmetrical nucleotide within either the U3 or U5 inverted repeats which significantly affected integrase for the full-site reaction. The A nucleotide at the 5th position of U3 (T for U5 at this position) appears to be responsible for the large preference for wt U3 over wt U5 LTR ends by integrase for half-site and full-site strand transfer. The 8th and 12th nonsymmetrical nucleotides appear to have little effect on strand transfer. Taken together or independently, the U3 and U5 mutational analyses demonstrate that the 5th nucleotide of both LTR termini have a significant role in regulating integrase recognition and catalysis for full-site strand transfer. Significantly, it appears that the LTR mutations affect both the half-site and full-site reactions in nearly a parallel quantitative fashion.

Both recombinant RSV (Schmitt-Ruppin B strain) integrase (16) as well as AMV integrase (14, 15) produce the avian 6-bp host site duplication at a high frequency. The specific activities of our RSV PrA integrase and AMV integrase preparations are similar for half-site and full-site strand transfer with wt and mutant donor substrates. However, compared with AMV integrase, RSV PrA integrase appears to prefer the donor US5P5,6-TT/AA, although both proteins prefer this substrate over the wt U3/U5 donor. The mutation in the U5 end of donor US5P5,6-TT/AA produced the sequence 5′-GGCAACA-9OH on the processed strand compared with the wt U5 sequence of 5′-GGCT-TCA-9OH. Cleavage by integrase does not occur at the internal CA dinucleotide with donor US5P5,6-TT/AA (data not shown), which is consistent with only the terminal CA dinucleotide being used for full-site strand transfer (Table 1). Further studies are under way to determine if this mutation or any of the other LTR mutations studied in this report will allow us to determine the number of integrase subunits involved in full-site reactions, as yet undefined.

It was reported previously that changing the 5th and 6th nucleotides on the U5 LTR processed strand from TT to AA completely inhibited 3′-OH processing and strand transfer reactions using double-stranded oligonucleotide substrates and purified bacterial expressed RSV integrase (Schmidt-Ruppin B strain) (23, 24). Substitution of this U5 LTR mutation into an infectious RSV clone demonstrated that the virus was still infectious, showing that this U5 LTR mutation was not lethal. It was suggested that there were cooperative interactions between the wt U3 LTR terminus and the mutated U5 LTR terminus to permit virus replication. With either AMV or RSV integrase, the donor substrate containing the same TT to AA mutation at the U5 LTR terminus (donor US5P5,6-TT/AA) showed a significant increase in strand transfer activities relative to the wt U5 end. The mutated U5 LTR end of donor US5P5,6-TT/AA is fully capable of full-site strand transfer with the wt U3 end (Fig. 3, fifth column). Sequence analysis of the donor-target junctions in these recombinants verified that this mutation was still present in the donor after genetic selection in our CA244 cells. The blunt ended donor US5P5,6-TT/AA substrate was also trimmed correctly by integrase to produce a dinucleotide. Chemical degradation of the fill-in donor showed that it also contained the correct mutation. The reason for the difference between our in vitro data and those reported with oligonucleotide substrates containing the TT to AA mutation is unknown.

The 5′ 2-bp overhang on the unprocessed donor strand appears to play a major stabilizing role for integrase-DNA complexes capable of half-site strand transfer reactions with oligonucleotide substrates and recombinant human immunodeficiency virus type 1 integrase (5). Although the 3′-OH trimming reactions using blunt ended donor substrates with AMV integrase are observed (Fig. 6), the resulting integrase-recessed DNA complexes are not used in an efficient matter for full-site reactions compared with reaction mixtures containing 3′-OH recessed donors (14). Similar data for the inefficient use of blunt ended donor substrates for the unimolecular full-site strand transfer reaction with AMV (6, 15, 18) or RSV integrase (16) have been reported. The wt and several mutant recessed donor substrates used in our study which contained the 5′ 2-bp overhang are inserted efficiently into target DNA by a full-site reaction mechanism. These results suggest that the 5′ 2-bp overhang on recessed LTR substrates may induce conformational changes in integrase which allow integrase to form stable protein-DNA complexes both in vitro and in vivo (5). Possibly, the molecular crowding and osmotic pressure effects of PEG and organic solvents (25–27) modify the ability of both AMV and RSV integrase to recognize and to use substrates with the 5′ 2-bp overhang but not blunt ended substrates efficiently for the subsequent strand transfer activities. Whether the lack of efficient coupling of the 3′-OH processing reaction to the subsequent full-site integration reaction (but not half-site reactions) (14) is the result of the lack of physical contact between processed donors in the reaction mixture or other reasons is unknown. The potential role of the organic solvents in destabilizing the donor termini (14) thus allowing for more efficient strand transfer is also unknown. A recent report suggests that end fraying or DNA distortion of the blunt ended LTR termini by integrase is a required step in the 3′-OH processing reaction in vitro (28).

Mutations introduced into one LTR terminus influenced the ability of integrase to use the other wt LTR end for the 3′-OH processing reaction in vivo (29). It is difficult to compare this in vivo result with the interactions between wt and mutant recessed LTR ends in our study for full-site strand transfer. Our results with recessed LTR substrates suggest that a wt LTR substrate can increase the ability of a mutant LTR substrate to participate in full-site catalysis. Apparently, the wt U5 LTR end of donor US3P5-AT diminishes the inhibitory effect of the mutated U3 LTR end on the same molecule to produce the 3.3-kbp U3/U5 recombinant (Fig. 3, third column). Comparison of this U3/U5 reaction with donor US5P5-AT with the same reaction with the wt U3/U5 donor (Fig. 3, first column) is necessary to support this possibility. What is apparent with the wt U3/U5 donor reactions is that the homologous U3/U3 full-site reaction is significantly better (~5-fold) than the homologous U5/U5 reaction (set to 1), with the normal in vivo U3/U5 reaction at an intermediate level (~3.6-fold). Whether there is a preferential or leading role of the U3 LTR end over the U5 LTR end for formation of stable preintegration complexes (30) in vivo is unknown. The above observations suggest that there may be significant interactions between the different LTR ends that are presumably coupled by integrase.

REFERENCES

1. Varmus, H. E. (1983) in Mobile Genetic Elements (Shapiro, J. A., ed) pp. 411–503, Academic Press, New York
2. Goff, S. P. (1992) Annu. Rev. Genet. 26, 527–544
3. Bushman, F. D., and Craige, R. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 1339–1343
4. Bushman, F. D., Fujiwara, T., and Craige, R. (1990) Science 249, 1555–1558
5. Ellison, V., and Brown, P. O. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7316–7320
Inverted Repeat Mutations Affecting Full-site Integration

6. Katz, R. A., Merkel, G., Kulkosky, J., Leis, J., and Skalka, A. (1990) Cell 63, 87–95
7. Lee, S. P., Kim, H. G., Censullo, M. L., and Han, M. K. (1995) Biochemistry 34, 10205–10214
8. Reicin, A. S., Kalpana, G., Paik, S., Marmon, S., and Goff, S. (1995) J. Virol. 69, 5904–5907
9. Van Den Ent, F. M. I., Vink, C., and Plasterk, R. H. A. (1994) J. Virol. 68, 7825–7832
10. Vink, C., van der Linden, K. H., and Plasterk, R. H. A. (1994) J. Virol. 68, 1468–1474
11. Katz, R. A., and Skalka, A. (1994) Annu. Rev. Biochem. 63, 133–163
12. Vink, C., and Plasterk, R. H. A. (1993) Trends Genet. 9, 433–4348
13. Balakrishnan, M., and Jonsson, C. B. (1997) J. Virol. 71, 1025–1035
14. Vora, A. C., and Grandgenett, D. P. (1995) J. Virol. 69, 7483–7488
15. Vora, A. C., McCord, M., Fitzgerald, M. L., Inman, R. B., and Grandgenett, D. P. (1994) Nucleic Acids Res. 22, 4454–4461
16. Aiyar, A., Hindmarsh, P., Skalka, A., and Leis, J. (1996) J. Virol. 70, 3571–3580
17. Fitzgerald, M. L., and Grandgenett, D. P. (1994) J. Virol. 68, 4314–4321
18. Fitzgerald, M. L., Vora, A. C., Zeh, W. G., and Grandgenett, D. P. (1992) J. Virol. 66, 6257–6263
19. Fitzgerald, M. L., Vora, A. C., and Grandgenett, D. P. (1991) Anal. Biochem. 196, 19–23
20. Grandgenett, D. P., Inman, R. B., Vora, A. C., and Fitzgerald, M. L. (1993) J. Virol. 67, 1628–1636
21. Grandgenett, D. P., Vora, A. C., and Schiff, R. (1978) Virology 99, 119–132
22. Mumma, S. R., and Grandgenett, D. P. (1991) J. Virol. 65, 1160–1167
23. Cobrinik, D., Aiyar, A., Ge, Z., Katzman, M., Huang, H., and Leis, J. (1991) J. Virol. 65, 3864–3872
24. Katzman, M., Katz, R. A., Skalka, A., and Leis, J. (1989) J. Virol. 63, 5319–5327
25. Robinson, C. R., and Sligar, S. G. (1996) Protein Sci. 5, 2119–2124
26. Sidorova, N. Y., and Rau, D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12272–12277
27. Timasheff, S. N. (1993) Annu. Rev. Biophys. Biomol. Struct. 22, 67–97
28. Scottoline, B. P., Chow, S., Ellison, V., and Brown, P. O. (1997) Genes Dev. 11, 371–382
29. Murphy, J. E., and Goff, S. P. (1992) J. Virol. 66, 5092–5095
30. Brown, P. O., Bowerman, B., Varmus, H. E., and Bishop, J. M. (1987) Cell 49, 347–356
Avian Retrovirus U3 and U5 DNA Inverted Repeats: ROLE OF NONSYMMETRICAL NUCLEOTIDES IN PROMOTING FULL-SITE INTEGRATION BY PURIFIED VIRION AND BACTERIAL RECOMBINANT INTEGRASES

Ajaykumar C. Vora, Roger Chiu, Mark McCord, Goodarz Goodarzi, Stephen J. Stahl, Timothy C. Mueser, C. Craig Hyde and Duane P. Grandgenett

J. Biol. Chem. 1997, 272:23938-23945.
doi: 10.1074/jbc.272.38.23938

Access the most updated version of this article at http://www.jbc.org/content/272/38/23938

Alerts:

- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 29 references, 17 of which can be accessed free at http://www.jbc.org/content/272/38/23938.full.html#ref-list-1