A novel assay for the DNA strand-transfer reaction of HIV-1 integrase

Daria J.Hazuda*, Jeffrey C.Hastings, Abigail L.Wolfe and Emilio A.Emini
Department of Antiviral Research, Merck Research Laboratories, West Point, PA 19486, USA

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An essential step in the replication of retroviruses is the integration of a DNA copy of the viral genome into the genome of the host cell. Integration involves a series of well defined DNA cleavage and strand-transfer steps that are catalyzed by the virally encoded enzyme integrase (1–4). The sequence-specific endonucleolytic activity of integrase removes two nucleotides from the 3′ end of each LTR sequence at either end of the double-stranded DNA provirus. In the subsequent strand-transfer reaction, integrase non-specifically nicks the host cell DNA, creating a staggered break whose 5′ ends are coordinately ligated to the processed 3′ ends of each viral LTR. Although repair of the resultant integrated product may require host-encoded cellular enzymes, integrase is the sole protein responsible for both the specific and non-specific endonucleolytic steps as well as DNA strand transfer. The enzyme's absolute requirement for propagation of HIV-1 in cell culture defines integrase as an attractive target for antiviral chemotherapeutic intervention (5). We designed the microtiter plate assay shown in Figure 1 with the specific aim of developing a high volume, non-radioisotopic assay for HIV-1 integrase which uses unique oligonucleotides to represent the LTR donor and target DNA substrates.

For these studies, we used recombinant HIV-1 HXB2 integrase purified from E.coli (4). However, to increase the scale and overall yield, the procedure for purification of integrase was modified as follows: A 20 gm frozen pellet resuspended in 25 ml of buffer A (50 mM Tris–HCl pH 7.6, 10 mM MgCl₂) was lysed using sonication and lysozyme (final concentration of 0.5 mg/ml). Following centrifugation for 30 min at 4°C (15,000 rpm Sorval SS34 rotor), the pellet was resuspended in 25 ml of buffer A, and treated with DNase I (4,000 U, Boehringer Mannheim) for 20 min at 37°C. DNAse I was used to aid in solubilization, facilitating purification by improving the chromatographic profile of the enzyme, and reducing the number of steps required to achieve homogeneity.

Integrase was solubilized by the addition of 5 M NaCl to a final concentration of 1.25 M and solid CHAPS to a final concentration of 10 mM. The slurry was stirred gently on ice for 30 min and then centrifuged as above. The supernatant was diluted to reduce the NaCl concentration to 250 mM prior to chromatography on Heparin agarose (4). Purity of the enzyme was assessed by SDS–PAGE and enzymatic activity was demonstrated using the assay for 3′ end-processing of LTR substrates (4) as well as the microtiter strand-transfer assay. For the microtiter strand transfer assay, donor substrate oligonucleotides representing the HIV-1 (strain HXB2) US LTR (Figure 1A) were immobilized onto CovaLink polystyrene microtiter plates (NUNC, Naperville, IL) (6). The oligonucleotides were synthesized (Midland Certified Reagent Co., Midland, Texas) to include a 5′ phosphorylated three base overhang on the 5′ end of the strand which is specifically processed on the 3′ end by integrase. As illustrated in Figure 1B, carbodiimide mediated condensation of this phosphate with the secondary amino group on the surface of the microwell orients the double-stranded LTR oligonucleotide such that oligonucleotide strand which is cleaved and eventually ligated by integrase is immobilized on the plate with the 3′ end distal to the surface. The 5′ overhang is required for maximum efficiency of the condensation reaction (6).

The condensation reaction using the LTR donor oligonucleotide (75 µl of 0.067 µM in 10 mM 1-methylimidazole, pH 7) and 25 µl freshly prepared EDC (0.2 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in 10 mM 1-methylimidazole) was performed as described (6). Plates were stored for several months at 4°C in blocking buffer, PBS, 1% BSA (CalBiochem) and 0.2% sodium azide. Coupling efficiency as estimated using a radio-labelled version of the phosphorylated LTR strand, generated with T4 polynucleotide kinase (Boehringer Mannheim, Indianapolis, IN) (7), was comparable to that published (6). Using 0.067 µM oligonucleotide for condensation, the amount of substrate immobilized per well was determined to be 1.25 pMol.

The standard microtiter DNA strand-transfer assay outlined in Figure 1B was performed directly in these oligonucleotide coated microtiter wells using the immobilized 30 bp US LTR donor substrate and the biotinylated 20 bp target sequence shown in Figure 1A. Each reaction of 100 µl included 20 mM Tris–HCl pH 7.8, 25 mM NaCl, 3 mM MnCl₂, 5 mM Beta-mercapto-ethanol, and 50 µg/ml BSA, conditions comparable to published endonuclease and integration gel assays (7). The microtiter strand transfer assay reaction exhibited requirements for salt and MnCl₂ which were indistinguishable from those previously determined (data not shown).

Upon the addition of integrase (20 µl, final concentration 50 nM), specific cleavage of the 3′ end of the immobilized strand processed the LTR donor for subsequent strand transfer. After 30 min at 37°C, 5 µl of the heterologous, biotinylated oligonucleotide added to the reaction provided the target substrate for

* To whom correspondence should be addressed
strand transfer. Integrase nicks the target DNA ‘randomly’ and mediates the formation of a new phosphodiester linkage between the 5’ end of this nick and the processed 3’ end of the immobilized LTR donor oligonucleotide, thereby transferring the 3’ biotin group of the target oligonucleotide to the plate.

The strand transfer reaction was performed for 30 min at 37°C. The wells were washed three times with 200 μl each of PBS containing 0.05% Tween 20. After two washes with PBS to eliminate residual detergent, plates were blocked with 1% BSA in PBS, and the strand transfer products were detected and quantified using a colorimetric avidin-linked alkaline phosphatase reporter (alkaline phosphatase-conjugated avidin, Boehringer Mannheim, Indianapolis, IN, and PNP substrate, Sigma Chemical Co., St Louis, MO, as per the manufacturer’s directions).

The microtiter integration assay displayed hyperbolic saturation with respect to both the concentration of immobilized donor LTR oligonucleotide and added target DNA substrate (Figure 2). Either the blunt-ended US LTR 30-mer oligonucleotide or the analogous preprocessed US LTR oligonucleotide (which does not contain the terminal dinucleotide normally removed by integrase, ref. 8) could be employed as donor substrates in this assay. As shown in Figure 2A, both DNAs were equally efficient donor substrates in the reaction and can, therefore, be used to discriminate between the specific 3’ processing and non-specific nicking and strand-transfer activities of the enzyme.

In addition, since the microtiter assay uncouples the requirements for donor and target DNAs, the assay can also be used to investigate the sequence requirements for each substrate. As shown in Figure 2B, mutation of the donor oligonucleotide at the −2 position of the 5’ overhang reduced the strand-transfer activity by approximately three-fold. This suggests that the enzyme may interact with this strand even though this portion of the substrate is not directly involved in the reaction. The result is consistent with previous studies which demonstrated that strand transfer requires at least a one base 5’ overhang on the unprocessed strand (9). In Figure 2C, four oligonucleotides of equivalent length, but different composition, were tested as target substrates. Comparison of these target substrates demonstrates that strand transfer occurs preferentially on target DNAs containing GC base pairs. Poly(dA)/poly(dT) 20-mers were poor integration target substrates when compared to either the ‘random’ sequence target (Figure 2A and B) or alternating copolymers containing GC base pairs (Figure 2C).

In conclusion, we have developed a novel rapid assay for the DNA strand-transfer activity of HIV-1 integrase. Since the assay is designed to uncouple donor and target DNA selection, it can be used for the detailed characterization of both donor and target requirements. The assay requires the 3’ end processing, non-specific nicking and strand-transfer activities of the enzyme. The ease with which this assay can be performed using readily available reagents should facilitate the identification of selective inhibitors for any of these processes. Moreover, the general format of the assay can easily be adapted to the study of similar enzymes such as other retroviral integrases as well as recombinases, transposases and ligases.

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