HIV-1 Preintegration Complexes Preferentially Integrate into Longer Target DNA Molecules in Solution as Detected by a Sensitive, Polymerase Chain Reaction-based Integration Assay*

Alexei Brooun‡‡, Douglas D. Richman‡‡, and Richard S. Kornbluth‡‡

From the Departments of §Medicine and ¶Pathology, University of California San Diego, La Jolla, California 92093 and the Veterans Affairs San Diego Healthcare System, San Diego, California 92161

After entering a cell and undergoing reverse transcription, the retroviral genome is contained in a preintegration complex (PIC) that mediates its integration into host cell DNA. PICs have been shown to prefer torsionally strained DNA, but the effect of target DNA length has not been previously examined. In this report, concatemerization of a repeating 105-base pair unit was used to vary target DNA length independently from basic DNA sequence, while maintaining both PICs and target DNAs in solution. Integration junctions were quantified by real-time fluorescence-monitored polymerase chain reaction amplification using primers in the viral long terminal repeat and the target DNA. Unreacted target DNA severely inhibited the post-reaction polymerase chain reaction detection step, requiring its removal using a exonuclease digestion. Integration into a 32-unit concatemer of target DNA was markedly more efficient than integration into a monomeric unit, indicating that longer target DNA was preferred. This substrate was used to construct a simple, robust, and adaptable assay that can serve as a method for studying the host cell factors that enhance PIC integration, and as a drug discovery platform for integration inhibitors active against PICs.

The integration of HIV into the host cell genome requires the integrase enzyme (1, 2) and mutations which destroy integrase activity block viral replication (3). These observations established integration as an important target for the development of new antiretroviral drugs (4). Recently, Hazuda et al. (5) found a new class of integrase inhibitors by screening a library of 250,000 compounds with a strand-transfer assay. Several di-keto compounds were found which inhibited HIV infection in CD4+ T cells in vitro, igniting the hope that integration inhibitors could become a new treatment modality (5).

In the strand-transfer assay used by Hazuda et al. (5), an artificial, preassembled complex was formed between recombinant integrase protein and oligonucleotides designed to model the ends of the viral cDNA. Then, candidate inhibitors were added, followed by the target DNA (6). Although more reliable than the original strand transfer assay (7), this assay still scores as positive occasional compounds which fail to inhibit integration in vitro by authentic preintegration complexes (PICs) isolated from the cytoplasm of infected cells (5, 8) or virus-mediated integration in cultured cells (5). This suggests important roles for the other components present in PICs, which include viral proteins (reverse transcriptase, MA, NC, and Vpr) (9, 10) together with cellular factors that are essential for the efficient integration of retroviral cDNA (11–15). These host cell factors may provide additional drug targets or otherwise influence the ability of a putative inhibitor to successfully inhibit integration. However, relatively little is known about PIC-mediated integration as an enzymatic process, and it has been difficult to construct a high-throughput assay for integration by PICs.

Several groups have studied the influence of the target DNA upon PIC integration. PICs prefer to integrate into regions of distorted DNA (16) such as nucleosomes (17, 18) and tend to avoid integration sites upstream of a pyrimidine nucleotide (19). Not addressed in these studies, however, is the issue of target DNA size, which has inherent effects on substrate mobility. The present report describes an entirely fluid phase assay in which integration junctions are directly detected using PCR amplification. The only molecules that score as positive in this assay result from an actual joining of viral cDNA and target DNA. A uniquely designed DNA substrate is used to maintain amplicon size within the optimum limits for real-time fluorescence-monitored PCR detection. By comparing a single target DNA sequence of 105 bp with longer concatemers of the same sequence, the preference of PICs for longer target DNA was clearly demonstrated. A model is presented which suggests that long DNA is preferred because its decreased rate of diffusion allows more time for the rate-limiting “target commitment” stage of PIC integration. Incorporating this information into the assay yielded a sensitive, robust, and adaptable platform for the discovery of drugs that inhibit integration by PICs. This assay was used to detect host cell factors that reconstituted the integration competence of salt-stripped PICs.

* This work was supported by National Institutes of Health Grants HL57911, AI25316, and AI07384, American Foundation for AIDS Research Grant R29550-30-RCT; State of California’s Universitywide AIDS Research Program Grant R95-SD-165, the University of California, San Diego, Center for AIDS Research (supported by National Institutes of Health Grant AI36214), the Department of Veterans Affairs, and the Research Center on AIDS and HIV Infection of the Veterans Affairs San Diego Healthcare System. 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.

‡ Current address: Syrrx, Inc., San Diego, CA.

¶ To whom correspondence should be addressed: Dept. of Medicine, 0679, University of California at San Diego, 9500 Gilman Dr., La Jolla, CA 92093-0679. Tel.: 858-552-8585 (Ext. 2620); Fax: 858-552-7445; E-mail: rkornbluth@ucsd.edu.

† The abbreviations used are: PIC, preintegration complex; IPI, integrase-directed PIC inhibitor; LTR, long terminal repeat; NIPI, non-integrase-directed PIC inhibitor; PCR, polymerase chain reaction; HIV, human immunodeficiency virus; bp, base pair(s).
**Target DNA Preferences of HIV-1 Preintegration Complexes**

**EXPERIMENTAL PROCEDURES**

*Integration Substrates—*Plasmid DNA containing head-to-tail concatamers encoding for drag-line silk protein was kindly provided by Dr. David Kaplan, Biotechnology Center, Tufts University (20, 21). Plasmid DNA was isolated from transformed *Escherichia coli* DH5α and purified using a Maxi Prep Kit (Qiagen Inc., Valencia, CA). The insert in the plasmid contained monomeric units of 105 bp repeated 32 times. Insert DNA was prepared by overnight digestion with *Bam*HI (which creates the protruding 5’ ends favored by λ exonuclease, described below), purified by electrophoresis in agarose, and isolated using a QIAquick Gel Extraction Kit (Qiagen). To prepare single monomeric units of the 105-bp sequence, insert DNA was digested with *Sst*I (which cleaves between each unit) and similarly gel purified.

*Oligonucleotide Primers—*The real-time PCR (TaqMan) detection of integration junctions used the following oligonucleotides: forward primer LTRTaq5, 5’-GGTGTTGCGCGCCTTTGTTG-3’; reverse primer SILKREV1a, 5’-CAGCAGGGCCCATGTTG-3’; and HIV2, 5’-AGTGGTGGCCCTCCTTTGTTG-3’.

*Preparation of PIC-containing Cytoplasmic Extracts—*PICs were prepared from the detergent lysates of cells acutely infected by HIV-1_LAV (LAV-1 strain) (22, 23). Briefly, CEM cells were infected by HIV-1_LAV under BS-3 conditions and cultured for 7–10 days until cytopathic changes (ballooning and apoptosis) were evident in the majority of cells by phase microscopy examination. Then, the cells were counted and co-cultured with a 10-fold excess of SupT1 cells (American Type Culture Collection, Manassas, VA) in RPMI 1640 with 10% fetal bovine serum at 5% CO₂ for 10 days until cytopathic effects were barely visible. A barely visible pellet was then resuspended in 170 μl of water and re-amplified in an identical manner. The resulting extract was added to the integration assay.

*In Vitro Integration Reactions—*Integration reactions (15 μl total volume) were prepared on ice in 200-μl thin-walled PCR tubes (either in 8-tube strips or in 96-well plates) by adding 5 μl of a master mixture consisting of 1 μl of target DNA (3.3–100 ng/μl with 11–33 ng/μl S32 concaner being optimal for general use), 1.5 μl of 10 × integration buffer (200 mM HEPES, pH 7.4, 50 mM MgCl₂, 10 mM dithiothreitol), and 2.5 μl of 30% PEG-8000 (Sigma). Then, 10 μl of PIC-containing cytoplasmic extract was added and the tube contents were mixed by pipetting up and down. The tubes were kept on ice and placed in a thermocycler (GeneAmp 9600, Applied Biosystems, Foster City, CA) and incubated at 4 °C for 10 min, 37 °C for 45 min, and then at 60 °C for 5 min.

*Post-reaction Processing—*Unreacted target DNA was removed by adding 3–5 units of λ exonuclease in 15 μl of a 2 × concentration of its supplied buffer (New England Biolabs, Beverly, MA). The tubes (now containing inserts and unreacted cytoplasmic extracts) were then returned to the thermocycler plates at 37 °C for 45 min (which allows the λ exonuclease to degrade the target DNA) followed by 75 °C for 10 min (to heat-inactivate the λ exonuclease). The processing was completed by adding 20 μl of proteinase K (1 mg/ml in water) and incubating in the thermocycler at 60 °C for 30 min, followed by heat inactivation at 95 °C for 15 min. The processed integration reactions (now 50 μl in volume) could be stored at 4 °C for at least 1 week prior to TaqMan analysis. In some experiments, processed and unprocessed reactions were analyzed by polyacrylamide gel electrophoresis, staining with SYBR Gold (Molecular Probes, Eugene, OR), and visualization with UV light.

*Real-time Fluorescence-monitored PCR Detection of Integration Junctions—*In some experiments, the λ exonuclease and proteinase K-provided integration reactions were digested with 20 μl of a master mix containing 12.5 μl of TaqMan Universal PCR Master Mix 2X (Applied Biosystems), 2.5 μl each of 9 μM LTRTaq5 and SILKREV1a amplification primers (900 nm final concentration), and 2.5 μl of 2 μM LTRTaq5 probe (200 nM final concentration). Five μl of the processed integration reaction was added to each well, and the tube contents were mixed by pipetting up and down. Real-time fluorescence-monitored PCR reactions (TaqMan) were performed on an Applied Biosystems Model 7700 Sequence Detection System. The temperature profile for the reaction was: 50 °C for 2 min, 95 °C for 10 min, and then 95 °C for 15 and 60 °C for 1 min for 45 cycles. Using the manufacturer’s software, the cycle number at which fluorescence exceeded background (Cₚ) was determined for each well. For each real-time PCR analysis, a standard curve was generated using a dilution series of a cloned integration junction (below) calculated to provide 3, 10, 100, and 1000 integration junctions per well. The reactions were set up as 2- or 3-fold replicates and typically differed by less than 0.4 Cₚ.

*Data Analysis of the Integration Assay—*To deduce the number of integration junctions in each sample, its Cₚ value was compared with the cloned integration junction standard curve following linear regression analysis. Because each TaqMan reaction used only 5 μl of the 50 μl of processed integration reaction, the number of integration junctions was then multiplied by 10 to arrive at the number of integration events produced by the original 10 μl of PIC-containing cytoplasmic extract. If the processed reaction was diluted prior to TaqMan analysis, then an additional correction was made for the dilution factor. No correction was made, however, for the presumably equal number of integrations into the other, unmeasured strand of the target DNA. Statistical calculations were performed using the Instat software program (GraphPad Software Inc., San Diego, CA).

*Hemi-nested PCR for Cloning and Sequencing of Representative Integration Junctions—*Hemi-nested PCR was used to isolate PCR products in order to confirm that the method identifies integration junctions. For the first round of PCR, integration reactions were diluted 1:100 in water and 5 μl was amplified in a 25-μl reaction using HIVout and SILKREV1a (above) and HotStartTaq (Qiagen) according to the manufacturer’s instructions. The reactions were heated to 95 °C for 15 min; then 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s for 25 cycles; and finally 72 °C for 10 min. The first round reaction products were then diluted 1:100 in water and re-amplified in an identical manner using HIVin2 and SILKREV1v. The first-round reaction products were then electrophoresed in agarose, isolated using a QIAquick Gel Extraction Kit (Qiagen), and cloned into the pCR4-TOPO vector using a TOPO TA Cloning Kit (Invitrogen Corp., Carlsbad, CA). Dye-terminator sequencing of eight clones was performed by the Molecular Biology Core of the UCSD Center for AIDS Research using the kit-supplied primers. One of the sequenced clones was used to create an integration junction standard curve in the TaqMan reaction above.

*Preparation of Salt-stripped PICs—*Salt-stripped PICs were prepared by a modification of the methods of Lee and Craige (26) and Chen and Engelmann (15). 500 μl of PIC-containing cytoplasmic extract was diluted in an equal volume of Buffer A (above) containing 0.025% digitonin but formulated without KCl to reduce the salt concentration to 75 mM. The diluted PICs were incubated at 4 °C for 30 min and then pelleted at 8,000 × g for 20 min in a refrigerated microcentrifuge. The barely visible pellet was then resuspended in 170 μl of Buffer A with 1.2 M KCl and incubated for 30 min on ice. Then, the hypertonic PIC solution was loaded onto a Sepharose CL-4B column (2.2 ml bed volume) that had been pre-equilibrated in the same buffer (but not pre-cleared). The column was then washed with a tube and centrifuged at 800 × g for 3 min at 4 °C. About 1,500 μl was retrieved from each column and then concentrated to about 40 μl by centrifugation at 800 × g in a Microcon-100 ultrafiltration unit (Millipore), taking care not to allow the sample to concentrate to dryness. The amount of HIV LTR cDNA was then quantified by TaqMan (above) so that samples could be adjusted to equal numbers of PICs before placing them in the integration assay.
Reconstitution of Salt-stripped PICs—Cytoplasmic extracts were prepared from uninfected SupT1 cells using exactly the same protocol used to prepare PIC-containing extracts. 5 μl of this extract was added to 2 μl of salt-stripped PICs and incubated on ice for 15 min. Then, 5 μl of Buffer A without salt was added to bring the KCl concentration down to about 262 mM, and the mixture was incubated on ice for another 15 min to allow the cytoplasmic proteins to assemble onto the salt-stripped PICs. Then, 10 μl of this mixture was analyzed in the integration assay above.

RESULTS

Design of a Concatemeric Target DNA and Primers for the Detection of Integration Junctions

To investigate the effect of target DNA length on the efficiency of integration, we chose a concatemeric DNA substrate containing 32 head-to-tail monomeric units encoding dragline silk, a natural protein composed of repeating units (27). To amplify integration junctions, PCR primers were designed to hybridize to the 3’ LTR of HIV-1 (forward primer) and the silk target DNA (reverse primer). The concatemeric arrangement of the target DNA places a binding site for the reverse primer within a short distance (≤183 bp) from the LTR forward primer and effectively limits amplicon size to a length that is optimal for TaqMan detection. Although both the 5’ and 3’ LTRs integrate into the target DNA during concerted integration, only the integration of the 3’ LTR was analyzed in these studies. Also, although HIV can integrate into the target DNA in either orientation, the assay only detects integration junctions in one of the two target DNA strands (shown as the top strand in Fig. 1).

Integration Junctions Are Correctly Amplified by the PCR Assay System

As a first test of the PCR system depicted in Fig. 1, amplicons containing putative integration junctions were isolated by hemi-nested PCR and cloned into a plasmid. Sequencing analysis revealed that the integrations had occurred into various sites of the target DNA, with a possible “hot spot” immediately 5’ to the reverse primer (data not shown). In several cases, the cloned amplicons contained an additional monomeric unit of target DNA on their 3’ end resulting from the hybridization of the reverse primer to a binding site in the next unit adjacent to the integration site.

Real-time PCR Detection of Integration Events

To validate the real-time PCR detection step, control reactions were set up lacking either PICs or target DNA. As expected for a 45-cycle real-time PCR, these negative control integration reactions yielded C_t values equal to 45, indicating that no integration junctions were detected. To quantify positive reactions, dilutions of a cloned integration junction were prepared to create the standard curves used to relate the C_t values obtained from an unknown sample to the number of integration events that occurred. When reaction mixtures were diluted prior to detection, the final number of integration events reported was calculated by multiplying by the dilution factor. No correction was performed to account for the presumably equal number of integrations that occurred into the opposite, unmeasured strand of the target DNA.

Fig. 2 presents data representative of five experiments. When PICs and target DNA were both present in an integration reaction and the λ exonuclease processing step was omitted, a signal corresponding to 500–1,000 integration events was detected. If the reaction mixtures were diluted prior to PCR, however, the number of integration events detected (corrected for the dilution factor) increased, indicating that the reaction mixtures contained an inhibitor for the PCR detection step. This disturbing nonlinearity likely explains why there has been no previous report of a quantitative PIC assay based upon the PCR quantification of integration junctions. After an exhaustive analysis, it was determined that the principal cause of this inhibition of detection is the carryover into the PCR detection step of unreacted target DNA, which contains a very large number of binding sites for the reverse primer (black box) is within 165 bp downstream of the integration junction.

However, in part because the great excess of binding sites for the reverse primer interferes with the subsequent PCR reaction, unreacted target DNA is removed using an exonuclease which digests DNA in the 5’ → 3’ direction. Proteins bound to the LTR protect the integration junction from the exonuclease digestion (see text for discussion). Next, proteinase K is used to remove the exonuclease and other proteins, following which the proteinase K itself is inactivated by heat treatment. Bottom panel depicts the PCR detection step. The first cycle is critical as it depends solely on the reverse primer to create the strand complementary to the integration junction. Once the double-stranded amplicon has been formed (containing a binding site for the forward primer at the position shown by the open box in the LTR), succeeding cycles of PCR can utilize both primers and exponential amplification results. During the real-time PCR detection process, a TaqMan probe binds to an additional site in the LTR (shown as the hatched box). The 5’ exonuclease activity of Taq polymerase then cleaves the fluorophore (F) in the probe away from the quencher (Q), generating the signal measured by fluorescence detection.
Real-time fluorescence PCR detection of integration events. Integration reactions were performed as described under “Experimental Procedures” using 11 ng of 32-mer target DNA. The − or + symbols indicate if the sample was analyzed either without or with λ exonuclease digestion prior to the detection step. Samples with a dilution factor of 1 were tested undiluted, whereas samples with a dilution factor of 10 were diluted 10-fold prior to the detection step as described under “Experimental Procedures.” The number of integration events produced by the original 10 μl of PIC-containing cytoplasmic extract was calculated by multiplying by the dilution factor. The improvement in the assay resulting from dilution and/or λ exonuclease treatment results from the depletion of target DNA which interferes with the subsequent PCR detection step (see text for discussion). The data shown are the means of duplicate samples and are representative of five experiments.

primer creating the complementary strand to which the forward primer binds (whereas this strand pre-exists when a cloned integration junction is used). In this case, a simple increase in the reverse primer concentration was not sufficient to eliminate the nonlinearity of the assay (data not shown).

A close examination of the amplification plots revealed an additional problem caused by the carry-over of target DNA into the TaqMan reaction. In these cases, the slope of the amplification plot was markedly less than that of the cloned integration junction that was used as a standard. In effect, the target DNA prevented the amount of product from doubling with each cycle of PCR. The atypical shape of the amplification plots from the samples containing target DNA reduced the sensitivity of the TaqMan analysis by raising the apparent Ct, and also precluded the possibility of converting the assay to an end point detection format.

Fortunately, two strategies were found which succeeded in removing the nonlinearity of the assay by reducing or eliminating the carryover of target DNA into the PCR detection step. The first strategy is simply to dilute the integration reaction mixtures prior to PCR detection. However, the drawback of diluting the reaction mixtures is that this also dilutes the integration junctions that the real-time PCR aims to detect. For example, diluting the reaction mixtures beyond 100-fold prior to TaqMan detection generally resulted in Ct values outside of the dynamic range of the assay. A second strategy is to selectively remove the target DNA in order to prevent it from inhibiting the PCR detection step. This approach was not associated with the loss of sensitivity that occurred when the dilution method is employed. λ Exonuclease, which degrades double-stranded DNA containing phosphorylated, protruding 5′ ends, was employed for target DNA removal prior to the PCR detection step. An examination of the λ exonuclease-treated reactions by polyacrylamide gel electrophoresis and SYBR Gold nucleic acid staining showed that all visible DNA in the reactions had been removed (data not shown). However, integration junctions evidently survive this λ exonuclease step because PIC proteins bound to the DNA block the 5′ → 3′ digestive action of this exonuclease as it approaches the integration site (12). These PIC proteins may be part of the complex (the “intasome”) that has been detected on the ends of integrated Moloney murine leukemia virus LTR DNA using a sensitive DNA footprinting method (28). Supporting the concept that λ exonuclease effects the selective removal of target DNA, pretreatment of an integration reaction with λ exonuclease prior to detection consistently resulted in a 4–10-fold increase in detection sensitivity (Fig. 2). Also, the amplification plots of the λ exonuclease-treated samples showed a normal slope in parallel to the cloned integration junction standards, which could be important for converting the assay into a simplified end point detection format.

A Longer, Concatemerized Target DNA Permits the Detection of a Higher Number of Integration Events

To study the effects of target DNA length on the efficiency of PIC integration in vitro, target DNAs consisting of either 1 (S1) or 32 (S32) monomeric units of the same silk coding sequence were used in the integration assay system (Fig. 3). Two major conclusions were drawn from these experiments: 1) for a given number of monomeric units of target DNA present in the integration reaction, the concatemerization of those units into a longer molecule resulted in a 2- to >10-fold increase in the integration efficiency. 2) Overall, integration efficiency rises with an increase in the amount of target DNA molecules (in terms of monomeric units) (Fig. 3). This indicates that the amount of target DNA in the integration reaction must be kept high in order to maximize the number of integration events, even though the carryover of target DNA inhibits the subsequent real-time PCR detection step. As a counterbalancing factor, the linearity of the assay for PICs is best at ≈9.27 × 1010 monomers/15 μl reaction (see below and data not shown), a concentration where only the 32-mer can be used.

Applications of the PIC Integration Assay

Quantifying Integration-competent PICs—To validate that the integration assay system using long target DNA concatemers can actually measure integration-competent PICs, PIC extracts were serially diluted and then assayed. Based on the data obtained in the experiments described above, the number of integration events was quantified in undiluted integration
reactions using 32-mer concatemeronic target DNA and λ exonuclease treatment in order to maximize detection efficiency. As shown (Fig. 4), the number of integration events decreased proportionally to PIC dilution. For the PIC extract tested, dilutions beyond 9-fold produced Ct values that were outside of the linear range of the assay. In other experiments, the target DNA concentration was reduced resulting in less inhibition of the real-time PCR detection step. In these cases, integration events were detectable in PIC extracts diluted 27-fold, even though the absolute number of integration events at each dilution was lower. These results indicate that the PIC assay system using long concatemeric target DNA can reliably quantify the number of integration competent PICs in a sample. The system is so sensitive that a single 50-ml culture of infected SupT1 cells generates enough PICs for over 10,000 assays, which makes it possible to use authentic HIV-1 PICs in a drug discovery program. Additionally, the assay can be used to guide the purification of PICs by ultracentrifugation in a sucrose gradient (data not shown).

Detection and Characterization of Integrase-directed PIC Inhibitors (IPIs)—A number of compounds have been shown to inhibit HIV-1 integration in vitro. To determine whether PICs could be preincubated with candidate inhibitors, the stability of PIC extracts at 37 °C was first examined. As shown Fig. 5 (inset), PIC-containing cytoplasmic extracts were stable at 37 °C for about 20 min, but became unable to integrate into target DNA by 1 h. Next, two known inhibitors of HIV-1 integrase, purpurin and quinalizarin, were studied using the PIC integration assay system. As expected, both integrase inhibitors were clearly identified as integration inhibitors using PICs (Fig. 5), indicating that an assay based on long, concatemeric target DNA can be used to identify IPIs.

Detection of Host Cell Factors Capable of Enhancing PIC Integration and Their Inhibitors—Lee and Craigie (11) introduced the technique of removing necessary factors from PICs under conditions of high salt. These “salt-stripped” PICs can then be recombined with extracts from uninfected host cells in order to identify cellular factors that may be important for retroviral integration. Consequently, HIV-1 PICs were salt-stripped and found to have no detectable integration competence using the PIC integration assay system with long, concatemeric target DNA. However, when cytoplasmic extracts of

The presence of an amplification product in a PCR reaction with primers specific to viral and target DNA molecules participating in an integration reaction would indicate that an integration event had occurred. Recently developed, highly sensitive, real-time fluorescence-monitored PCR technology provides a way to obtain accurate quantification of a PCR product within a 2–3-h period. This approach has maximum amplification efficiency and sensitivity for small amplions. However, host genomic DNA, the natural target of retroviral integration, has substantial length and structures that are characteristic for longer DNA molecules. To mimic this long target DNA, plasmid DNA was previously employed as a target DNA in conjunction with PCR-based detection using LTR-specific and plasmid DNA-specific oligonucleotides (29). However, the
length of the PCR product amplified as a result of integration varies significantly in this format, since the retroviral genome integrates nearly randomly into the target DNA. Thus, an integration assay utilizing plasmid DNA as a target DNA could not be adopted for the real-time PCR detection of integration events.

Alternatively, a short DNA molecule could be utilized as a target DNA in an integration assay. In this case, one can place an upper bound on the length of the PCR amplicon containing the integration junction. On the other hand, a short DNA molecule has little resemblance to host genomic DNA and is a very poor substrate for PIC integration (Fig. 3). This contrasts with the strand-transfer assay for integrase, where short target DNA molecules are typically used for the integration reaction (6, 7).

Utilization of long, concatemeric DNA as an integration target overcomes these limitations. By designing a forward oligonucleotide primer near the very end of the 3’ LTR and a reverse primer for a site in each 105-bp monomeric unit, amplicon size was maintained within the limits optimal for efficient real-time PCR detection (Fig. 1). Several thousand integration events were routinely detected in 10 µl of PIC extract using real-time fluorescence-monitored PCR detection based on measurements for 3’ LTR integration only.

Concatemeric target DNA provided an opportunity to conduct a systematic study of the relationship between target DNA length and the efficiency of HIV-1 cDNA integration. This analysis was performed under conditions where the target DNA sequence remained constant but only the degree of multimerization varied. The results of these experiments were normalized in terms of an absolute number of monomeric units present in an integration reaction. From this analysis, concatenation of the target DNA resulted in a 2- to >10-fold increase in the number of integration events detected, which also depended on the concentration of target DNA (number of monomers) present in the integration reaction (Fig. 3). In this context, a recent study on the diffusion coefficients of double-stranded DNA molecules showed that they were highly dependent on DNA length and the media in which the DNA was tested. In the cytoplasm of HeLa cells, a large DNA fragment (>2,000 bp) diffused 20 times slower than a smaller, 100-bp DNA fragment (30). This raises the possibility that PICs prefer to integrate into a longer 32-mer target DNA (3,360 bp) because a slowly moving target DNA favors the formation of a PIC-target DNA complex. Maintaining target DNA in solution may be vital for optimum sensitivity, however. In a previous high-throughput PIC assay, target DNA was immobilized to the bottom of 96-well plates to capture integrated PICs which were subsequently detected by real-time fluorescence-monitored PCR with primers specific solely for the viral LTR, not the integration junctions (31). In this assay, which we estimate is 20 times less sensitive than the assay in the present report, PICs must diffuse to the bottom of the wells in order to interact with the immobilized target DNA, making diffusion a rate-limiting factor for integration.

Although it was originally reported that excess DNA does not affect PCR amplification reactions including real-time PCR (32), recent studies have shown that large amounts of double-stranded DNAs can completely suppress the PCR reaction (33, 34). In the present study, the excess target DNA that was required for the integration reaction was carried over to the PCR detection step, where it became the major inhibitor of PCR amplification. This complication is the likely reason why there has been no previous report of a quantitative PIC integration assay using PCR to detect integration junctions. Two independent ways were devised to overcome the inhibition of PCR detection caused by target DNA carryover: 1) dilution of the integration reaction mixture prior to detection; and 2) selective degradation of target DNA by λ exonuclease. Overall, the combination of λ exonuclease treatment and 10-fold dilution resulted in the highest number of integration events detected in a given sample, after adjustment was made for the dilution.

This study also demonstrated that the ability of PICs to integrate decays within 1 h at 37 °C under the conditions studied (Fig. 5, inset). This may be due to autointegration by PICs resulting from intramolecular or intermolecular interactions with HIV-1 cDNA (35). In this context, the preference for longer target DNA may reflect an improvement in target commitment caused by decreased diffusion of the target DNA away from the PIC. Through target DNA competition studies, Miller et al. (36) established that cytoplasmic extracts contain factors which interfere with the ability of PICs to quickly integrate into a target DNA molecule with which they may come into contact. The decreased diffusion of a long target DNA molecule may act to prolong the period of contact with a PIC, thereby facilitating the proposed target-induced conformational changes in the arrangement of integrase molecules on the LTR ends (37), before the decay of PIC activity becomes a limiting factor.

The PIC integration assay was successfully used to quantify the number of integration-competent PICs in a sample over a dynamic range of 1–1.5 orders of magnitude, depending on amount of target DNA used. Integration events from 10-µl samples containing as few as 5 × 10^4 copies of cDNA could be measured with a high degree of reproducibility.

The reliable measurement of integration events is particularly important for the development of integration inhibitors and for studies on the mechanism of retroviral integration. The activity of two previously described integrase inhibitors, purpurin and quinalizarin, was demonstrated in this assay, with IC_{50} values that were similar to previously published values (8). In addition, the experiments showing the reconstitution of salt-stripped PICs by cytoplasmic extracts from uninfected host cells (Fig. 6) demonstrate the possibility of using this assay to detect NIPIs, a putative new category of antiviral drugs.

In conclusion, we have developed a quantitative assay for the direct detection of authentic integration junctions. The advantage of utilizing a long, concatemeric target DNA was shown. In its final form, the assay consists of only three steps performed in a single tube and yields quantitative data within 4–5 h. The
assay was successfully applied to measure the number of integration-competent PICs, to guide the purification of PICs, to detect host cell factors which restore the integration competence of salt-stripped PICs, and to characterize integration inhibitors. Converting from real-time PCR detection to an end point detection system will enable this assay to be used in a high-throughput screen for novel retroviral integration inhibitors of either the IPI or NIPI categories.

Acknowledgments—Janette D. Rhodes contributed to initial experiments designed to construct a concatemeric target DNA. We thank Dennis Sheeter and Jacques Corbeil (UCSD CFAR Genomics Core) for assistance in setting up the TaqMan assay.

REFERENCES

1. Hansen, M. S., Carteau, S., Hoffmann, C., Li, L., and Bushman, F. (1998) Genet. Eng. (N. Y.) 20, 41–61
2. Asante-Appiah, E., and Skalka, A. M. (1999) Adv. Virus Res. 52, 351–369
3. Wiskerchen, M., and Muesing, M. A. (1995) J. Virol. 69, 376–386
4. Moore, J. P., and Stevenson, M. (2000) Nat. Rev. Mol. Cell. Biol. 1, 40–49
5. Hazuda, D. J., Felock, P., Witmer, M., Wolfe, A., Stillmock, K., Grobler, J. A., Espeseth, A., Gabryelski, L., Schleif, W., Blau, C., and Miller, M. D. (2000) Science 287, 646–650
6. Hazuda, D. J., Wolfe, A. L., and Emini, E. A. (1994) Nucleic Acids Res. 22, 1121–1122
7. Craigie, R., Mizuuchi, K., Bushman, F. D., and Engelman, A. (1991) Nucleic Acids Res. 19, 2729–2734
8. Farnet, C. M., Wang, B., Lipford, J. R., and Bushman, F. D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9742–9747
9. Bukinsky, M. I., Sharova, N., McDonald, T. L., Pushkarskaya, T., Tarpley, W. G., and Stevenson, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6125–6129
10. Heinzinger, N. K., Bukinsky, M. I., Haggerty, S. A., Ragland, A. M., Kewalramani, V., Lee, M. A., Gendeiman, H. E., Ratner, L., Stevenson, M., and Emerman, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7311–7315
11. Lee, M. S., and Craigie, R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9823–9827
12. Miller, M. D., Farnet, C. M., and Bushman, F. D. (1997) J. Virol. 71, 5382–5390
13. Farnet, C. M., and Bushman, F. D. (1997) Cell 88, 483–492
14. Kalpana, G. V., Marmon, S., Wang, W., Crabtree, G. R., and Goff, S. P. (1994) Science 266, 2002–2006
15. Chen, H., and Engelman, A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15270–15274
16. Bor, Y. C., Bushman, F. D., and Orgel, L. E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10334–10338
17. Przybyla, P. M., and Varmus, H. E. (1992) Cell 69, 769–780
18. Pruss, D., Bushman, F. D., and Wolffe, A. P. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5913–5917
19. Bor, Y. C., Miller, M. D., Bushman, F. D., and Orgel, L. E. (1996) Virology 222, 283–288
20. Prince, J. T., McGrath, K. P., DiGrolamo, C. M., and Kaplan, D. L. (1995) Biochemistry 34, 10879–10885
21. Winkler, S., Wilson, D., and Kaplan, D. L. (2000) Biochemistry 39, 12739–12746
22. Farnet, C. M., and Haseltine, W. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4164–4168
23. Ellison, V., Abrams, H., Roe, T., Lifson, J., and Brown, P. (1990) J. Virol. 64, 2711–2715
24. Terai, C., Kernbluth, R. S., Panza, C. D., Richman, D. D., and Carson, D. A. (1991) J. Clin. Invest. 87, 1715–1715
25. Rossini, J. L., Eser, M. T., Suryanarayana, K., Schneider, D. K., Bess, J. W., Jr., Vasquez, G. M., Wiltout, T. A., Chertova, E., Grimes, M. K., Sattentau, Q., Arthur, L. O., Henderson, L. E., and Lifson, J. D. (1998) J. Virol. 72, 7992–8001
26. Lee, M. S., and Craigie, R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1528–1533
27. Winkler, S., Suda, S., Avtges, P., Valluzzi, R., Kirschner, D. A., and Kaplan, D. L. (1999) Int. J. Biol. Macromol. 24, 265–270
28. Wei, S. Q., Mizuuchi, K., and Craigie, R. (1997) EMBO J. 16, 7511–7520
29. Shibagaki, Y., and Chow, S. A. (1997) J. Biol. Chem. 272, 8361–8369
30. Lukacs, G. L., Haggie, P., Sekseck, O., Lechardeur, D., Freedman, N., and Verkman, A. S. (2000) J. Biol. Chem. 275, 1625–1629
31. Hansen, M. S., Smith, G. J., 3rd, Kafri, T., Molteni, V., Siegel, J. S., and Bushman, F. D. (1999) Nat. Biotechnol. 17, 578–582
32. Holland, P. M., Abramsen, R. D., Watson, R., and Gelfand, D. H. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 7276–7280
33. Kainz, P., Schmiedlechner, A., and Strack, H. B. (2000) Biotechniques 28, 278–282
34. Kainz, P. (2000) Biochem. Biophys. Acta 1494, 23–27
35. Farnet, C. M., and Haseltine, W. A. (1991) J. Virol. 65, 6942–6952
36. Miller, M. D., Bor, Y. C., and Bushman, F. (1995) Curr. Biol. 5, 1047–1056
37. Gao, K., Butler, S. L., and Bushman, F. (2001) EMBO J. 20, 3565–3576
HIV-1 Preintegration Complexes Preferentially Integrate into Longer Target DNA Molecules in Solution as Detected by a Sensitive, Polymerase Chain Reaction-based Integration Assay
Alexei Brooun, Douglas D. Richman and Richard S. Kornbluth

J. Biol. Chem. 2001, 276:46946-46952. doi: 10.1074/jbc.M108000200 originally published online October 10, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M108000200

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 37 references, 21 of which can be accessed free at http://www.jbc.org/content/276/50/46946.full.html#ref-list-1