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Molecular interactions between HIV-1 integrase and the two viral DNA ends within the synaptic complex that mediates concerted integration⁺

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Summary

A macromolecular nucleoprotein complex in retrovirus-infected cells, termed the preintegration complex, is responsible for the concerted integration of linear viral DNA genome into host chromosomes. Isolation of sufficient quantities of the cytoplasmic preintegration complexes for biochemical and biophysical analyses is difficult. We investigated the architecture of human immunodeficiency virus type-1 (HIV-1) nucleoprotein complexes involved in the concerted integration pathway in vitro. HIV-1 integrase (IN) non-covalently juxtaposes two viral DNA termini forming the synaptic complex, a transient intermediate in the integration pathway and shares properties associated with the preintegration complex. IN slowly processes two nucleotides from the 3' OH ends and performs the concerted insertion of two viral DNA ends into target DNA. IN remains associated with the concerted integration product, termed the strand transfer complex. The synaptic complex and strand transfer complex can be isolated on native agarose gels for biochemical and biophysical analyses. In this report, in-gel fluorescence resonance energy transfer measurements demonstrated that the energy transfer efficiencies between the juxtaposed Cy3 and Cy5 5'-end labeled viral DNA ends in the synaptic complex (0.68 ± 0.09) was significantly different than observed in the strand transfer complex (0.07 ± 0.02). The calculated distances were 46 ± 3 Å and 83 ± 5 Å, respectively. DNaseI footprint analysis of the complexes revealed that IN protects U5 and U3 DNA sequences up to ~32 bp from the end, suggesting two IN dimers were bound per terminus. Enhanced DNaseI cleavages were observed at nucleotide positions 6 and 9 from the terminus on U3 but not on U5 suggesting independent assembly events. Protein-protein cross-linking of IN within these complexes revealed the presence of dimers, tetramers, and a larger-size multimer (>120 Kd). Our results suggest a new model where two IN dimers individually assemble on U3 and U5 ends prior to the non-covalent juxtaposition of two viral DNA ends, producing the synaptic complex.

Keywords

HIV-1; preintegration complex; integrase; synaptic complex

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Introduction

Retroviruses such as human immunodeficiency virus type-1 (HIV-1) replicate in cells by integrating a linear DNA copy of their RNA genome into host cell chromosomes. After reverse transcription, integrase (IN) binds to ~200 bp at the very ends of the viral DNA long terminal repeats (LTR) sequences forming cytoplasmic preintegration complexes (PIC)^{1–5}. Integration of viral DNA by IN requires two catalytic steps known as 3'-OH processing and strand transfer. Processing involves removal of the terminal GT dinucleotides from both the U3 and U5 blunt-ends in an independent manner^{6,7}. During 3'-OH processing, IN remains bound to the LTR ends and juxtaposes the ends in a non-covalently fashion². In the second step, the recessed 3'-OH ends are attached by IN in a concerted fashion into opposite DNA strands of the target chromosome with a canonical small size bp stagger, 5 bp in case of HIV-1. Finally, the 5'-overhang dinucleotides of the viral DNA are removed, and the single-stranded DNA gaps are repaired by cellular enzymes producing the host-site duplication^{8,9}.

Structural investigations of IN from different species of retroviruses show IN possesses three similar structural domains even though their amino acid sequences differ significantly⁸. The N-terminal domain (residues 1–49) is a helical bundle stabilized by the coordination of single zinc atom and is involved in IN dimerization. The catalytic core domain (residues 50–212) contains the D,D-35-E motif which binds Mg^{+2} or Mn^{+2} and plays the critical role in catalysis. The C-terminal domain (residues 213–288) also binds the LTR DNA and is involved in dimerization.

X-ray analyses of one domain or two domain structures of HIV-1 or other retrovirus IN domains show IN is dimeric in nature. The distance between the two active sites in the catalytic core domain is about 35 Å^{10,11}. The 5 bp duplication of target sequences upon concerted integration of HIV-1 LTR ends suggests that the two points of insertion must be ~18 Å apart. Thus, concerted integration must be mediated by two different dimers¹². No co-crystals structures are available for IN-viral DNA complexes. Site-directed mutagenesis, DNA-protein cross-linking, and other structural data were used for molecular modeling for IN-DNA complexes^{13–20}. Several experiments studying IN-viral DNA interactions and concerted integration suggest that a dimer of dimers or a tetramer of IN at the DNA ends prompts concerted integration^{21–23}.

To further understand the properties of HIV-1 IN within the PIC, we assembled and identified the synaptic complex (SC) on native agarose gels wherein IN non-covalently juxtaposes two 1.6 kb U5 blunt-end substrates^{24,25} (Fig. 1a). Kinetics of the integration reaction demonstrate that SC is the transient intermediate in the concerted integration pathway²⁴ and is the precursor to the strand transfer complex (STC)²³, the end product of concerted integration in vitro. The transient nature of SC appears related to the slow processing of a dinucleotide from each LTR end by IN²⁴ prior to binding supercoiled DNA and concerted integration, producing the STC.

In the present study of HIV-1 SC and STC, we performed in-solution fluorescence resonance energy transfer (FRET) using fluorophore-labeled viral DNAs. In-gel FRET analyses employing native agarose gels was used to measure FRET efficiencies (E) between viral DNA ends in these complexes. The E values between the two 5'-fluorophore-labeled LTR ends within SC and STC by in-gel FRET measurements were 0.68 ± 0.09 and 0.07 ± 0.02 , respectively, suggesting significantly different arrangements of DNA ends within these complexes. The calculated distances were 46 ± 3 Å and 83 ± 5 Å, respectively. We observed a ~32 bp DNaseI protective footprint on both the U3 and U5 ends in SC and STC suggesting a similar global arrangement of IN subunits within these complexes. Using several different protein-protein cross-linkers, we identified dimers, tetramers, and a large-size IN multimer in both nucleoprotein complexes. Our results suggest that IN dimers exists as independent multiple

species within SC and that two IN dimers align on each individual LTR end covering ~32 bp prior to juxtaposition of the two LTR ends to form SC.

Results

Fluorophore-labeled viral DNA substrates do not affect assembly of HIV-1 SC and concerted integration activity

The assembly pathway to form SC and STC using IN and viral DNA substrate is depicted in Fig. 1. The STC contains the concerted or full-site (FS) integration product. Fluorophores, either Cy3 or Cy5, were placed at the catalytic 5' end of 1.6 kb U5 blunt-ended DNA substrate. For a typical experiment, Cy3-U5 DNA (3 nM) was assembled with 60 nM IN at 14°C for 15 min, followed by addition of supercoiled DNA (3 nM). The samples were incubated at 37°C for 20 min for SC assembly (Fig. 1b, lane 1) and 120 min for STC (Fig. 1b, lane 2), prior to electrophoresis on a 0.7% native agarose gel. A higher-order form of SC (H-SC)²⁴ was also produced which is probably due to DNA looping by IN (Fig. 1a, lane 1). It is evident from time course experiments, that both SC and H-SC are transient structures which peak at ~30 min and decrease significantly by 120 min, being converted to the STC (Fig. 1b, lanes 1 and 2)^{24,25}. Other studies also demonstrated that Cy3- or Cy5-U5 DNA (data not shown) do not interfere with assembly of SC compared to ³²P-5'-end labeled U5 DNA^{24,25}. In summary, equivalent assembly was observed with SC and STC using Cy5-U5 DNA (data not shown) and Cy3-U5 DNA (Fig. 1).

Deproteinization of the above samples yielded the expected strand transfer products (Fig. 1c, lanes 3 and 4). The integration products are: FS, circular half-site (CHS) and donor-donor (D-D)²⁴. Concerted integration activities of Cy3-U5 or Cy5-U5 DNA substrates, calculated from Cy3 or Cy5 fluorescence scans, respectively, were identical to those calculated from PhosphorImager analysis of these same substrates, when labeled with ³²P on the non-LTR end (data not shown). In summary, fluorophores at the 5' end of LTR substrates do not affect assembly of SC and the concerted integration reaction.

Specificity of HIV-1 IN-LTR DNA complexes as analyzed by in-solution FRET

We investigated the FRET properties of complexes formed with varying concentrations of HIV-1 IN and equal molar concentrations (1.5 nM) of each 1.6 kb U5 blunt-ended DNA labeled with Cy3 (donor fluorophore) and Cy5 (acceptor fluorophore) (Fig. 2). IN-DNA complexes were assembled at 0.1 M NaCl in the presence of target DNA and incubated for 2 h at 37°C, to form the STC²⁴. The samples were then adjusted to 0.5 M NaCl concentration prior to measuring the emission spectra in the range of 560 to 720 nm while exciting the donor fluorophore at 550 nm at 14°C (Fig. 2a). The STC is stable at 0.5 M NaCl and eliminates IN non-specific interactions with DNA^{23,26} (data not shown). Upon increasing IN concentrations, the enhancement of the acceptor emission fluorescence in DNA complexes containing the donor and acceptor-labeled DNAs was associated with a concomitant quenching of the donor fluorescence (Fig. 2a and 2b). This reciprocal spectral change is qualitative evidence of resonance energy transfer. Similar FRET data were obtained with other time-dependent studies of STC formation at a constant IN concentration (60 nM) (data not shown). With in-solution analysis, HIV-1 IN binding to the fluorophore-labeled DNAs did not affect the quantum yield and peak positions of Cy3- or Cy5-U5 DNA substrates (data not shown), as previously observed with Rous sarcoma virus (RSV) IN complexed with Cy3- and Cy5-labeled 93 bp LTR DNAs²⁷. With nonspecific 1.6 kb DNA substrates labeled at the 5' end with Cy3 and Cy5, there was no detectable energy transfer suggesting that IN is unable to juxtapose non-specific DNA ends (Fig. 2b) nor promote strand transfer activities (data not shown). In summary, in-solution FRET measurements established energy transfer occurred between Cy3-U5 and Cy5-U5 DNA substrates. But, the observed FRET signal is an cumulative effect of several IN-U5

DNA complexes besides the STC (Fig. 1b). As shown below, separation of these nucleoprotein complexes on native agarose gels allowed FRET analysis of these individual complexes.

In-gel FRET analysis of SC and STC

FRET efficiency depends on the inverse-sixth-power of the distance between donor and acceptor fluorophores and provides distance information if the labeled biomolecules are within 10 to 100 Å range^{28,29}. In-gel FRET analysis of SC and STC, separated on a native agarose gel, allowed us to determine the E values between the viral DNA ends in these complexes and calculate their distances.

For in-gel FRET analysis, 3 nM Cy3-U5 DNA (Fig. 3a, lanes 1–6), 1.5 nM each of Cy3-U5 and Cy5-U5 (lanes 7–12), and 3 nM Cy5-U5 (lanes 13–18) were incubated with increasing concentrations of HIV-1 IN. Figure 3a and 3b show the laser scans of sensitized and quenched FRET of SC and STC after electrophoresis on a 0.7% native agarose gel. Sensitized and quenched FRET signals, followed by ImageQuant analysis, were calculated (see Materials and Methods for details). The observed sensitized FRET signal for SC at 670 nm (Fig. 3d) was due to excitation of the donor at 532 nm (Fig. 3a, lanes 7–12) when normalized and compared with lanes 1–6 (donor) and lanes 13–18 (acceptor). Increasing concentrations of IN demonstrated an increased sensitized FRET signal, as shown by in-solution analysis (Fig. 2). The quenched FRET signal for SC (Fig. 3d) was also observed in lanes 7–12 of Fig. 3b (donor and acceptor), when normalized and compared with lanes 1–6 (donor). The quantities of SC and STC formed in each corresponding lane (without and with acceptor) were determined from SYBR Gold staining of the same gel (Fig. 3c), to normalize the fluorescence intensities. Using equation (1), the calculated E values (Table 1) from the quenched FRET signal remained the same for a particular nucleoprotein complex (see below) at different concentrations of IN. Energy transfer was detectable in H-SC also (Table 1). No energy transfer was detectable within the CHS complex on the native gel (Fig. 3) since CHS is the result of a single insertion of either a Cy3-U5 DNA or a Cy5-U5 DNA end into DNA. With independent experiments, the intensities of sensitized and quenched in-gel FRET for STC using the same IN concentrations were calculated (Fig. 3e). In summary, the results demonstrate that energy transfer is occurring between Cy3-U5 and Cy5-U5 DNA within SC, H-SC, and STC.

FRET efficiency (E) between two juxtaposed U5 ends within SC, H-SC, and STC

The E values for the different nucleoprotein complexes were determined from quenching of donor fluorescence. The quenching of donor fluorescence from different experimental protocols was calculated; e.g., protein titration (Fig. 3) and acceptor titration (data not shown). The E values for a particular complex (SC, H-SC, STC) were similar with either experimental protocol (Table 1). Since the labeling of donor and acceptor were not stoichiometric, the following equation was used to determine the E value for a particular nucleoprotein complex³⁰:

$$\frac{I^D - I^{DA}}{I^D} = 1 - \frac{[\text{complex}]^{DA}}{[\text{complex}]^D} (1 - fE) \quad (1)$$

where I^{DA} and I^D are the fluorescence intensities of the donor fluorophore in the presence and absence of acceptor fluorophore, respectively. The $[\text{complex}]^{DA}$ and $[\text{complex}]^D$ are the total amount of the product in the complexes in presence and absence of acceptor fluorophore, respectively. The value for f , which equaled 0.8, is the degree of acceptor fluorophore labeling.

The distance R between the two U5 blunt-ends labeled with donor and acceptor fluorophores

in these complexes was calculated from the relation: $R = R_0 \sqrt[6]{\frac{1}{E} - 1}$, where R_0 is the Forster distance for Cy3 and Cy5 fluorophores. The R_0 value (52.8 ± 1 Å) was determined from triplicate experiments for quantum yield (Supplemental Table S1), absorption, and emission spectra of the 1.6 kb blunt-ended fluorophore-labeled substrate. Table 1 shows the calculated E values and corresponding distances between Cy3-U5 and Cy5-U5 ends within each complex. In SC and H-SC, the two U5 ends are non-covalently juxtaposed by IN²⁴. The E values for SC and H-SC were 0.68 ± 0.09 and 0.52 ± 0.08 , respectively. The distances were 46 ± 3 Å and 52 ± 3 Å, respectively. Importantly, the same E values were observed if the complexes were formed for either 20 min or 30 min at 37°C (Table 1) or for 120 min (data not shown). IN remains stably bound in the STC where both of the 3' OH recessed DNA ends are inserted in a concerted fashion into target DNA^{23,26}. The E value was 0.07 ± 0.02 between the fluorophores on the non-attached 5' two nucleotide overhangs, at 120 min (Table 1) or for 60 min (data not shown). The distance was 83 ± 5 Å in the STC. The experimental determined E values and the calculated distances suggest major structural rearrangements have occurred between the two U5 ends in SC and STC, but not between SC and H-SC.

Strand transfer inhibitor modifies the E value between LTR ends in SC

We determined that HIV-1 IN strand transfer inhibitors, with IC_{50} values of ~40 nM, prevent binding of target DNA to SC thus inhibiting concerted integration^{24,25}. We determined if the E value between the 5' DNA ends in SC, formed in the presence of an inhibitor, was changed. SC were formed for 30 min at 37°C in the presence of inhibitor (L-870,810) ranging from 200 nM to 2000 nM, prior to electrophoresis on native gels. At the inhibitor concentrations of 200, 500, 1000, and 2000 nM, we observed that the production of the full-site integration product was inhibited between 85% to 98%, as previously shown²⁴. The calculated E values (0.10 ± 0.04) were similar at all of the above inhibitor concentrations and were ~15% of the value observed in the SC without inhibitor (0.68 ± 0.09) (Table 1), indicating a DNA structural perturbation associated with SC formed in the presence of L-870,810. The calculated distance between the Cy3- and Cy5-U5 ends within SC in the presence of inhibitor was 77 ± 6 Å after 30 min (Table 1) or at 120 min (data not shown). Importantly, the fluorescence properties of Cy3- and Cy5-U5 were unaltered in SC formed in the presence of L-870,810 (data not shown). The results suggest that the strand transfer inhibitors changed the positions of the two U5 ends in SC.

DNaseI protection of LTR ends by IN in HIV-1 SC and STC

The fluorophore-labeled DNA allowed us to investigate the relative positioning of the DNA ends in SC without and with inhibitors and as well as to the STC. We used DNaseI protective footprint analysis to probe the global interactions of IN with the entire LTR region. HIV-1 IN protects ~200 bp from the ends on both U5 and U3 in PIC isolated from virus-infected cells^{3,5}. HIV-1 IN mutant (W235F) protects ~16 nucleotides of the terminal U5 DNA end from DNaseI digestion in strand transfer inhibitor-induced stable synaptic complex²³; the same size footprint was also observed with a 3' OH recessed U5 substrate which possessed a 3' dda that block strand transfer. RSV IN protects ~16 bp of the U3 terminal sequences³¹. We investigated the interactions of wild type HIV-1 IN at the U5 and U3 LTR DNA ends within the different IN-DNA complexes by the DNaseI protective footprint technique.

IN was incubated with 1.6 kb 5'-³²P-U5 blunt-ended DNA for 15 min at 14°C and then for 20 min at 37°C in presence of target DNA. Subsequently, the samples were equilibrated at 14°C for 10 min and digested with DNaseI (0.3 units) for 3 min (Fig. 4a, lanes 3 and 4) or without treatment (Fig. 4a, lane 1), prior to electrophoresis on a native gel. DNaseI treated SC and H-SC were isolated from the gel. The DNA was extracted from both complexes and subjected to

denaturing 15% polyacrylamide gel electrophoresis (Fig. 4b). Both SC and H-SC displayed an ~32 nucleotide DNaseI protective footprint (marked by rectangle on right)(Fig. 4b, lanes 5 and 6, respectively) when compared with naked DNA treated with DNaseI (Fig. 4b, lane 7). The DNaseI digestion pattern is uniform above nucleotide 32-G up to at least nucleotide 60-C and upstream (see bracket on right side) in all of the digested samples (Fig. 4b, lanes 5 to 7) suggesting, little or no IN interactions upstream of nucleotide 32-G. At ~32 bp, there is slight DNaseI enhanced cleavages in both complexes in comparison to the control digestion pattern (see Fig. 5 also). Two minor bands in SC and H-SC near nucleotides 24-A and 28-A were observed in SC and H-SC (Fig. 4b, lanes 5 and 6). PhosphorImager analysis showed that these two bands were reduced 92% in comparison to the same two bands in the naked DNA digested sample (Fig. 4b, lane 7). The results suggest that HIV-1 IN produces an extended DNaseI footprint protection pattern ~32 nucleotides from the U5 end in both SC and H-SC.

IN remains associated with the STC²³ and therefore, this complex may also produce the same ~32 bp DNaseI footprint observed with SC and H-SC. For preparative analysis, the STC was formed for 2 h at 37°C (for example see Fig. 1b, lane 2). After DNaseI treatment (0.3 units) at 14°C, the STC was isolated from a native agarose gel or a gel containing 1 M urea²³. The same ~32 bp DNaseI protection pattern was observed with STC from the 1 M urea gel (Fig. 4c, lane 1) or without urea in the gel (Fig. 4c, lane 2), in comparison to naked DNA treated with 0.3 units (Fig. 4c, lane 3). An additional control using a higher concentration of DNaseI (1 unit) for digestion of naked DNA (Fig. 4c, lane 4) demonstrated the same cleavages had occurred when using 0.3 units of DNaseI (Fig. 4c, lanes 1 to 3, respectively). The results support the conclusion that IN also protects ~32 bp at the U5 end in the STC.

IN multimerizes individually on a single U5 DNA end prior to the juxtaposing of two U5 ends to form SC

What is the association of IN on a single U5 DNA end that is inserted into supercoiled DNA producing the CHS product? IN-DNA complexes were formed in the presence of 1.6 kb U5 blunt-ended DNA, IN, and target for 2 h at 37°C. The samples were then digested with DNaseI (0.3 units) at 14°C for 3 min, stopped with EDTA, and treated with SDS-proteinase K. The DNA was subjected to analytical gel electrophoresis (Fig. 5a) and to a preparative gel for isolation of both CHS and FS products. Surprisingly, the IN-DNA complex that produces the CHS product possessed the same ~32 bp DNaseI protection pattern as the FS product derived from the STC (Fig. 5b, lanes 5 and 6, respectively). The enhanced DNaseI cleavages observed in the CHS and FS products just above 32-G in comparison to the naked DNA digestion pattern, highly suggests IN is tightly anchored here and distorts this immediate upstream DNA structure permitting easier access to DNaseI. The data also suggests that IN assembles on each individual U5 end in an independent manner prior to the juxtaposing of both U5 ends by IN to form SC (see Fig. 6 also).

SC formed in the presence of L-870,810 modulates IN interactions within the internal U5 DNA sequences

Strand transfer inhibitor L-870,810 decreased the observed E value ~85% within SC or presumably increased the distance ~1.7 times between the two 5'-U5 ends (Table 1). Are the interactions of IN, within the very upstream internal viral DNA sequences, modified in SC treated with inhibitor? In presence of 750 nM L-870,810, we observed the same size ~32 bp DNaseI protection pattern on the U5 end in both H-SC and SC (Fig. 5c, lanes 6 and 7, respectively); the enhanced DNaseI cleavages just upstream of 32-G were also evident. The DNaseI digestion patterns observed with the internal sequences from ~5-C to 32-G (Fig. 5c, lanes 6 and 7) appear slightly modified at several positions relative to the control DNA digestion pattern (Fig. 5c, lane 5). In additional experiments with L-870,810, the internal DNA digestion patterns in SC were altered at two or three other positions that were different then what was

observed in Fig. 5 (lanes 6 and 7)(data not shown). The results suggest that the inhibitor also modifies the internal binding of IN to U5 DNA but does not completely disrupt IN binding to the viral DNA in SC and H-SC.

HIV-1 IN interactions with U3 ends are independent of and different with U5 ends

IN binding to U3 DNA ends, which are ~40% as active as U5 DNA ends for strand transfer activities^{26,32}, was investigated by the DNaseI protective footprint technique. A 2.4 kb ³²P-5'-U3 DNA alone or in combination with unlabeled 1.6 kb U5 DNA were used; both substrates were blunt-ended. As previously described for U5 (Fig. 5), the reaction mixtures with only U3 DNA were incubated for 2 h at 37°C prior to DNaseI footprint analysis and isolation of the CHS and FS products from agarose gels (Fig. 6). An ~32 bp protective profile was observed on U3 ends in the FS product found in the STC, in the absence (Fig. 6, lane 5) and in the presence of unlabeled U5 DNA (data not shown), summarized in Fig. 7. No DNaseI enhanced cleavages were observed on the FS product immediately upstream of 31-A on U3 (Fig. 6, lane 5) or the CHS product (Fig. 6, lane 4). A reproducible minor area of protection was observed at ~39-A in the FS product with U3 suggesting that IN multimerization may be extended under other conditions. The DNaseI digestion patterns were altered near nucleotides 28 and 29 in both the CHS and FS products in comparison to the control DNA pattern. Interestingly, there were enhanced DNaseI cleavages at nucleotides 6-A and 9-G on U3 in the FS product (Fig. 6, lane 5) in comparison to naked DNA digestions (Fig. 6, lanes 6 and 7). The DNaseI footprint on the CHS product of U3 demonstrates a similar protection pattern as the FS product except the DNaseI enhancements at 6-A and 9-G were more apparent (Fig. 6, lane 4). In summary, the different DNaseI protection pattern observed between U3 and U5 indicates that IN assembles differently and independently on U3 in comparison to U5 (Fig. 4 and 5). The presence of the ~32 bp DNaseI footprint and the enhanced cleavages at 6-A and 9-G on the CHS with U3 supports the possibility that two IN dimers assemble on each LTR prior to forming the SC.

Multimeric structures of IN exist in SC, H-SC, and STC as determined by protein-protein cross-linking studies

To determine the multimeric forms of IN in SC, H-SC, and STC, IN (50 nM) was assembled on 1.6 kb blunt-ended U5 DNA (3 nM) at 14°C for 15 min, followed by 30 min at 37°C for SC and H-SC, and 120 min for STC. The reactions were stopped by the addition of EDTA, prior to protein-protein cross-linking at 14°C for 60 min. The optimum concentrations for each cross-linker for maximum isolation of the IN-DNA complexes using BS³, glutaraldehyde, DFDNB, and DSS were empirically determined (data not shown). The optimum concentrations were from 25 µM to 50 µM that allowed ~70–80% of SC, H-SC, and STC to enter the agarose matrix and migrate at their respective positions, as defined by controls without cross-linkers. Higher concentrations of cross-linker resulted in large-size IN-DNA complexes unable to enter the agarose matrix. As expected, the production of the FS product at the low concentration range of cross-linkers was normal in comparison to untreated reactions.

SC and H-SC were formed for 30 min without target, cross-linked with 25 µM BS³ (Fig. 8a) or 0.0005 % (50 µM) glutaraldehyde (data not shown) and subjected to electrophoresis on a native agarose gel. SC and H-SC were excised from the gel and the proteins were recovered by elution. The samples were subjected to SDS-PAGE followed by Western Blot analysis using N-terminal anti-HIV-1 IN (Fig. 8b, lanes 2 and 4 with BS³; lanes 7 and 8 with glutaraldehyde). The STC was independently prepared in the presence of target DNA for cross-linking and isolation of IN (Fig. 8b, lane 9). IN extracted from SC, H-SC, and STC exhibited monomer, dimer, tetramer, and a large-size multimeric IN forms. Dimers and tetramers were the predominant forms in all of the complexes. Minimal amounts of monomers were present suggesting efficient cross-linking at these low cross-linker concentrations. The distribution of

IN towards the anticipated dimers, tetramers, and large-size multimer (>120 kd) in the IN-DNA complexes suggests specific interactions with the U5 ends (Fig. 8, lanes 2, 4, 7 to 9). The additional cross-linking of an adjacent dimer with an already cross-linked tetramer may produce the large-size IN multimer (Fig. 9). A similar distribution of IN forms were observed at 50 μ M BS³ (data not shown). Control samples of IN (50 nM) cross-linked with 25 μ M BS³ in the absence of viral DNA substrate (Fig. 8b, lane 1) or 50 μ M glutaraldehyde (Fig. 8b, lane 5) also produced the same size multimeric structures as observed in the IN-DNA complexes, however at different proportions. In summary, the results suggest that besides the active tetramer at the very LTR ends responsible for strand transfer^{22,23}, a presumed dimer is also located just upstream of the active tetramer in SC (Fig. 9), thus producing the ~32 bp DNaseI protective footprint.

Differential recognition of cross-linked IN subunits in SC and H-SC using N-terminal and C-terminal antibodies

The presence of a large-size IN multimer (>120 Kd) was evident in the IN-DNA complexes (Fig. 8b and 8c). The possibility exists that cross-linking destroys antibody recognition sites on IN preventing an evaluation of the relative concentrations of each cross-linked species by Western Blotting. To address this question, equivalent quantities of cross-linked IN from SC and H-SC were subjected to gel electrophoresis on the same SDS-PAGE gel, one set each for antibodies directed against the N-terminus or C-terminus of IN (Fig. 8c). The N-terminal antibody reacted predominantly with the dimer and tetramer forms of IN (Fig. 8c, lanes 2 and 4). The C-terminal antibody reacted primarily with the dimer and a larger-size multimer in SC and H-SC (Fig. 8c, lanes 7 and 8). An independent experiment with the C-terminal antibody verified the presence of the large-size multimer and that it did not readily detect the tetramer in H-SC (Fig. 8c, lane 9). The differential recognition of multimeric forms of IN in SC, H-SC, and STC with two different antibodies suggests different residues are cross-linked in these complexes destroying one or more antibody recognition site. The mass of the large-size multimer and the actual molar ratio of each cross-linked species in the IN-DNA complex are currently unknown. In summary, the data suggest that multiple contacts exist between IN dimers located on the U5 ends resulting in a cross-linked tetramer and a large-size multimeric structure in SC, H-SC, and STC.

Discussion

This study defines the biochemical and biophysical properties of HIV-1 IN within the transient SC and the terminal STC in the concerted integration pathway in vitro. Using in-gel FRET measurements, the E value and the calculated distance between the DNA ends in the STC are significantly different than those observed in SC and H-SC, suggesting a DNA structural rearrangement had occurred in the STC. The same size ~32 bp DNaseI protective footprint observed on U3 and U5 ends in SC, H-SC, and STC suggests that once IN organizes itself correctly on each LTR end, it is stable. The initial association of IN at the LTR ends appears to occur individually on U3 and U5 because the same size ~32 bp DNaseI footprint is observed in a nucleoprotein complex that gives rise to the CHS, a single viral DNA insertion event. Thus, the data suggests that the juxtaposition of two independent LTR ends with bound IN produces SC (Fig. 9). The DNaseI enhanced cleavages that occur near the very end of U3, not observed on U5, suggests that the interactions of IN with U3 are slightly different. Protein-protein cross-linking established that a dimer, tetramer, and a large-size multimer are observed in SC, H-SC, and STC. In summary, these results and others²⁴ suggest that the association of IN with the U5 and U3 DNA ends within SC mirror catalytic and physical properties associated with the HIV-1 PIC^{2,3,5,7,33}.

Energy transfer occurs between Cy3- and Cy5-U5 substrates in SC and STC demonstrated by in-solution (Fig. 2) and in-gel FRET experiments (Fig. 3). In-solution FRET analysis of SC produced with RSV IN also displayed energy transfer between Cy3- and Cy5-labeled LTR DNA substrates and not with non-LTR substrates²⁷. In-gel quenched FRET (Fig. 3b) allowed us to calculate the distances between the two 5'-ends of the viral DNA within SC, H-SC, and STC (Table 1). The distance between the 5' ends of Cy3- and Cy5-DNA in SC was 46 ± 3 Å (see Supplemental Fig. S1 for model). After 20 min of incubation at 37°C, ~16% of the ends are processed by IN in SC²⁴ and ~50% after 50 min (data not shown). Distance measurements in SC at 20 min and 30 min thus include a mixture of juxtaposed blunt-end DNAs (~80%) and, one processed and one blunt-end DNA juxtaposed by IN. It is not possible to know the extent of the unpairing of the blunt-ends for 3' OH processing by IN³⁴ in SC although it probably does not affect the position of the 5' overhang, located adjacent to Y143 of IN³⁴⁻³⁶. Thus, the cumulative energy transfer may be an average distance measurement of several possible DNA structural configurations within SC.

Strand transfer inhibitors block the binding of target DNA to HIV-1 IN-viral DNA complexes^{33,37,38}. We suggested that inhibitor binding to IN within SC occurs prior to or during 3' OH processing²⁴ and, that only one blunt-end with one recessed DNA end in the SC is sufficient for effective inhibition²⁵. We determined that inhibitor L-870,810 altered the E value between the two LTR ends within SC. The calculated distance was 77 ± 6 Å suggesting some movement of the DNA ends in SC with inhibitor (Table 1). SC was formed for 30 min at 37°C in the presence of inhibitor thereby allowing inhibitor binding to IN^{36,39}. Incubation times of 30, 60, or 120 min at 37°C at inhibitor concentrations between 200 nM to 2000 nM ensured that a major fraction of SC was subjected to the inhibitory effects of L-870,810^{24,25}. The significant decrease in energy transfer in SC with inhibitor presence suggests that the two DNA ends were shifted apart in the presence of inhibitor. A reasonable explanation for the decreased energy transfer is that the L-870,810 slightly modified the location of the DNA ends in SC, thus altering viral DNA binding by IN although insufficient to destroy SC^{24,25}. The altered DNaseI footprint of U5 in SC and H-SC formed in the presence of L-870,810 (Fig. 5, lanes 6 and 7) in contrast to its absence (Fig. 4b, lanes 5 and 6) also supports that the inhibitor modestly perturbs viral DNA binding. In summary, the apparent binding of the strand transfer inhibitor within the active site of IN^{36,39} is sufficient to inactivate the PIC that results in a significant increase in the 2-LTR circle junction viral DNA in the nucleus of virus- infected cells^{33,38,40}.

Both DNA blunt-ends are sequentially processed during maturation of HIV-1 SC resulting in the capture of the target DNA for concerted integration²³⁻²⁵. The distance between two processed 3'-OH ends in SC should be ~18 Å apart for generation of the HIV-1 5 bp host-site staggered insertions. After concerted insertion of the viral DNA ends into target producing the STC, the distance is 83 ± 5 Å (Table 1). Model building shows the donor and acceptor fluorophore on the 5'-end of the two unpaired nucleotides in the STC (Supplemental Fig. S1). The observed E values and subsequent distance calculations suggest significantly different viral DNA end structural configurations between these two nucleoprotein complexes.

The calculated distances between Cy3- and Cy5-U5 ends are based on the orientation factor of 2/3, assuming there is free movement of the fluorophores^{30,41}. Anisotropy values of 0.28 to 0.30 for Cy3- and Cy5-1.6 kb U5 blunt-ended DNA suggest that the movements of the fluorophores are hindered (Supplemental Table S1). In addition, Cy3 and Cy5 with 3-carbon linkers at 5' DNA ends generally stack on the top of the double strand DNA resulting in constraint of the fluorophore movement⁴². We performed additional control experiments to determine anisotropy values and quantum yields using blunt-ended and recessed-ended substrates containing either Cy3 or Cy5 (Supplemental Table S1). The anisotropy and quantum yield values suggest that the movement of Cy3 and Cy5 attached to the blunt-end and recessed-ended DNAs are nearly equivalent. In summary, although we do not know the absolute distance

between the Cy3- and Cy5-5' ends in these complexes, the same Forster distance (R_0) for all DNA substrates allows a relative distance comparison between the complexes⁴³.

The HIV-1 and murine leukemia virus PIC display an ~200 bp protective footprint defined by a Mu transposase-mediated polymerase chain reaction assay^{3–5}. This large-size footprint in the PIC requires an active IN that promotes viral DNA integration implying that the protective footprint requires IN multimers³ and, this multimerization promotes the stable juxtapositioning of LTR ends in the PIC². Most IN-viral DNA models using IN-DNA cross-linking and other structural data infer HIV-1 IN binding up to ~15 bp from the LTR end. An ~16 bp DNaseI footprint is observed using HIV-1 W235F IN in both a inhibitor-induced stable synaptic complex and the STC²³. A cross-linked tetramer is found in the stable synaptic complex²³ and a cross-linked tetramer formed in-solution is also capable of promoting concerted integration²², arguably at a very low rate. Our data confirms that a cross-linked tetramer exists in SC and STC. However, the ~32 bp DNaseI protective footprint observed on U5 and U3 DNA ends in SC, H-SC, and STC using IN suggests more than one dimer is aligned on each LTR end in these complexes, summarized in Fig. 7 and modeled in Fig. 9. Of particular note, DNaseI enhanced cleavages occur at the outer boundary of U5 (~32 bp) suggesting that IN distorts the DNA at these positions for easier access to DNaseI. The presence of same ~32 bp DNaseI footprint on the CHS product (Fig. 5) supports the alignment of two IN dimers per LTR end, prior to their juxtaposition to form SC (Fig. 9). In summary, the active tetramer at the LTR ends responsible for integration may be the consequence of two independently assembled LTR ends containing two parallel aligned IN dimers per DNA end (Fig. 9).

Similarly, IN protects ~32 bp on the U3 LTR end (Fig. 6, lanes 4 and 5), even in the presence of U5 (Fig. 7). The interactions of IN with U3 are different than observed with U5. First, there is no apparent enhanced DNaseI cleavages near the ~32 bp protective boundary on U3 implying that IN binding at this boundary is different with U5 (Fig. 5). The DNA is apparently not distorted by IN at the U3 boundary but is on U5. A nucleotide position (~39-A) in U3 is always protected in the FS product suggesting that IN binding may be extended even further upstream on U3 although it is not apparent on the entire region beyond ~32 bp (Fig. 6). Second, there is significant DNaseI enhanced cleavages at 9-G and 6-A on U3 (Fig. 6, lanes 4 and 5) while no enhanced cleavages are observed with U5 in this same region (Fig. 4, 5 and 7). IN apparently distorts U3 DNA in this region allowing DNaseI cleavages in SC in comparison to naked DNA (Fig. 6, compare lanes 4 and 5 to lanes 6 and 7). The presence of these same DNaseI cleavages on the CHS product using U3 (Fig. 6, lane 4) and the ~32 bp footprint further supports the parallel alignment of two IN dimers per LTR end prior to forming the SC (Fig. 9). Differences also occur in the U3 and U5 footprint patterns within the first ~15 nucleotides in the HIV-1 PIC⁵. In summary, IN dimers apparently form multimeric structures on U5 and U3 DNA ends independently.

In summary, we observe multiple contacts between IN dimers located at the LTR ends in SC, H-SC, and STC that produce a cross-linked tetramer and large-size multimer. The relative low portion of monomers to cross-linked dimers and higher forms of IN suggests that dimers may be the minimal functional forms of IN at the LTR ends in the HIV-1 SC (Fig. 8). These same cross-linked IN multimers are observed with molar ratios of DNA substrate ends to IN dimers between 1 to 18 and 1 to 25, respectively (data not shown)²⁴. The actual molar ratios of these cross-linked IN species and the mass of the large-size multimer of IN in SC and STC is currently under investigation. Complementation studies using the above assembly system for SC with HIV-1 IN mutants whose functions are unknown, mainly non-catalytic Class II IN mutants⁴⁴, may allow a more functional understanding of HIV-1 IN.

Materials and Methods

Preparation of IN

HIV-1 IN was expressed and purified as described^{24,45,46}.

HIV-1 DNA substrates and ³²P-5'-end DNA labeling

DNA substrates possessing HIV-1 U5 or U3 blunt-ended terminal sequences were prepared as described²⁴. The ScaI linearized and dephosphorylated DNA was 5'-end labeled using [γ -³²P] ATP and T4 polynucleotide kinase. For integration and DNaseI footprint experiments, the labeled DNA was digested by NcoI to produce 1.6 kb single U5 blunt-ended and 2.4 kb single U3 blunt-ended substrates. The fragments were isolated from agarose gels and purified.

Fluorophore labeling of U5 DNA substrates

Fluorophore-labeled 1.6 kb DNA containing a single U5 end was produced by PCR using Cy3- or Cy5-labeled and unlabeled primers and template DNA (4731 bp plasmid with HIV-1 U5/U3 LTR circle junction)^{24,47}. The primers were Cy3 or Cy5 5'-ACTGCTAGAGATTTTCCACACTGACTAAAAGGGTC (35 mer reverse primer 1189-1155) and 5'-CATGGGGCGGAGAATGGGCGGAAC (24 mer forward primer 4352-4375). The fluorophore-labeled primers and unlabeled primers were synthesized and purified using HPLC/PAGE by Integrated DNA Technologies Inc. (Coralville, IA) and Operon Technologies (Santa Clarita, CA), respectively. PCR cycling parameters included initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 30 sec, and extension at 72°C for 4 min, followed by a final extension at 72°C for 10 min. The DNA product (1.6 kb) containing Cy3- or Cy5-DNA was purified on 0.7% agarose gels. The degree of fluorophore labeling was between 0.75 to 0.82.

Integration assay and assembly of IN-viral DNA complexes

The standard integration assay using U5 and U3 blunt-ended substrates was performed as described^{25,26}. In brief, the DNA substrate and IN were preassembled at 14°C for 15 min in 20 mM HEPES buffer (pH 7.0), 5 mM DTT, 10 mM MgCl₂, 25 μ M ZnCl₂, 100 mM NaCl, and 10% polyethylene glycol (6000 Daltons). The DNA substrate (3 nM) and the IN concentrations (10 to 80 nM) are described for each experiment. The strand transfer reactions were initiated by addition of supercoiled DNA (3 nM). Samples were incubated for 20 or 30 min at 37°C for SC and 120 min for STC, respectively. EDTA was added to 25 mM and the mixtures were briefly stored on ice. To identify the IN-DNA complexes, aliquots were subjected to electrophoresis on pre-cooled 0.7% native agarose gels at 4°C for 16 h. Aliquots of the same reaction mixtures were treated with SDS (0.5%) and proteinase K (1 mg/ml) for 1 h at 37°C and, the ³²P-labeled strand transfer products were separated by agarose gel electrophoresis to determine strand transfer activities²⁴. The quantities of each product for FRET analysis (see below) was accomplished by SYBR Gold staining of the gels and analysis by ChemiDoc XRS (BioRad) Quantity One software.

In-solution FRET

All fluorescence measurements were performed on a Fluoromax-3 spectrofluorometer (Jobin Yvon, Inc., Edison, NJ) with a temperature-regulated cell holder. The band pass was 5 nm for excitation and emission monochromator. The samples were excited at 550 nm (λ_{ex} for Cy3) and fluorescence emission collected from 560 to 720 nm using 4 mm path length semi-microcell (200 μ l). After formation of STC at 37°C for 2 h, all fluorescence measurements were performed at 14°C in same integration assay buffer. The absorbance of solutions used for all fluorescence measurements at the wavelength of excitation was always below 0.1 to minimize any inner filter effects. Q_D , the quantum yield of the donor in the absence of acceptor

was calculated using $Q_D = Q_{RF} (I^D/I^{RF})(A_{RF}/A_D)$ where I^D and I^{RF} are the respective buffer and instrument corrected integrated fluorescence intensities over the entire emission wavelength range of the donor and reference compound. A_D and A_{RF} is the absorbance of donor fluorophore and reference at excitation wavelengths; Q_{RF} for rhodamine 110 was 0.91 in 10 mM HEPES, pH 7.5 containing 15% ethanol⁴⁸ and fluorescein was 0.95 in 0.1 N NaOH⁴⁹.

In-gel FRET data analysis of SC and STC

To determine FRET signal intensities, three sets of reaction mixtures were prepared: 1) contains 3 nM of Cy3-U5 (donor fluorophore) substrate; 2) contains 1.5 nM of each Cy3-U5 and Cy5-U5 (donor-acceptor fluorophore pair); 3) contains 3 nM Cy5-U5 (acceptor fluorophore)(Fig. 3). As an activity control, 3 nM of unlabeled U5 was also used concurrently.

For FRET signal measurements, the gel was scanned in a Typhoon Trio variable image laser scanner with green laser (532 nm) and a 580 nm emission filter with band pass 30 nm for detection of Cy3 fluorophore (donor) quenching. For the sensitized FRET signals, the gels were scanned with a green laser (532 nm) and a 670 nm emission filter with band pass 30 nm. The latter scan produced the sensitized emission of Cy5-U5 (acceptor) via resonance energy transfer. The fluorescence intensity of SC and STC were determined by using ImageQuant 5.2 software. The quenched FRET signals were determined from the extent of donor fluorescence quenching in the complex containing the acceptor compared with donor fluorescence in absence of acceptor fluorophore. With proper control experiments, the sensitized FRET signal for acceptor fluorophore was also calculated.

The gel was scanned for acceptor emission at 670 nm ($I^{D'}$, I^{AD} and I^A for sensitized FRET) (Fig. 3a) and donor emission (I^D and I^{DA} for quenched FRET)(Fig. 3b) by excitation of the donor. $I^{D'}$ is the contribution of fluorescence emission intensity of the donor fluorophore at 670 nm due to excitation at 532 nm in absence of acceptor fluorophore. I^A and I^{AD} are the fluorescence emission intensities of acceptor fluorophore at 670 nm in absence and presence of donor fluorophore, respectively, when excited at 532 nm. I^D and I^{DA} are the fluorescence emission intensities at 580 nm of donor fluorophore in absence and presence of acceptor fluorophore, respectively, when excited at 532 nm. After the laser scans, the same gel was stained with SYBR Gold Stain (Fig. 3c) for quantitative analysis of the FRET data. Fluorescence intensities were normalized for the differences in amounts of donor-only ($[\text{complex}]^D$) and donor-and acceptor-labeled complex ($[\text{complex}]^{DA}$) by multiplying with $[\text{complex}]^D/[\text{complex}]^{DA}$. In Fig. 3d, quenched FRET was determined for SC, produced after 20 min at 37°C, as the difference between corrected fluorescence intensities of donor fluorophore in absence and presence of acceptor fluorophore. Similarly, sensitized FRET is equal to $I^{AD} - I^A - I^{D'}$ (corrected intensities). When calculating FRET intensities and hence energy transfer efficiency (E), we account for the appropriate concentrations of donor and acceptor fluorophore-labeled DNA substrates present in the reaction mixture. Formation of SC with Cy3-U5 and Cy5-U5 produces four kinds of complexes. They are: Cy3-U5 & Cy3-U5, Cy3-U5 & Cy5-U5, Cy5-U5 & Cy3-U5, and Cy5-U5 & Cy5-U5, all with equal probability of being produced. Only two complexes (donor-acceptor and acceptor-donor) are responsible for energy transfer. With independent experiments, Fig. 3e shows the quantitative in-gel FRET analysis for STC produced after 120 min at 37°C. The same IN concentrations for SC (Fig. 3a to 3d) were used for the in-gel FRET analyses of STC.

The Förster distance R_0 was calculated for donor-acceptor pairs by the method of Wu and Brand⁵⁰. Quantum yields of Cy3-U5 were nearly identical in the electrophoresis Tris-Borate-EDTA (TBE) buffer and in 0.7% agarose in TBE buffer (37°C). We observed approximately 5% less fluorescence emission intensity in solid agarose at 14°C when compared with liquid agarose at 37°C. The quantum yields of Cy3-U5 for blunt and recessed ended DNAs were

nearly identical (Supplemental Table S1). Our measured anisotropy values of these two fluorophore-labeled DNAs in TBE buffer (with or without protein) and 0.7% agarose (liquid) in the same buffer were between 0.27 and 0.30. These data imply constrained rotational motions of both fluorophores^{29,51}. We assumed our measured anisotropy values were low enough to justify the use of $2/3$ for κ^2 , the value accepted with the same anisotropies ranges of fluorophore-labeled DNA^{30,41}. Therefore, the R_0 values are similar for any combination of donor-acceptor fluorophore labeled blunt-ended and recessed-ended substrate used in this study.

DNaseI footprint analysis of IN-LTR DNA complexes

For SC formation, we assembled the IN-DNA complexes with 1.6 kb ^{32}P -5'-U5 blunt-ended DNA (3 nM), IN (60 to 80 nM), and target DNA under standard reaction conditions for 30 min at 37°C. The samples were immediately equilibrated for 10 min at 14°C³¹. DNaseI (0.3 units) (New England Biolabs) was added and further incubated for 3 min at 14°C. Reactions were stopped by adding 25 mM EDTA and the samples were subjected to native agarose gel electrophoresis. SC and H-SC were excised. The STC was produced after 2 h of incubation followed by DNaseI treatment as described above, prior to gel electrophoresis. The DNAs from the excised complexes were electro-eluted, concentrated by Centricon-3K filter device, and ethanol precipitated. The naked DNA substrate (without IN) was treated with DNaseI as a digestion control. In some experiments, the nucleoprotein complexes formed for 2 h at 37°C were treated with DNaseI, deproteinized, and the FS and CHS products were purified on agarose gels. Equivalent cpm of each ^{32}P -labeled DNA sample were analyzed on 15% denaturing PAGE gels along with Maxim-Gilbert chemical sequence markers (G + A), C and (C + T).

For U3 DNA footprint analysis, a 2.4 kb blunt-ended DNA containing a single ^{32}P -5'-U3 end, without or with unlabeled 1.6 kb U5 DNA, was used. The two different size substrates allowed us to detect and extract the different size STC containing the U3-U3 or U3-U5 DNA ends on native agarose gels by SYBR Gold staining. The nucleoprotein complexes containing only U3 DNA and treated with DNaseI for evaluation of FS and CHS products is described above using U5 DNA.

Chemical cross-linking of IN subunits in SC, H-SC, and STC

Protein-protein chemical cross-linkers were used to identify the multimeric structures of IN in the different IN-DNA complexes. The IN-DNA complexes were assembled at 37°C for 30 min or 120 min and the reactions were stopped with 25 mM EDTA. Chemical cross-linking was performed with either homobifunctional cross-linker BS³ (11.4 Å, Pierce) or a zero-length bis-aldehyde homobifunctional cross-linker glutaraldehyde (Sigma) at final concentrations of 25 μM to 50 μM or 0.0005% (50 μM), respectively, at 14°C for 60 min. Cross-linkers were quenched by addition of Tris-HCl (pH 7.6) to 50 mM and further incubation for 15 min at 14°C. Cross-linked complexes were subjected to electrophoresis on 0.7% agarose (SeaPlaque GTG, Lonza Biosciences) at 100 V for 16 h at 4°C. Gels were stained with SYBR Gold (Invitrogen) and the IN-DNA complexes were excised. The cross-linked IN was eluted from chopped gel slices in elution buffer (20 mM HEPES pH 7.0, 50 mM NaCl, 50 mM DTT, 0.1 mM AEBSF and 0.1% SDS) for 6 hr at room temperature. The concentrated samples were subjected to electrophoresis on 4–12% Bis-Tris gels (Invitrogen) and were blotted onto a nitrocellulose membrane. The membranes were blocked in 5% fat-free milk in Tris-saline buffer (0.05% Tween 20) for 1 h at room temperature. The blot was incubated overnight at 4°C with rabbit polyclonal HIV-1 IN peptide directed-antisera (1:1000 dilution)⁵², followed by incubating with HRP conjugated anti-rabbit antibody (1:125,000 dilution, Pierce). N-terminal (1–16 residues) and C-terminal (276–288) peptide antisera were used. Bands were detected

using Pierce SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) and photographed in Bio-Rad ChemiDoc XRS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

HIV-1	human immunodeficiency virus type-1
IN	integrase
LTR	long terminal repeats
PIC	preintegration complex
SC	synaptic complex
STC	strand transfer complex
FS	full-site
CHS	circular half-site
D-D	donor-donor
RSV	Rous sarcoma virus
FRET	Fluorescence Resonance Energy Transfer
E	energy transfer efficiency
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

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(a) Concerted integration pathway **(b) Native gel (c) Strand transfer products** **Fig. 1.**

HIV-1 IN-LTR DNA complexes that produce the concerted integration product. (a) The assembly pathway for SC and STC that results in the concerted or FS integration product is shown. Other strand transfer side-reactions produce the CHS, Y-type, and D-D products (see c). (b) The integration reaction with 5'-Cy3-U5 blunt-ended DNA (3 nM) and 60 nM IN was carried out for 30 min for SC (lane 1) and 120 min for STC (lane 2). Samples were subjected to native agarose electrophoresis. The Typhoon laser scan was set for excitation and emission band for Cy3. (c) The same samples were deproteinized and subjected to electrophoresis, followed by laser scanning. Lanes 3 and 4 are the 30 min and 120 min reactions, respectively. The percentage of Cy3-U5 DNA incorporated into the FS, CHS, and D-D products after 120 min were 16%, 7%, and 6%, respectively.

Fig. 2.

Fluorescence emission spectra and FRET signal intensities of HIV-1 IN-viral DNA complexes. (a) Spectra of quenched emission of donor fluorophore (Cy3) and sensitized emission of acceptor fluorophore (Cy5) resulting from energy transfer is shown. An equimolar (1.5 nM each) mixture of 1.6 kb Cy3-U5 and Cy5-U5 blunt-ended DNA substrates were titrated with IN (0, 20, 30, 40, 50, and 60 nM; black, red, green, yellow, blue and magenta lines, respectively). After a 2 h reaction at 37°C and adjustment of the mixture to 500 mM NaCl and 14°C, the spectra of the mixture was taken in the region 560 nm to 720 nm. All spectra are instrument and buffer corrected. b. Quenched FRET at 568 nm and sensitized FRET signal intensities at 670 nm were determined at each IN concentration by subtracting corresponding

emission intensity from the sample with no IN (black line spectra shown in (a)). The data at each IN concentration were plotted (20, 30, 40, 50, and 60 nM IN). Similar analyses using fluorophore labeled non-LTR DNA at 20, 40 and 60 nM IN were performed (spectra not shown).

Fig. 3.

Energy transfer occurs in HIV-1 SC and STC as analyzed by in-gel FRET. Panel a, b, and c show the sensitized FRET, quenched FRET, and a SYBR Gold stained photograph of the same gel, respectively. The 5'-end of 1.6 kb U5 blunt-ended substrate was labeled with Cy3 or Cy5. Assembly and incubation conditions were described in Fig. 1b. For FRET analysis, 3 nM Cy3-U5 (lanes 1–6), 1.5 nM Cy3-U5 and 1.5 nM Cy5-U5 (lanes 7–12), and 3 nM Cy5-U5 (lanes 13–18) were incubated with increasing concentrations of IN (top triangles). Lanes 19–22 were with U5 substrate (3 nM) without fluorophore label. To detect SC, the samples in lanes 2–5, 8–11, 14–17, 20–21 were incubated for 20 min at 37°C. As an activity control to detect STC, lanes 6, 12, 18, and 22 were incubated for 120 min. In each of the following sets of lanes: 1–6, 7–12 and 13–18, the IN concentrations were 0, 20, 40, 60, 80, and 80 nM, respectively. The IN concentrations in lanes 19 to 22 were 0, 40, 80 and 80 nM, respectively. The fluorescence intensity of a particular nucleoprotein complex band was calculated using ImageQuant 5.2 software. In c), T1 and T2 on the right are supercoiled and nicked circular forms of target DNA, respectively. Two kb DNA marker (M) lanes are shown. Sensitized and quenched FRET signal

intensities were plotted for SC (d) and STC (e)(see details in Materials and Methods). The FRET data for STC were derived from independent experiments using the same assay conditions but incubation was for 120 min at 37°C.

Fig. 4.

DNaseI footprint analysis of SC, H-SC, and STC formed with U5 DNA. (a) IN-³²P-DNA complexes were assembled for 30 min at 37°C in the presence of target and then treated with DNaseI prior to electrophoresis on the native agarose gel at 4°C. The complexes (marked on right) were identified by SYBR Gold staining. Lane 1 (30 µl) was not digested with DNaseI (-) while preparative lanes 3 and 4 were digested (+). A kb marker (M) ladder is in lane 2 and the sizes are marked on the left. Supercoiled, nicked circular and linear target DNA along with donor DNA are indicated on the right. SC and H-SC were excised from the preparative gel and the DNAs were purified. (b) DNA isolated from SC and H-SC was subjected to denaturing 15% polyacrylamide gel electrophoresis. Lane 1 is input naked U5 DNA without DNaseI treatment. Lanes 2 to 4 contain Maxium-Gilbert chemical sequence markers of the labeled DNA. Lanes 5 and 6 are DNaseI treated SC and H-SC, respectively. Lane 7 contains naked DNA digested with DNaseI at 0.3 units (U). Nucleotide positions are shown on the left and the bar on the right shows the ~32 bp protected region. The star indicates slight DNaseI enhanced digestion in the samples compared to the control digestion. All of the samples contain equivalent cpm. (c) The same DNaseI treatment procedure was performed on preparative amounts of STC produced after 2 h of incubation at 37°C. After DNaseI digestion (0.3 U), the STC was either isolated from an agarose gel with 1 M urea (+)(lane 1) or without urea (-)(lane 2). The isolated DNA was subjected to denaturing gel analysis. Lanes 3 and 4 contain naked DNA at 0.3 and 1 unit of DNaseI, respectively. Lane 5 is input naked viral DNA without DNaseI treatment.

Fig. 5. DNaseI footprint analysis of nucleoprotein complexes that produce the CHS and FS products and, SC and H-SC formed in the presence of L-870,810. (a) The IN-DNA complexes were assembled for 2 h at 37°C and treated with DNaseI (3 min) at 14°C. An aliquot of the deproteinized sample was analyzed on a native agarose gel. The percent U5 DNA incorporated into the FS and CHS products were 29% and 11%, respectively. (b) The FS and CHS products obtained in (a) were excised and subjected to denaturing gel electrophoresis. Lanes 1 to 3 contain the U5 DNA sequence markers. Lane 4 is naked DNA digested with 0.3 U of DNaseI. Lanes 5 and 6 contain CHS and FS products, respectively. DNaseI protection is indicated by the rectangle on the right and the star indicates enhanced DNaseI cleavages in comparison to the naked DNA. Nucleotide positions are indicated on the left. (c) IN-DNA complexes were formed in the presence of 750 nM of L-870-810 for 2 h at 37°C. Samples were then treated with DNaseI and SC and H-SC were separated on an agarose gel. DNA purified from SC and H-SC was analyzed on a denaturing gel. Lane 1 is input naked viral DNA without DNaseI treatment. Lanes 2 to 4 are the chemical markers of U5 DNA. Lane 5 contains naked DNA treated with DNaseI (0.3 units). Lanes 6 and 7 are DNA purified from H-SC and SC formed in presence of 750 nM of inhibitor, respectively. Protection is indicated by rectangle on the right and the star indicates enhanced DNaseI cleavages. The nucleotide positions are shown on the left.

Fig. 6.

DNaseI footprint analyses of nucleoprotein complexes with U3 DNA as substrate. The 2.4 kb ^{32}P -U3 blunt-ended DNA (3 nM) was assembled with 80 nM of IN, followed by the addition of supercoiled DNA and incubation for 2 h at 37°C. DNaseI footprint analysis and isolation of CHS and FS products was described in Fig. 5. The percent of U3 DNA incorporated into the FS and CHS products were 16% and 12%, respectively. Lanes 1 to 3 contain chemical markers for U3 DNA. Lanes 4 and 5 contain the CHS and FS products, respectively. Lanes 6 and 7 contain naked DNA treated with DNaseI (0.3 U) for 3 and 4 min, respectively. Lane 8 contains the input DNA. The protected region in the FS product is indicated by the rectangle on the right and the enhanced DNaseI digestions at 9-G and 6-A are indicated by the stars. The small block on the right indicates a minor area that was protected by IN (lane 5) from DNaseI digestion in comparison to naked DNA (lane 6).

Fig. 7.

Summary of DNaseI protective footprint analyses of U5 and U3 ends in SC, H-SC, and STC. U5 and U3 terminal sequences are shown. The protected regions are indicated by the thick dark underline and the enhanced DNaseI cleavages are indicated by the arrows. The thickness of the arrow indicates the degree of enhancement observed over multiple experiments. The DNaseI footprint data for U3 were derived from both U3 alone for FS and CHS products (Fig. 6) and from the different size STC isolated on gels formed with U3 only or with unlabeled U5.

Fig. 8.

Multimeric structures of HIV-1 IN in SC, H-SC, and STC. (a) IN-DNA complexes were assembled for 30 min at 37°C without target DNA. The cross-linked (25 μ M BS³) samples were subjected to native agarose gel electrophoresis. SC and H-SC were excised. The cross-linked IN subunits were eluted and concentrated. (b) Western Blot analysis of cross-linked IN. IN was detected using rabbit polyclonal antiserum directed against an N-terminal peptide of HIV-1 IN (residues 1–16). Lane 1 contains ~25 ng of BS³ (25 μ M) cross-linked IN at 50 nM without DNA; no polyethylene glycol was present in the reaction mixture. Lanes 2 and 4 are SC and H-SC, respectively. Lane 3 is molecular weight protein markers (M) whose sizes are indicated on the left. The IN monomer, dimer, tetramer, and larger-size multimer are indicated in the middle. Lanes 7, 8, and 9 contain the SC, H-SC, and STC, respectively, obtained after cross-linking the IN-DNA complexes with glutaraldehyde (50 μ M). Lane 5 contains glutaraldehyde cross-linked IN (~25 ng) without DNA. The molecular weight protein markers (lane 6) are identified on the extreme left. (c) The same protocol as described above with BS³ was used except equal aliquots of SC and H-SC from the same reaction were subjected to electrophoresis and transfer. The nitrocellulose sheet was cut into half and analyzed. The sheets were probed with N-terminal antiserum (lane 2 and 4) or C-terminal antiserum (lane 7 and 8) directed against IN residues 276–288. Lanes 1 and 6 were cross-linked IN without DNA. Protein markers (lanes 3 and 5) were described above. An independent experiment of H-SC cross-linked with BS³ is shown in lane 9.

Assembly Pathway of Synaptic Complex (SC)

Fig. 9.

Assembly model for HIV-1 SC. The HIV-1 IN dimer is shown as a single blue sphere and the 1.6 kb U5 blunt-ended DNA as a red box with an extended black line. The assembly of two adjacent IN dimers on each of two LTR substrates is required prior to their juxtaposition forming the transient SC, which is a time and temperature-dependent process^{24,25}. The active tetramer of IN (circled by a pink oblong loop) responsible for catalysis is formed by the juxtaposition of two LTR ends in the SC. The large-size multimer presumably arises by cross-linking of a tetramer to one or more dimers.

Table 1

FRET efficiencies between Cy3-U5 and Cy5-U5 ends within IN-DNA complexes

Complexes	FRET Efficiency (E) ^a	Distance (R) ^b in Angstroms
SC	0.68 ± 0.09	46 ± 3
H-SC	0.52 ± 0.08	52 ± 3
STC	0.07 ± 0.02	83 ± 5
SC + L-870,810	0.10 ± 0.04	77 ± 6

^aFRET efficiency (E) of donor-acceptor pairs within the different complexes were determined using equation (1) from six independent protein titration experiments (Fig. 3) and two acceptor fluorophore titrations. The concentration of the total U5 DNA substrate (3 nM) was held constant in the acceptor titration experiment (varied from 1 nM to 1.5 nM) through the use of unlabeled U5 DNA (0.5 nM to 0) and constant donor fluorophore (1.5 nM). The data obtained for SC with L-870,810 were obtained from six independent experiments where IN was at 60 nM and the total substrate concentrations were 3 nM (Fig. 3).

^bDistances (R) were calculated from the relation $R=R_0(1/E-1)^{1/6}$, where R_0 is the Forster radius ($52.8 \pm 1 \text{ \AA}$) determined from triplicate experiments for quantum yield (Supplemental Table S1), absorption and emission spectra of 1.6 kb blunt ended fluorophore-labeled substrates in our assay conditions.