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A stable complex between integrase and viral DNA ends mediates human immunodeficiency virus integration *in vitro*

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ABSTRACT **Retroviral replication depends on integration of the viral genome into a chromosome of the host cell. The steps in this process are orchestrated *in vivo* by a large nucleoprotein complex and are catalyzed by the retroviral enzyme integrase. Several biochemical properties of the *in vivo* nucleoprotein complex were reproduced *in vitro* with purified integrase of human immunodeficiency virus type 1 and model viral DNA substrates. A stable complex between integrase and viral DNA was detected as an early intermediate in the integration reaction. After formation of this initial complex, the enzyme processively catalyzed the 3′ end processing and strand transfer steps in the reaction. Complexes containing only purified integrase and the model viral DNA end were stable under a variety of conditions and efficiently used nonviral DNA molecules as integration targets. These complexes required a divalent cation for their formation, and their stability was highly dependent on the 5′-terminal dinucleotide of the viral DNA, for which no functional role has previously been defined. Thus, interactions between integrase and the extreme ends of the viral DNA molecule may be sufficient to account for the stability of the *in vivo* integration complex.**

Integration of viral genomic DNA into a chromosome of the host cell occurs by a transpositional recombination reaction and is an essential step in the retroviral life cycle (1). Integrase—a viral protein encoded at the 3′ end of the *pol* gene (2, 3)—and specific sequences located at the viral DNA termini (4) are required for integration. Integration occurs in three steps: (*i*) endonucleolytic removal of the terminal bases 3′ to the highly conserved CA dinucleotide by a reaction referred to as 3′ end processing; (*ii*) strand transfer, a concerted cleavage and ligation reaction in which the recessed 3′ ends of viral DNA are joined to 5′ staggered sites in the target DNA; and (*iii*) repair of the short gaps flanking the viral DNA intermediate by DNA synthesis and subsequent joining of the 5′ ends of viral DNA to the target DNA (5, 6). The product of this highly regulated, precise set of reactions is a mature provirus flanked by direct repeats of short sequences from the target site. The first two steps are apparently executed *in vivo* by large, fast-sedimenting, nucleoprotein complexes (5, 7). These integration complexes, when isolated from infected cells, can faithfully reproduce the *in vivo* integration reaction *in vitro* and are stable through multiple steps of purification. Thus far, only a subset of viral proteins have been identified as constituents of the integration complex along with viral genomic DNA (7–9).

Although purified integrase can mediate the catalytic steps of the reaction *in vitro* (10–12), the role played by integrase in other key properties of the integration complex has not been rigorously investigated. In this study, we employ a modified version of the standard *in vitro* integration assay to identify and characterize a stable, functional complex between human immunodeficiency virus type 1 (HIV-1) integrase and viral DNA ends. After this stable complex is formed, the two catalytic steps in the reaction occur processively. Thus, the ability to form a stable complex with viral DNA is intrinsic to HIV-1 integrase.

MATERIALS AND METHODS

Oligonucleotides and Preparation of Substrates. Oligonucleotides were purchased from Operon Technologies (Alameda, CA) and purified by electrophoresis through a denaturing 15% polyacrylamide gel. The standard integration substrate consisted of the terminal 20 bp from the U5 end of viral DNA and was prepared by using the oligonucleotides C220 (5′-ATGTGGAAAATCTCTAGCAGT-3′) and C120 (5′-ACTGCTAGAGATTTTCCACAT-3′). C220 was phosphorylated with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (ICN) and the specific activity (typically ≈700,000 cpm/pmol) was determined. The radiolabeled oligonucleotide was then annealed to C120 and the unincorporated nucleotide was removed by centrifugation through a Sephadex G-15 column with a 1-ml bed volume. The 5′ deletion (ΔAC) viral substrate was prepared as described above by annealing C220 to B1-1 (5′-TGCTAGAGATTTTCCACAT-3′).

To prepare the competitor/target DNA, a duplex molecule without free 5′ or 3′ ends, the oligonucleotide 5′-AGCTGGCTAACGGCCCTTGGGCCGTTAGCCAGCTATAGACGGCGCTTGCGCCGTCTAT-3′ was phosphorylated and then incubated at 120 nM in 50 mM NaCl to favor intramolecular annealing. The DNA was then ligated by T4 DNA ligase (New England Biolabs) under standard conditions, extracted with phenolchloroform (50:50, vol/vol), and then purified in a denaturing 10% polyacrylamide gel.

In Vitro Integration Assays. HIV integrase was expressed in *Escherichia coli* using the T7 expression system and was purified as described (13). In a typical experiment, 100 nM integrase (≈70 ng) was incubated at 37°C with 10 nM viral substrate in a 25-μl reaction containing 20 mM Hepes (pH 7.5), 1 mM dithiothreitol, 0.05% Nonidet P-40, 0.5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 20 mM NaCl, and 5 mM $MnCl_2$ (all references to molar concentrations of integrase refer to integrase protomers). All incubations were performed at 37°C. The reaction tubes containing the reaction buffer were prewarmed for 5–10 min; then all DNA and competitor substrates (when indicated) and finally integrase were added. Reactions were stopped by adding an equal volume of gel loading buffer (95% formamide/30 mM EDTA/0.1% xylene cyanol/0.1% bromophenol blue) and analyzed by electrophoresis in a denaturing 15% polyacrylamide gel. Time courses were performed by scaling up the reaction 2-fold and taking 5-μl aliquots at indicated time points. Reaction products were quantitated with a Molecular Dynamics PhosphorImager. In pulse–chase

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; HIV, human immunodeficiency virus; ISC, initial stable complex; PSC, processed stable complex.

experiments, reaction mixtures were incubated at 37°C for 10 min, then the competitor/target DNA was added (500 nM) and the incubation was continued for 15 min. To measure the dissociation kinetics, polylysine (P6143; Sigma) was used to block further productive interactions between integrase and viral DNA substrates. After a 10-min incubation of 100 nM integrase with 10 nM unprocessed viral DNA substrate, the resulting complexes were incubated for the indicated time in the presence 1 μM polylysine, then 500 nM competitor/target DNA was added, and the incubation has continued for 15 min. In prebinding experiments, 100 nM integrase was incubated with 10 nM unprocessed viral substrate for 30 sec (or the specified time) at 37°C, then either 10 μM poly-(Asp^{50},Glu^{50}) (P1408; Sigma) or 500 nM competitor/target DNA was added to further block binding to viral DNA, and the reaction mixture was incubated for an additional 30 min. Poly(Asp, Glu), like polylysine, blocks the functional interactions between integrase and viral DNA.

RESULTS

Stable Complex Formation Precedes Viral DNA End Processing and Requires a Divalent Cation. Integration was assayed *in vitro* by using oligonucleotides containing the terminal sequences from the U5 end of viral DNA. Integration proceeds in two distinct catalytic steps (Fig. 1 *Top*). The first, 3′ end processing, results in the removal of the terminal dinucleotide 3′ to the highly conserved CA dinucleotide. In the second step, termed strand transfer, the recessed 3′ end of the processing product is joined to a second, often identical, oligonucleotide serving as the target. To test for the presence of a stable interaction between integrase and viral DNA, we used a modified version of the standard integration assay (Fig. 1 *Bottom*). If assembly of a stable complex between integrase and viral DNA is an initial step in the reaction, binding of integrase to viral DNA should become resistant to competition before 3′ end processing activity is detected. In the experiment of Fig. 2, integrase was allowed to bind unprocessed viral DNA ends in a brief preincubation at 37°C (from 15 sec to 5 min). Poly(Asp^{50},Glu^{50}) was then added as a competitor to block further binding, and the incubation was continued for 30 min. The presence of the competitor during the preincubation prevented 3′ end processing (Fig. 2, lanes 19–21). However, processing activity was observed when the competitor was added after the preincubation (lanes 14–18). Thus, a stable complex that was resistant to competition [henceforth referred to as the initial stable complex (ISC)] was formed between integrase and unprocessed viral DNA ends. ISC formation required a divalent cation (here Mn^{2+} was used, but Ca^{2+} as well as Mg^{2+} also suffice; data not shown) and proceeded at a much slower rate at 4°C than at 37°C (data not shown). In addition, the ISC was formed before 3′ end processing activity was detectable (Fig. 2, lanes 4 and 5 vs. lanes 14 and 15). Thus, the formation of a stable complex between integrase and viral DNA precedes the chemical steps in the integration reaction.

Viral DNA Ends Remain Stably Associated with HIV-1 Integrase Between the 3′ End Processing and Strand Transfer Steps. We next asked whether the ISC was competent for both 3′ end processing and strand transfer by performing the same prebinding experiment depicted in Fig. 1 *Bottom* using nonviral DNA, instead of poly(Asp,Glu), as a competitor.

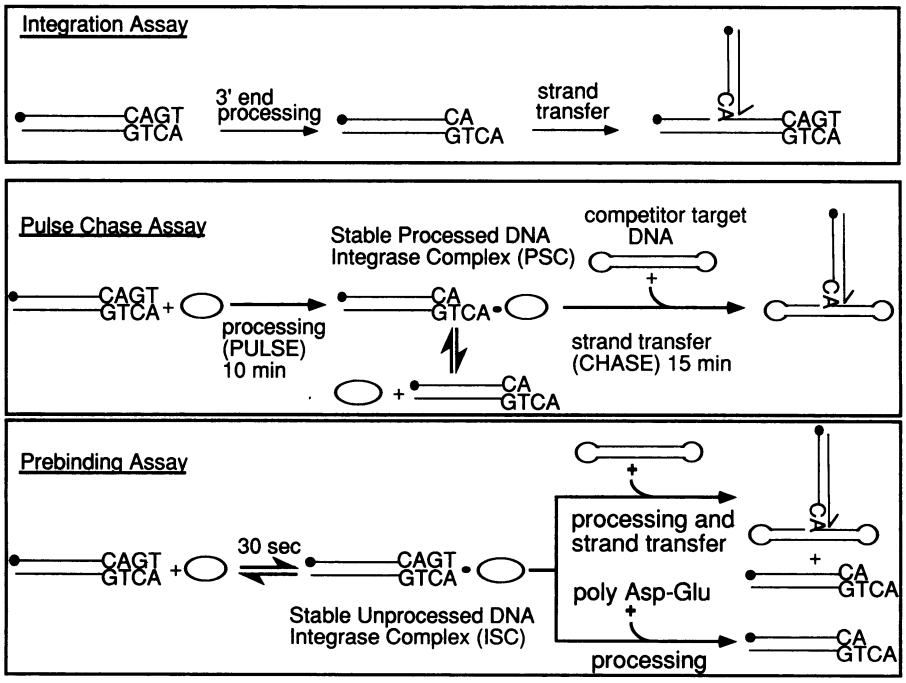

FIG. 1. *In vitro* integration assays. (*Top*) Standard *in vitro* assay for integration. An oligonucleotide mimicking one end of viral DNA is substrate in the reaction. The integration reaction consists of two steps: 3′ end processing and strand transfer. In the 3′ end processing reaction, integrase acts as an endonuclease, removing the two terminal bases distal to the highly conserved CA dinucleotide. In the second step, called strand transfer, integrase catalyzes a concerted cleavage and ligation reaction in which the recessed 3′ end of viral DNA is joined to target DNA. The two lower panels depict the modified versions of the standard integration assay used to test for stable integrase–viral DNA intermediates in the reaction. (*Middle*) Pulse–chase assay. Processed ends were generated during a brief incubation (pulse) after which further processing activity was inhibited by adding a large excess of nonradioactive nonviral DNA, which competitively blocks further binding of integrase to the viral DNA substrate. The activity of complexes formed during the pulse was monitored by assaying integration into the nonspecific competitor (chase). (*Bottom*) Prebinding assay. This assay was similar to the pulse–chase assay, except that the competitor was added before any detectable end processing had occurred. Any activity detected after the addition of competitor should be due to stable complexes formed during the preincubation, because the addition of competitor blocks any further binding to viral DNA. In these experiments, either nonspecific DNA, a poly(amino acid) [poly(Asp,Glu), poly(Glu,Tyr) poly(lysine)], or heparin was used as a competitor for viral end binding.

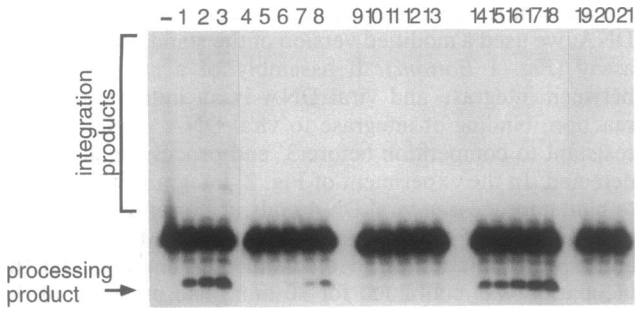

FIG. 2. Stable complexes between integrase and unprocessed viral DNA ends. The prebinding experiment was performed as described in *Materials and Methods*. Preincubation was for the indicated time in the presence or absence of 5 mM $MnCl_2$. When omitted from the prebinding step, the divalent cation was added to the reaction simultaneously with the competitor, poly(Asp,Glu). Integrase was omitted from the reaction in lane −. Lanes 1–8 are, respectively, time points of 10, 20, 30, 0.25, 0.5, 1, 2.5, and 5 min from a standard integration reaction. For lanes 9–18, integrase was preincubated with viral DNA for 0.25 min (lanes 9 and 14), 0.5 min (lanes 10 and 15), 1 min (lanes 11 and 16), 2.5 min (lanes 12 and 17), or 5 min (lanes 13 and 18), then poly(Asp,Glu) was added, and the incubation continued for 30 min. The prebinding step was performed either in the presence (lanes 14–18) or in the absence (lanes 9–13) of the divalent cation. Lanes 19–21 show products of standard reactions in which the competitor [target DNA in lane 19, poly(Asp,Glu) in lanes 20 and 21] and the viral substrate were added simultaneously, and the reaction mixtures were incubated for 30 min (lanes 19 and 21) or 0.25 min (lane 20).

Integration into this competitor DNA should be detected only if dissociation does not occur after processing. Indeed, after forming the initial stable complex, integrase processively catalyzed both the 3′ end processing and strand transfer reactions (Fig. 3, lanes 5 and 6). Thus, under the appropriate experimental conditions, purified integrase displays two properties previously recognized for the *in vivo* integration complexes: (*i*) formation of a stable complex with viral DNA end sequences and (*ii*) stable association with its viral DNA substrate between the two essential chemical steps in the integration process.

Stable Complexes Between HIV-1 Integrase and Viral DNA Ends: Distinct Conditions for Formation, Activity, and Maintenance. In integration-competent nucleoprotein complexes isolated from murine leukemia virus- or HIV-infected cells, integrase maintains a very stable interaction with processed viral DNA. The isolated complexes retain integration activity through multiple steps of purification and prolonged exposure to EDTA and to high concentrations of KCl and detergents. We therefore investigated the stability of the processed stable complex (PSC) formed *in vitro* between purified HIV integrase and model DNA ends, using the protocol outlined in Fig. 1 *Middle*. When added prior to the preincubation step (Fig. 4, lanes 5 and 7) 200 mM NaCl or 10 mM CHAPS blocked 3′ end processing and integration. However, the PSC was resistant to 200 mM NaCl and 10 mM CHAPS (Fig. 4, lanes 6 and 8). The complexes retained nearly all their activity after 3 hr at 0°C in the presence of excess competitor (Fig 4, lanes 9 and 10) and after incubation in 10 mM EDTA (compare lanes 12 and 13). The conditions required for assembly of the stable complex are, therefore, distinct from the conditions required for its maintenance and activity and are, in general, more restricted.

In contrast to the stable association we observed when viral DNA substrate molecules were functionally bound as viral DNA ends, the association between integrase and the same viral DNA substrates was unstable when they were functionally bound as target DNA. Competitor/target DNA exchanged rapidly with viral DNA at the enzyme's binding site for target DNA, providing a clear functional distinction between the viral and target DNA binding sites, and ruling out a trivial sequestration phenomenon as the explanation for the resistance of the stable complexes to competitor DNA.

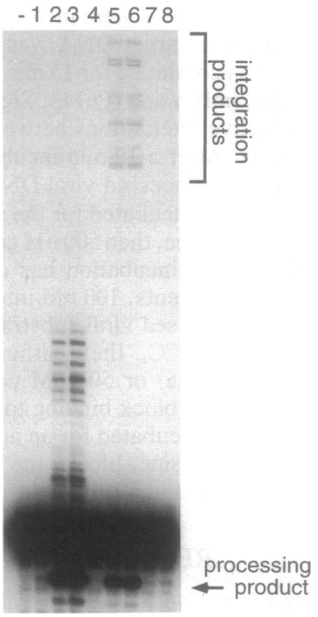

FIG. 3. The 3′ end processing and strand transfer steps occur processively after assembly of the ISC. In this prebinding experiment, the preincubation was only 30 sec and target DNA was used in place of 10 μM poly(Asp,Glu) as a competitor. The competitor DNA was a double-stranded oligonucleotide with no free 5′ or 3′ ends (preparation described in the *Materials and Methods*). Therefore, any single integration of the viral DNA substrate used into this molecule should yield a linear 77-nt product. However, the ability of the target to form very stable secondary structures led to the appearance of multiple product bands corresponding to different sites of integration. Integrase was omitted from the reaction in lane −. Lanes 1–3 are time points (0.5, 10, and 30 min) from a standard integration reaction, and lane 4 is a 30-min time point from a standard reaction in which viral DNA and competitor DNA were added at once. For the preincubations (lanes 5–8), 5 mM $McCl_2$ was either included (lanes 5 and 6) or omitted (lanes 7 and 8), and time points (10 min, lanes 5 and 7; 30 min, lanes 6 and 8) were taken after competitor/target DNA addition.

A 2-bp Deletion from the 5′ End of Viral DNA Destabilizes the PSC. One substrate alteration, a deletion of the 5′-terminal dinucleotide AC, was previously reported to compromise strand transfer but not processing (14). Paradoxically, the "pre-processed" blunt-ended counterpart of this substrate was fully competent for strand transfer when compared with a pre-processed "wild-type" viral DNA end (15). One possible resolution to this apparent paradox is that while both the processing and strand transfer reactions occur efficiently under saturating conditions, the two reactions are not executed processively by integrase using this modified substrate, because the 2-base 5′ overhang is required for the stability of the PSC. This hypothesis was tested by examining the stability of PSCs with the ΔAC substrate in a modified version of the pulse–chase assay. In this experiment (Fig. 5), polylysine was used as a nonspecific competitor because, unlike the ISC, the PSC that formed with the 5′ ΔAC substrate was unstable in the presence of poly (Asp,Glu) (unpublished results). Although polylysine could block ISC assembly when present during the preincubation (lanes C), integration by the PSC into the target DNA was not affected by polylysine (lanes 1). However, when PSCs were incubated with polylysine before target DNA addition, we observed a time-dependent decrease in strand transfer activity (Fig. 5, *Left*, lanes 2–6, time course of incubation of standard viral

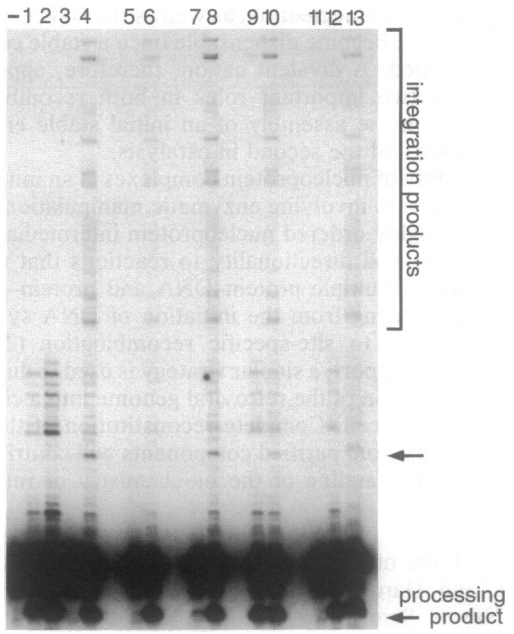

FIG. 4. Pulse–chase experiment: Conditions for complex formation, activity and stability. The pulse–chase assay is described in *Materials and Methods*. Integrase was preincubated with viral DNA for 10 min at 37°C, after which competitor/target DNA was added and the incubation continued for an additional 15 min. In this particular experiment only, the competitor/target DNA oligonucleotide was not gel purified. Therefore, along with the characteristic set of product bands typically observed, there were also smaller product bands resulting from integration into this substrate. Lanes 1 and 2, 10-min and 20-min time points from a standard integration reaction; lane 3, viral and competitor DNA were added to the reaction simultaneously; lane 4, integrase was preincubated with viral DNA and then competitor/target DNA was added; lanes 5–8, 200 mM NaCl (lanes 5 and 6) or 10 mM CHAPS (lanes 7 and 8) was added to the reaction mixture before (lanes 5 and 7) or after (lanes 6 and 8) the preincubation of integrase with viral DNA; lanes 9 and 10, following the preincubation of integrase, the reaction mixtures were incubated at 0°C for 3 hr, after which target DNA was added and the incubation continued for 20 min at either 0°C (lane 9) or 37°C (lane 10); lanes 11 and 12, 10 mM EDTA was added either before (lane 11) or after (lane 12) the preincubation of integrase with viral DNA; lane 13, after the preincubation of integrase with viral DNA, 10 mM EDTA was added to the reaction mixture, which was then incubated at 0°C for 5 min, diluted with fresh reaction buffer (final EDTA and $MnCl_2$ concentrations, 2 mM and 8 mM, respectively), and then incubated with target DNA at 37°C for 20 min. Integrase was omitted from the reaction in lane −.

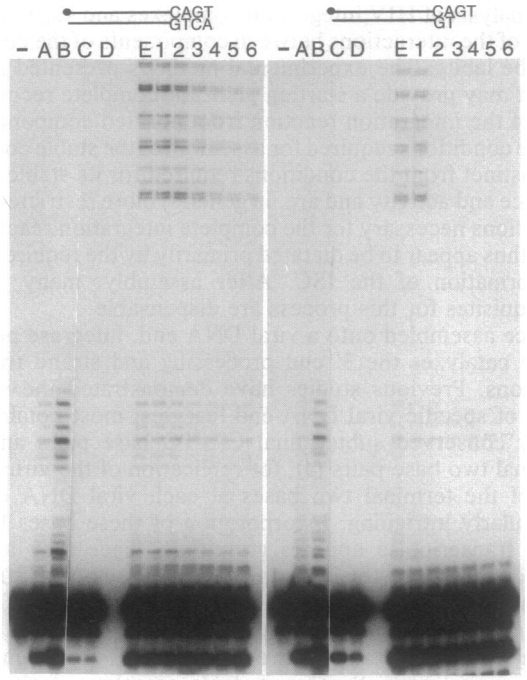

FIG. 5. Instability of complexes with processed ends lacking the 2-bp 5′ overhang. After a 10-min preincubation of integrase with viral DNA at 37°C, 1 μM polylysine was added and the incubation continued at 37°C for the specified time. Competitor/target DNA was then added and the incubation continued for an additional 15 min at 37°C. The reactions were performed with either the standard viral DNA substrate (*Left*) or the ΔAC viral DNA substrate (*Right*) and are as follows: lanes A and B, 10-min and 60-min time points from a typical integration reaction; lanes C, integrase was incubated with polylysine and viral DNA (added simultaneously) for 60 min and then competitor DNA was added; lanes D, a 60-min incubation of integrase with the viral DNA substrate and the competitor DNA (added simultaneously); lanes E, integrase was preincubated with viral DNA then competitor DNA was added to the reaction; lanes 1–6, after the preincubation of integrase with viral DNA, 1 μM polylysine was added and the reaction mixture was incubated for 0, 2.5, 10, 20, 40, or 60 min, respectively, after which competitor DNA was added and the reaction mixture was incubated for another 15 min. Integrase was omitted from the reaction in lane −.

DNA-end PSCs with polylysine) which we attribute to dissociation of the PSCs formed during the preincubation. From quantitation of this data and additional data not shown, we determined that PSCs with the standard viral DNA-end substrate had a half-life of ≈13 min under these conditions, whereas complexes assembled with the ΔAC substrate were significantly less stable (Fig. 5, *Right*, lanes 2–6), with ≈70% of the complexes dissociating in <2.5 min. Thus, recognition of a specific feature of the viral DNA end plays a critical role in the stability of the integrase–viral DNA end complex.

DISCUSSION

Integration of the retroviral genome is crucial for the establishment of a productive infection. This transpositional recombination reaction is executed by a nucleoprotein complex in which integrase is stably associated with viral DNA. The mechanism by which this functional, stable interaction is maintained during the lifespan of the complex, which may need to be many hours *in vivo* (16), has not previously been investigated. The purified enzyme binds nonspecifically not only to nucleic acids but also to other polyanionic molecules. In view of the abundance of such potential inhibitors in the cell, dissociation of integrase from the ends of viral DNA prior to the strand transfer step would most likely be fatal to the virus.

We have shown in this report that a stable interaction between integrase and viral DNA can be established *in vitro* with purified recombinant HIV integrase and small synthetic DNA substrates. This stable complex requires only purified integrase and specific sequences at the end of viral DNA for its formation and stability, and it processively executes the catalytic steps in the integration pathway. The stable association of the integration machinery with viral DNA, a fundamental feature of *in vivo* integration complexes, is therefore intrinsic to integrase. However, the possible involvement of other viral or cellular proteins in the integration pathway remains an open question.

Other retroviral proteins have been shown to be constituents of murine leukemia virus integration complexes isolated from infected cells (7). For HIV, Farnet and Haseltine (8) reported finding only integrase in HIV integration complexes, whereas Bukrinsky *et al.* (9) observed other viral proteins as components of the complex. This discrepancy may be due to the different conditions used for the isolation

and analysis of HIV integration complexes and suggests that some of the interactions between components of the complex may be labile. The experimental methods presented in this report may provide a starting point for complete reconstitution of the integration reaction from purified components.

The conditions required for assembly of the stable complex are distinct from the conditions required for its stable maintenance and activity and are, in general, more restricted. The conditions necessary for the complete integration reaction *in vitro* thus appear to be dictated primarily by the requirements for formation of the ISC. After assembly, many of the prerequisites for this process are dispensable.

Once assembled onto a viral DNA end, integrase processively catalyzes the 3′ end processing and strand transfer reactions. Previous studies have demonstrated the importance of specific viral DNA-end features, most notably the highly conserved subterminal CA/TG base pairs and the terminal two base pairs (4), for replication of the virus. The role of the terminal two bases at each viral DNA end is particularly intriguing. Incorporation of these bases by reverse transcriptase appears gratuitous, since they are removed in the next step in the life cycle. Based on our studies, we can assign to the 5′-terminal two bases a role in maintaining the association between integrase and viral DNA during the long interval between the 3′ end processing and DNA joining steps. Processed stable complexes lacking the 2-base 5′ overhang are destabilized, resulting in a reduction in the overall efficiency of strand transfer. The significance of these bases is also evident during the disintegration reaction—reintegration of processed viral DNA ends produced during disintegration is abolished by deletion of the 2-base 5′ overhang from the disintegration substrate (17). Thus, a specific interaction between integrase and the two terminal 5′ bases performs an essential role preceding the strand transfer step in the integration pathway, and its principal function may be to stabilize the PSC.

The relationship between the integrase protein's binding sites for its viral DNA and target DNA substrates remains unclear. Models proposing either independent DNA binding sites (13, 18) or a single binding site (19) for viral DNA and target DNA have been suggested. Our results are most consistent with the existence of clearly distinct binding sites for viral DNA and target DNA. The observation that at saturating substrate concentrations, DNA remains stably bound at the functionally defined viral DNA binding site, while in the same reaction, DNA bound at the functionally defined target DNA binding site exchanges rapidly (Figs. 4 and 5) is incompatible with a model in which the two sites are equivalent or rapidly interconvertible.

Assembly of a stable complex between integrase and viral DNA depends upon a divalent cation. Although Mn^{2+} was used exclusively in the experiments reported here, Ca^{2+} or Mg^{2+} also promoted stable complex formation (unpublished results). One possible role of the divalent cation is to promote specific interactions between integrase protomers leading to integrase multimerization (V.E., K. A. Vincent, J. Gerton, and P.O.B., unpublished work). The divalent cation plays a similar role in promoting the assembly of an important intermediate in the bacteriophage Mu transposition reaction, the stable synaptic complex (SSC) (20, 21). In Mu transposition, assembly of the active tetrameric form of MuA in the SSC requires not only the divalent cation, but also two types of MuA binding sites on a supercoiled substrate. After construction of the SSC, catalytic events can occur under relaxed conditions that dispense with many of the prerequisites for SSC formation. Likewise, in addition to the divalent cation, certain features of the viral DNA substrate, such as the conserved CA dinucleotide, as well as the HHCC domain of integrase (13), become dispensable once a stable complex has been formed. A divalent cation, therefore, appears to play at least two important roles in both recombination systems: one in the assembly of an initial stable enzyme–DNA complex and the second in catalysis.

The assembly of nucleoprotein complexes is an initial step in many processes involving enzymatic manipulation of nucleic acids. Highly ordered nucleoprotein intermediates impart specificity and directionality to reactions that require coordination of multiple protein–DNA and protein–protein interactions, ranging from the initiation of DNA synthesis and transcription to site-specific recombination (22). As described in this report, a similar strategy is used to direct the stable incorporation of the retroviral genome into a chromosome of the host cell. Complete reconstitution of the integration complex from purified components will contribute to a detailed understanding of the biochemistry of retroviral integration.

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