Displacement of Viral DNA Termini from Stable HIV-1 Integrase Nucleoprotein Complexes Induced by Secondary DNA-Binding Interactions†

Iain K. Pemberton,* Henri Buc, and Malcolm Buckle

Unité de Physicochimie des Macromolécules Biologiques, CNRS UMR 321, Institut Pasteur, Paris 75724, France

Received August 1, 1997; Revised Manuscript Received November 17, 1997

ABSTRACT: The human immunodeficiency virus type-1 (HIV-1) integrase is known to form a highly stable interaction with the termini of the linear, pre-integrated retroviral genome, where it catalyzes the 3′-OH processing and strand transfer processes required for their coordinated integration into host DNA. Here, we determine that the association of HIV-1 integrase with the viral DNA termini leads to the formation of two classes of nucleoprotein complexes with distinct properties *in vitro*. Both bound states are intrinsically stable and highly resistant to exonuclease digestion, but nonetheless they exhibit different stabilities in the presence of single-stranded polynucleotides. While a population of preassembled complexes tolerates elevated polynucleotide concentrations, the remainder forms an unstable ternary (integrase−substrate−polynucleotide) intermediate, leading to the rapid expulsion of the otherwise tightly bound substrate. The distribution of complexes between the two states is influenced by the preincubation time and temperature, increases in either of which favor the formation of the challenge-resistant species. Challenge-resistant complexes are formed more efficiently with Mn^{2+} than with Mg^{2+} and are sensitive to the length rather than the sequence of the DNA substrate. Due to the delayed appearance of the challenge-resistant form after the initial stable binding of the DNA substrate, our results may be indicative of a structural change in the preassembled complex which thereby modulates its response to exogenous DNA targets.

In the replicative cycle of the retroviruses, the establishment of a proviral state, where a DNA copy of the viral genome is stably inserted into a chromosome of the infected host cell, is essential for the production of subsequent generations of single-stranded RNA progeny. This reaction is catalyzed by the retroviral integrase which recognizes elements within the termini of the linear pre-integrated viral genome to, first, remove the two outermost bases from both 3′-OH ends (termed 3′-OH processing) and, second, coordinate the recessed 3′-OH ends in the strand transfer events which ultimately result in their covalent joining with 5′ phosphates of the host DNA. Detailed reviews of the general polynucleotidyl transfer mechanism (*1*, *2*) and retroviral DNA integration (*3*, *4*) are available.

Stable higher-order nucleoprotein assemblies play an important role in many DNA insertion mechanisms. In addition to the stability of isolated retroviral pre-integration complexes (*5*, *6*), several functionally analogous polynucleotidyl transfer reactions, such as the transposition of bacteriophage Mu (*7*), also proceed through a highly stable protein−DNA intermediate. The propensity of the human immunodeficiency virus type-1 (HIV-1)[1] integrase to remain tightly associated with viral long-terminal repeat (LTR) substrates throughout the course of an *in vitro* DNA integration reaction thus appears to reflect an intrinsic property of this retroviral protein. Previously, the stability of this interaction has been established by the resistance of the preassembled complex to the addition of poly(Asp^{50}, Glu^{50}) (*8*, *9*), competitor DNA (*8*, *9*), heparin (*10*), and an exonuclease (*11*). Stable binding has also been confirmed by the tight interaction observed with immobilized DNA substrates (*12*). While other retroviral (*5*, *13*) or host (*14*) proteins may be required to assemble a fully functional pre-integration complex *in vivo*, the minimal elements necessary to form a stable half-site (1-LTR) pre-integration intermediate are provided by short, synthetic viral DNA substrates and the HIV-1 integrase protein alone. However, whereas the stability of the *in vitro* complex is known to be influenced strongly by the presence of a divalent cation, notably Mn^{2+} in preference to Mg^{2+} (*9*, *11*), the underlying molecular mechanism involved in the assembly of these complexes remains poorly characterized.

Several DNA-binding and catalytic functions, such as disintegration (*15*), of the integrase can be reproduced *in vitro* with the isolated domains of this retroviral protein (*16*−*22*). In contrast, the formation of a stable complex *in vitro* requires that all of the HIV-1 integrase domains remain intact (*9*). This strict requirement may indicate the cooperation between these domains in this process. Recently, the activity of the HIV-1 integrase in Mg^{2+}-reaction buffers has been

† This work was supported by a fellowship grant from the Fondation pour la Recherche Medicale to I.K.P. and additional funding from The Royal Society.

* Author to whom correspondence should be addressed at Unité de Physicochimie des Macromolécules Biologiques, CNRS UMR 321, Institut Pasteur, 28 Rue du Dr., Roux, Paris 75724, France. Telephone: (331) 40613002. Fax: (331) 40613060. E-mail: iainpem@pasteur.fr.

[1] Abbreviations: HIV-1, human immunodeficiency virus type-1; IN, integrase; ssDNA, single-stranded DNA; LTR, long terminal repeat; EDTA, ethylenediaminetetraacetic acid.

S0006-2960(97)01893-X CCC: $15.00
Published on Web 02/06/1998

shown to be stimulated by Zn^{2+} (*23*, *24*). The stoichiometric binding of Zn^{2+} to the highly conserved N-terminal HHCC domain induces the oligomerization of the integrase (*24*, *25*). The HHCC domain has also been implicated in the Mn^{2+}-stimulated oligomerization of this protein (*10*). Metal ion-induced protein−protein interactions thus appear to play an important role in the assembly of the stable, higher-order nucleoprotein complex.

It may be surmised that at some stage in the process of retroviral DNA integration, the higher-order nucleoprotein complex must accommodate both viral DNA ends and a host target DNA site in a temporary integrase−(2-LTR)−target DNA complex, thereby enabling the concerted integration of the viral DNA ends at that site. Although the several reports cited above attest to the stability of the liaison formed between the integrase and viral DNA ends *in vitro*, the process by which the stable higher-order complex captures and orients the target DNA site for the ultimate purpose of integration is not known. Experiments with the pre-integration complex *in vitro* suggest that the integrase binds and commits quickly and stably to a nonspecific target DNA site (*26*). During the course of our studies, however, it was noticed that an early interaction with an exogenous polynucleotide target could in fact impair the activity of the preassembled stable complex *in vitro*. In the present study, we investigate the process underlying this inhibition mechanism. By this approach, we characterize a possible structural heterogeneity in the preformed nucleoprotein *in vitro*, resulting in the unexpected capacity of nonspecific polynucleotides to mediate the disassembly of a defined proportion of stable complexes via a transient ternary intermediate formed with the exogenous DNA target. Possible explanations for these observations are discussed in the context of a potential conformational change in the nucleoprotein assembly following the stable binding of the HIV-1 integrase to the viral DNA ends.

MATERIALS AND METHODS

Oligonucleotide Substrates. All oligonucleotides employed in this study were purchased gel-purified from Genset (Paris). Integrase assays were performed with a 21-bp duplex oligonucleotide derived from the terminal sequence of the U5 end of the viral DNA. The DNA fragment was prepared by 5′-end-labeling the oligonucleotide 5′-GTGTGCAAA-ATCTCTAGCAGT-3′ with [γ-^{32}P]ATP (3000 Ci/mmol, Amersham) and T4 polynucleotide kinase (New England Biolabs). The radiolabeled nucleotide was hybridized to its complement (5′-ACTGCTAGAGATTTTGCACAC-3′) and purified from nonincorporated nucleotides by gel filtration on a Sephadex G25 column (Pharmacia Biotech). The other duplex fragments used in this study were made by 5′-end-labeling the strands shown below and hybridizing them to their complements as described above: U5 LTR 42bp, 5′-TCCCTCAGACCCTTTTAGTCAGTGTGCAAAA-TCTCTAGCAGT-3′; nsp 23bp, 5′-ATTAATACGACTCAC-TATAGGGT-3′; nsp 45bp, 5′-TCGAAATTAATACGACT-CACTATAGGGAGACCACAACGGTTTCCC-3′.

Integrase Reactions. HIV-1 integrase was expressed in *Escherichia coli* under the control of a T7 polymerase promoter and purified as described (*11*). Standard DNA-binding and 3′-OH processing reaction conditions consisted of 400 nM HIV-1 integrase and 12.5 nM U5 LTR substrate in a 20 μL volume containing 30 mM Tris·HCl (pH 7.6), 1% glycerol, 1 mM dithiothreitol, 0.5 mM 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), 0.05% Tween 20, 50 mM NaCl, and 5 mM $MnCl_2$. Reactions were analyzed on 20% denaturing polyacrylamide gels.

Exonuclease Assays. Nuclease digestions using snake venom phosphodiesterase I (Pharmacia Biotech) were performed as previously described (*11*).

Sedimentation of the Stable Nucleoprotein Complex. The binding of HIV-1 integrase to DNA results in the formation of a sedimentable nucleoprotein complex (*27*). This property was used as a simple assay to monitor the occlusion of the DNA substrate into stable complexes under near DNA-saturating concentrations of the protein ligand (generally 400 nM HIV-1 integrase to 12.5 nM ^{32}P-end-labeled U5 LTR). After binding in a volume of 50 μL, the nucleoprotein was sedimented by centrifugation for 5 min at 13 000 rpm (at 4 °C). The supernatant was carefully removed, leaving a small but visible protein pellet. The pellet was then resuspended in an equal volume of reaction buffer prior to ^{32}P Cerenkov counting of both fractions.

Protein−DNA Cross-Linking. Rapid pulse (5 *ns*) UV cross-linking (*28*) of the HIV-1 integrase nucleoprotein was performed using a high-energy, monochromatic (266 nm) light source emitted by a pulsed Nd:YAG (neodymium-doped yttrium aluminum garnet) laser (Quanta-Ray DCR-11, Spectra-Physics) as described previously (*11*). Cross-linked adducts with ^{32}P-radiolabeled DNA were separated from non-cross-linked DNA by SDS−polyacrylamide gel electrophoresis on 8−18% gradient gels.

Quantification. Gel-separated products were quantified by PhosphorImage densitometry using ImageQuant software (Molecular Dynamics). In competition experiments, the IC_{50} value refers to the concentration of competitor for which there is 50% reduction in DNA-binding or 3′-OH processing activity compared to control incubations. For the preassembled nucleoprotein, the IC_{50} value refers to the concentration required to displace 50% of the challenge-sensitive complexes after a given time of exposure to the competitor.

RESULTS

Experimental Design. A duplex oligonucleotide composed from the sequence of the terminal 21-bp of the HIV-1 U5 LTR was employed as a substrate to monitor the stability of the liaison formed between the HIV-1 integrase (IN) and the viral DNA termini *in vitro*. Below, we establish that single-stranded (ss) DNA is an efficient inhibitor of this binding interaction, both pre- and postassembly of the integrase with the viral DNA ends. At the outset, two limiting mechanisms are possible to explain the influence of the competitor DNA, C, on the dissociation of the DNA substrate, S, from the preformed IN·S complex: (*1*) S must dissociate from the complex before C can associate (competitive), or (*2*) C can directly attack the IN·S complex, destabilizing it and leading to the chase of S (noncompetitive). These two mechanisms may thus be summarized

$$\text{IN•S} \xrightarrow[k_0]{\text{S}} \text{IN} \xrightarrow[\text{C}]{} \text{IN•C} \quad (1)$$

$$\text{IN•S} \xrightarrow[\text{C}]{} \text{IN•S•C} \xrightarrow[k_c]{\text{S}} \text{IN•C} \quad (2)$$

In the first case, the rate of displacement of S will not depend on the concentration of C, which is merely a trap for dissociated IN. In the second case, the displacement of S proceeds via a ternary (IN•S•C) species that is dependent on the concentration of C. In the absence of competitor, for both mechanisms, the spontaneous (unassisted) dissociation of the IN•S complex is expected to follow the first-order exponential decay expression

$$[\text{IN•S}]_t = [\text{IN•S}]_0 \exp^{-k_0 t} \quad (3)$$

where the concentration of IN•S at time t is defined by the rate, k_0, which represents the intrinsic dissociation rate of the preassembled complex.

To understand how ssDNA impairs the function of the preassembled complex, our experiments below were designed to distinguish between the competitive or noncompetitive mechanisms. Thus, to begin with, we introduce methods to detect the stability of the IN•S complex (defined by k_0) in the absence of competitor, prior to analyzing the influence of ssDNA on this parameter.

Measurement of k_0 by Dilution. For a reversible binding process, it should be possible to estimate the dissociation kinetics from the re-equilibration rate after dilution (i.e., to a level that minimizes rebinding). Thus, to detect if any fraction of the preformed IN•S complex remained in rapid equilibrium with the free IN and S species, the preformed complex was diluted with non-radiolabeled substrate (while keeping the concentration of the DNA and reaction components constant). Figure 1 shows the effect of this perturbation on the sedimentation of the IN•S complex after preassembly at 21 °C. When reactions were diluted without prebinding, a cooperative saturation curve was obtained with respect to the final integrase concentration (Figure 1A). This corresponds to a Hill coefficient of 2 and an apparent dissociation constant of 85 nM, consistent with previous observations of the binding equilibrium (*11*). However, after prebinding, less than 10% of the preformed complexes were observed to re-equilibrate with the cold DNA substrate. This confirms that >90% of the bound substrate DNA had been occluded into a stable complex in the initial 5 min preincubation step. By extending these measurements over a longer time period (Figure 1B), the first-order dissociation rate (k_0) of the preformed complex to the residual level (18%) was estimated to be on the order of 1.3×10^{-5} s^{-1} (half-life ~ 15 h).

Measurement of k_0 by Exonuclease Digestion. A property of the stable nucleoprotein complex is to protect the occluded viral DNA ends from degradation by exonucleases. We have used this principle to measure the release of the bound substrate DNA from the stable complex, as described previously (*11*). In reactions containing 5 mM Mn^{2+} (data not shown), k_0 is again estimated to be very slow (~7×10^{-6} and 1.5×10^{-5} s^{-1} at 21 and 37 °C, respectively). Furthermore, these measurements are independent of the fractional saturation (θ) of the DNA substrate (i.e., at θ = 0.15, 0.45, 0.78, and 0.9, $k_0 \sim 10^{-5}$ s^{-1} at 37 °C).

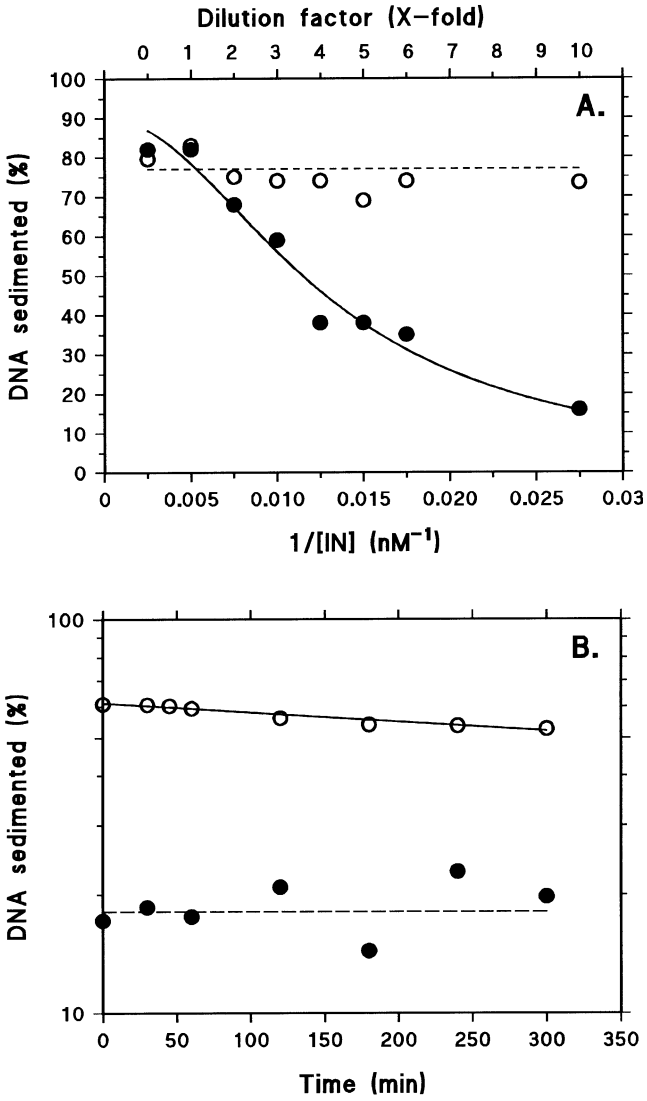


FIGURE 1: Effect of dilution on the binding of the viral DNA substrate. HIV-1 integrase was added to reactions containing the 21-bp ^{32}P-end-labeled U5 LTR substrate (12.5 nM) either before (○) or after (●) dilution with the unlabeled LTR substrate DNA. Preincubations were performed for 5 min at 21 °C, and DNA binding was monitored by sedimentation. (A) Complexes initially formed at 400 nM HIV-1 integrase were incubated for 15 min after dilution with the cold U5 LTR (12.5 nM) to a range of final integrase concentrations. (B) Complexes formed at 200 nM HIV-1 integrase and diluted 10-fold with unlabeled LTR.

Dissociation of the Stable Nucleoprotein Is Promoted by Secondary Interactions with Polynucleotides. Both estimates given above confirm that k_0 is slow. For mechanism 1, the addition of the competitor should not influence k_0 or, by consequence, the prolonged protection of IN•S from the exonuclease. In contrast, a significant increase in the nuclease-susceptibility of IN•S was detected. This effect is shown in Figure 2 for a range of ssDNA concentrations. Poly(dC)$_{50}$ is demonstrated to be an efficient inhibitor of stable complex formation when present during the binding reaction (IC_{50} ~ 30 nM; Figure 2A). However, after preassembly, the stable IN•S complex also remained partially sensitive to this inhibitor. Thus, a larger proportion of the prebound substrate was attacked in the presence of the ssDNA than in its absence (IC_{50} ~ 50 nM; Figure 2B).

The above effect might be explained by the displacement of the substrate from the stable IN•S complex. To test this,

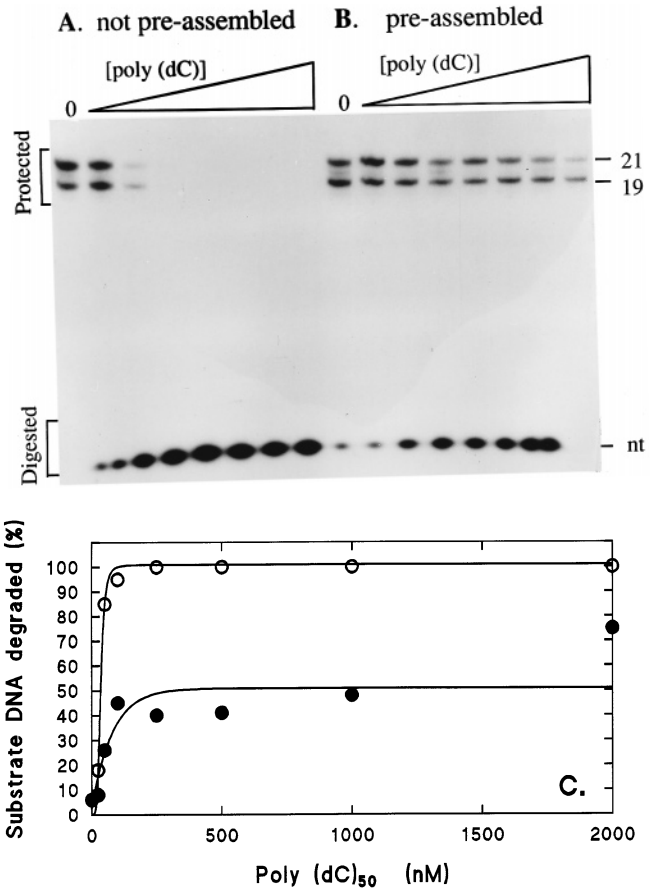


FIGURE 2: Diminished protection of the DNA substrate from degradation by exonucleases in the presence of poly(dC)$_{50}$. HIV-1 integrase−LTR complexes were formed under standard reaction conditions either (A) in the presence or (B) for a 5 min preincubation period in the absence of poly(dC)$_{50}$ at 37 °C. After addition of poly(dC), complexes were incubated for a further 5 min prior to exonuclease digestion for 30 min. The positions in nucleotides of the full-length substrate (21), 3′-OH processed product (19), and free nucleotides (nt) are shown. Controls performed in the absence of poly(dC) are indicated (lane 0). (C) Graphical representation of the data for the complexes formed either in the presence (○) or in the absence (●) of the competitor.

the complex was challenged with poly(dT)$_{50}$ prior to centrifugation of the nucleoprotein, as described in Figure 1. Single-stranded DNA was indeed found to induce the dissociation of the substrate DNA (Figure 3). Furthermore, the concentration dependence was similar to that observed for the displacement of nuclease-resistant complexes (IC_{50} ∼ 50 nM; Figure 3A). However, approximately 60% of the complexes survived the addition of ssDNA up to 100 μM (Figure 3B). These appear to have attained a challenge-resistant conformation. While poly(dC)$_{50}$ displaced an equivalent fraction of complexes, this effect was not observed with poly(dA)$_{50}$. Additionally, the displacement of the complex was dependent on the nucleotide chain length (N) of the ssDNA lattice. For poly(dT) or random polynucleotides of $N \leq 21$, no displacement of the prebound substrate was detected at concentrations up to 5 μM (data not shown). This provides an explanation to why ssDNA was not found to be an efficient competitor in a previous study (*29*).

Results similar to those above were obtained when the catalytic activity of the complexes was monitored. Here, the IN·S complexes were challenged with either poly(dC)$_{50}$ or heparin after a 5 min preincubation period, prior to incubation for a further 30 min at 37 °C. The displacement of the stable nucleoprotein [IC_{50} ∼ 50 nM for (dC)$_{50}$] was revealed in this case by a decrease in the nucleolytic processing of the viral LTR relative to the unchallenged control. Again, a substantial population was found to exhibit challenge resistance. An equivalent residual level of activity was obtained for the two polyanions (∼60% of the control). Hence, while the displacement of challenge-sensitive complexes is entirely due to nonspecific DNA-binding interactions, neither appears to affect the challenge-resistant population.

In summary, these observations support mechanism 2 above. That is, ssDNA does not act in the classical sense of a competitor (simply providing a trap for dissociated IN) but rather must play an active role in the displacement of the viral DNA ends from the otherwise stable nucleoprotein. However, this does not apply to all complexes. Our results also indicate that the stable nucleoprotein partitions into two classes of complexes with different sensitivities to this process.

Temperature Influences the Distribution between the Two Species. In Figure 5, the distribution between challenge-sensitive and challenge-resistant complexes was monitored as a function of the preincubation temperature. In the absence of ssDNA, DNA-binding was not significantly altered over this temperature range. Between 21 and 37 °C, approximately 90−95% of the LTR was incorporated into IN·S species under the reaction conditions employed. The IN·S complex is intrinsically stable at both these temperatures (see above). Nonetheless, the formation of the challenge-resistant species remained highly dependent on this parameter. When assessed by sedimentation, UV cross-linking, or catalytic activity, equivalent estimates for the population of challenge-resistant complexes were obtained. While the challenge-sensitive population predominates at low incubation temperatures (approximately 70−80% of the stable IN·S complex), at higher temperatures a greater proportion (approximately 50−60%) is found to attain the challenge-resistant state. At 42 °C or above, the challenge-resistant fraction decreases. However, this is most likely due to the relative instability of the viral DNA substrate (T_m ∼ 46 °C). Thus, at higher temperatures, the duplex would be partially dissociated into single-stranded DNA which, as shown previously (*11*), cannot be incorporated into stable complexes.

Accelerated Dissociation Kinetics of Challenge-Sensitive Complexes via a Transient Ternary Intermediate. We have shown that the ssDNA inhibitor, C, plays an active role in the displacement of the viral LTR substrate from the IN·S complex. As detailed in mechanism 2, this invokes the formation of an unstable ternary (integrase−LTR−polynucleotide, IN·S·C) intermediate. The alternative route to the dissociation of S from IN·S via displacement by C may thus be represented by the following general pathway:

$$\text{IN·S} + \text{C} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{IN·S·C} \xrightarrow{k_c} \text{IN·C} + \text{S} \qquad (4)$$

Since the spontaneous dissociation rate, k_0, of the prebound IN·S complex to yield free IN and S is negligible in comparison to k_c, IN·S can be considered as a unitary reactant in the pathway above. Thus, the rate equation is simplified

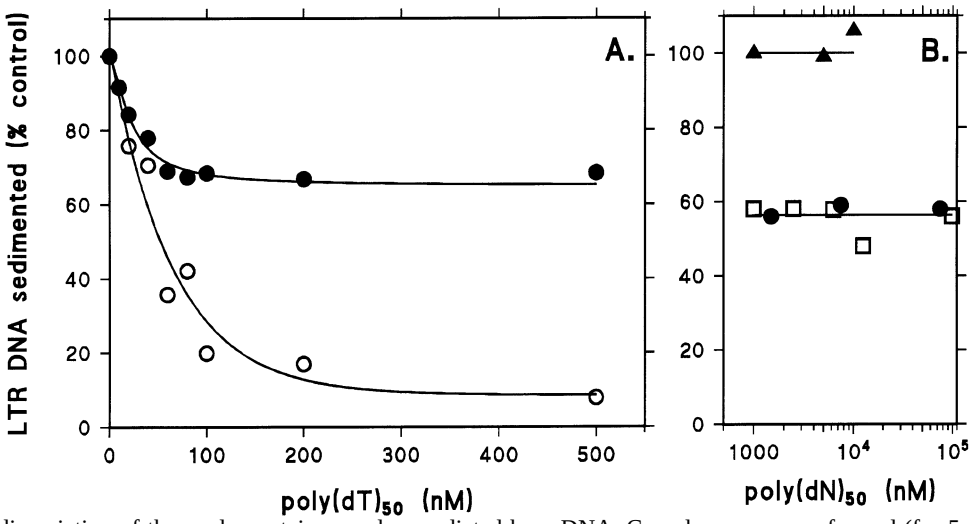


FIGURE 3: Partial dissociation of the nucleoprotein complex mediated by ssDNA. Complexes were preformed (for 5 min at 37 °C under standard reaction conditions) prior to addition of the single-stranded homopolymers [$(dN)_{50}$]. Incubations were continued for a further 5 min before sedimentation of the nucleoprotein. (A) Concentration dependence of poly(dT) on the sedimentation of the radiolabeled substrate when present during (○), or added after (●), assembly of the complexes. (B) Concentration dependence of poly(dA) (▲), poly(dC) (□), and poly(dT) (●) on the sedimentation of the radiolabeled substrate after preassembly.

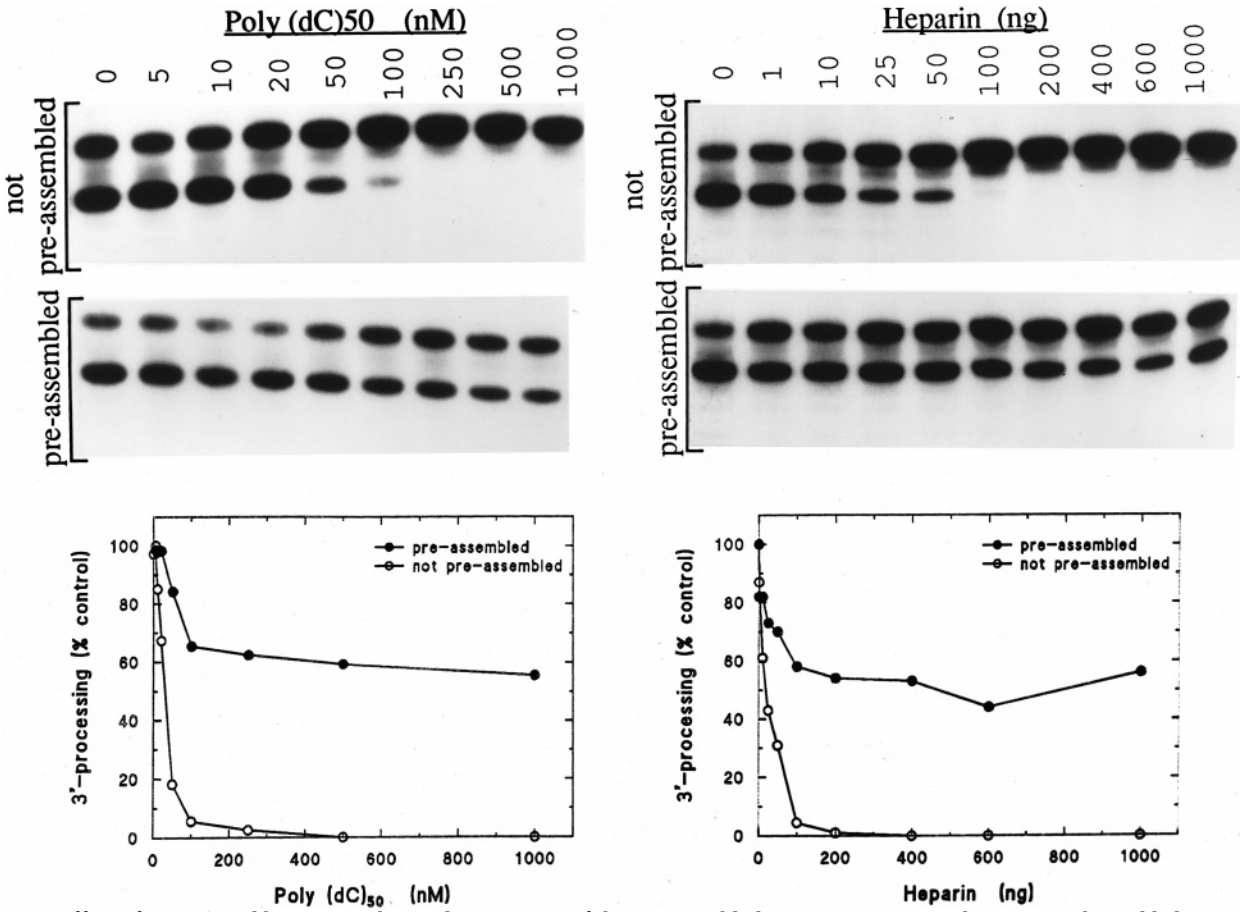


FIGURE 4: Effect of ssDNA and heparin on the catalytic activity of the preassembled HIV-1 integrase nucleoprotein when added to standard integrase reactions either directly (not preassembled) or after a 5 min preincubation period at 37 °C (preassembled). Incubations were continued for a further 30 min at 37 °C. The panels show the autoradiographs of the substrate (upper band) and -2 nucleotide product (lower band) separated on a 20% polyacrylamide gel. The quantification of these data is reported in the lower panels (relative to control activity).

to

$$d[S]/dt = k_c[IN{\cdot}S{\cdot}C] \quad (5)$$

This situation has been treated many times. In the present case, the concentration dependence of C on the observed dissociation rate, k_{obs} (τ_{obs}^{-1}), is predicted to follow the general pseudo first-order approximation where the time of displacement of the complex, τ_{obs}, is given by

$$\tau_{obs} = 1/k_c(1 + K_i/[C]) \quad (6)$$

and where K_i, the inhibition constant, is $(k_{-1} + k_c)/k_1$.

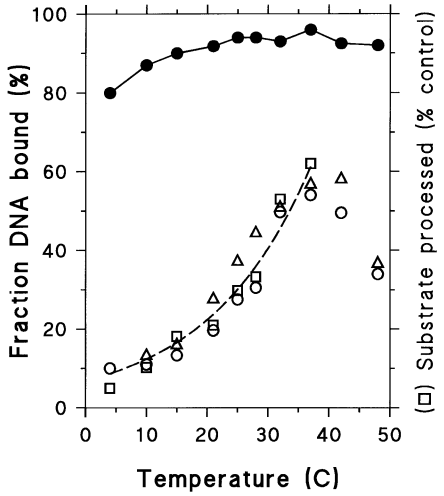


FIGURE 5: Influence of the incubation temperature on the appearance of ssDNA challenge-resistant complexes. Preincubations (5 min under standard conditions) were performed at various incubation temperatures prior to addition of 250 nM poly(dC)$_{50}$. DNA binding was assessed after a further dissociation period of 5 min. The residual bound DNA relative to unchallenged controls was measured by UV cross-linking (△) or sedimentation (○). Sedimentation of the U5 LTR in the absence of challenge is shown (●). To monitor 3′-OH processing (□), reactions were transferred to 37 °C for 30 min after the initial dissociation period.

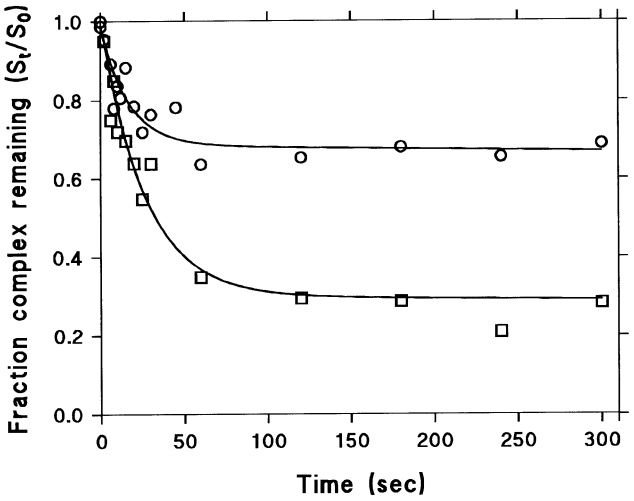


FIGURE 6: Biphasic dissociation kinetics of preassembled complexes after challenge with ssDNA. Complexes were preassembled for 10 min at 21 °C (□) or 37 °C (○) prior to the addition of 250 nM poly[d(T)$_{50}$]. DNA-binding was measured by rapid-pulse UV cross-linking. The relative occupancy of the DNA substrate is given by S_t/S_0, where S_0 refers to the percentage of DNA cross-linked at $t = 0$ and S_t to that measured at time intervals thereafter. Dissociation kinetics were fit to the biphasic first-order expression $S_t = S_1 \exp^{-k_1 t} + S_2 \exp^{-k_2 t}$, where the decay of the two forms of complex from the initial concentrations of S_1 and S_2 (at t_0) are given by rate constants k_1 and k_2, respectively.

Measurement of k_c was conducted by rapid-pulse UV cross-linking (Figure 6). Upon challenge, the nucleoprotein complex dissociated biphasically, as expected from the mixed population of complexes (Figure 6). Challenge-sensitive complexes dissociated quickly ($k_{obs} = 0.06$ s^{-1}) with a brief half-life of approximately 12 s at 37 °C. Challenge-resistant complexes dissociated at a much slower rate ($k_{obs} \sim 1.5 \times 10^{-5}$ s^{-1}, half-life $\sim$ 12.8 h). The latter measurement is consistent with the value of k_0 derived above. In agreement with the results shown in Figure 5, a greater proportion of

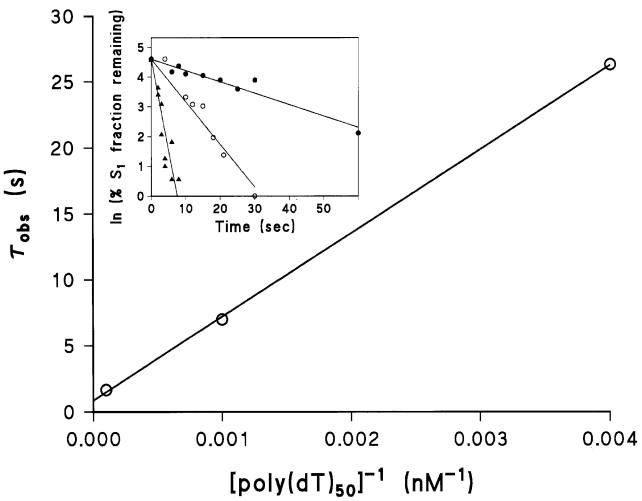


FIGURE 7: Kinetics of the attack and displacement of the challenge-sensitive nucleoprotein by ssDNA. Complexes were preassembled at 21 °C and treated as in Figure 6 to obtain the dissociation rate, k_{obs}, for the challenge-sensitive complexes for three concentrations of poly(dT)$_{50}$. (Inset) The decay of the challenge-sensitive complex is shown in semilogarithmic form as a function of the time after addition of 250 nM (●), 1 μM (○), and 10 μM (▲) poly(dT)$_{50}$.

the preformed complex was displaced at 21 °C. In this case, the corresponding half-lives for the two phases were 18 s and 1.5 h ($k_{obs} \sim 0.038 \pm 0.005$ and 1.4×10^{-4} s^{-1}), respectively.

In Figure 7, the concentration of the polynucleotide was varied and the influence of this on the observed dissociation rate (k_{obs}) of the challenge-sensitive complexes was monitored at 21 °C. When these data are plotted in double reciprocal form according to eq 5, a linear relationship is seen. Extrapolation of these data to the intercept with the vertical axis yields $1/k_c$ (0.8 ± 0.2 s), where k_c is the intrinsic rate of displacement of the LTR from the ternary complex. The observed dissociation constant, K_i, for the ternary intermediate approximated from these data is 5 μM.[2]

Delayed Appearance of the Challenge-Resistant Form after Initial Complex Formation. Two mechanisms can be postulated to explain the distribution of the complex between the two states *after* binding to the DNA ends. Either (i) the complexes may partition into two distinct and noninteracting species at the level of the elementary association between the HIV-1 integrase and the LTR substrate, or (ii) the two states may redistribute after initial complex formation, without dissociation, through a compositional or structural change in the stable nucleoprotein assembly. To distinguish between these possibilities, the kinetics of initial complex formation were determined and compared to the rate of appearance of the challenge-resistant species at two different temperatures. The results of these experiments, compared in Figure 8, demonstrate that the formation of the challenge-

[2] It is worth noting that $K_i \neq IC_{50}$ since the latter value merely refers to the concentration of inhibitor that accelerates the dissociation sufficiently to decrease the initial concentration of IN·S by half at the time of measurement, whereas at $C = K_i$ then $k_{obs} = 0.5k_c$, and multiple half-times of displacement would have been completed in the equivalent time period. The fact that $K_i \gg IC_{50}$ is consistent with the direct displacement mechanism. The IC_{50} should more closely approximate to the $K_d(k_{-1}/k_1)$ for the binding of C to IN·S, while K_i is the equivalent of a Michaelis−Menten constant in the form $(k_{-1} + k_c)/k_1$ since $k_c > k_{-1}$.

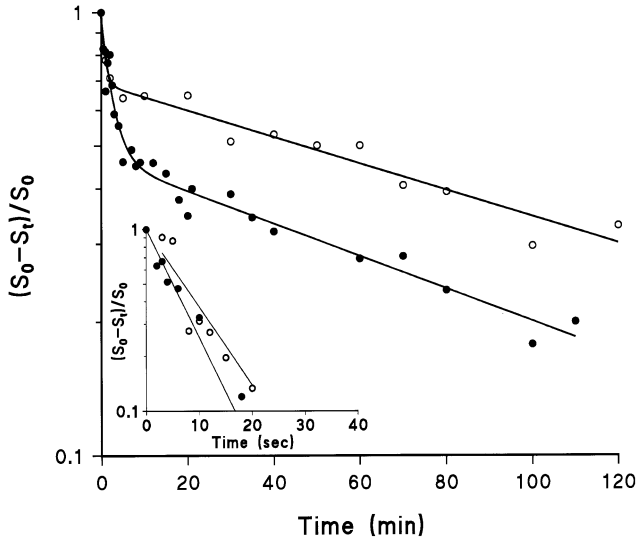


FIGURE 8: Kinetics of the appearance of challenge-resistant complexes. Complexes between the HIV-1 integrase (1 μM) and the 21-bp U5 LTR substrate (12.5 nM) were formed at 21 °C (○) or 37 °C (●) for defined time intervals before addition of 500 nM poly[d(C)$_{50}$]. Dissociation was for 10 min prior to sedimentation of the residual challenge-resistant population. The data are plotted in semilog form as a function of the disappearance of the challenge-sensitive fraction where S_0, the initial fractional saturation of the DNA substrate in the absence of challenge, was 0.95. (Inset) Initial appearance of complexes at 21 °C (○) and 37 °C (●) in the absence of challenge. Binding was monitored by rapid-pulse UV cross-linking, where S_0 refers to the maximal % DNA cross-linked at equilibrium and S_t to the % adduct formed at each appropriate time interval.

resistant species occurs more slowly than the elementary association with the DNA substrate and increases progressively over time rather than being committed to a pre-defined distribution.

Indeed, the initial formation of the nucleoprotein complex is relatively rapid. When measured by rapid-pulse UV cross-linking, the first-order rate of association at near-saturating integrase concentrations (1 μM) was 0.1 and 0.14 s^{-1} at 21 and 37 °C, respectively (and where binding is thus near complete in less than 0.5 min). Under the same reaction conditions, the rate of formation of the challenge-resistant form was considerably slower and occurred biexponentially, yielding two quite separate rates of 1.2 ± 0.8 min^{-1} and 6 ± 1 × 10^{-3} min^{-1} at 21 °C and 0.4 ± 0.1 min^{-1} and 8 ± 3 × 10^{-3} min^{-1} at 37 °C, respectively. In terms of challenge-resistant 3′-OH processing activity, a similar primary rate of 0.6 min^{-1} (half-time ∼ 1.2 min) was obtained for a similar partition (data not shown). In conclusion, the partition between challenge-sensitive and challenge-resistant complexes is not coincident with the initial binding of the LTR substrate, and thus a more complex mechanism is required to explain the rate-limiting appearance of the resistant species (see Discussion).

Both the Divalent Cation and DNA Substrate Length Influence the Partition between the Two States. DNA-binding reactions were thus far performed exclusively in 5 mM Mn^{2+} using a 21-bp U5 LTR substrate. While Mn^{2+} stimulates the tight binding of the integrase to this substrate, efficient binding and 3′-OH processing only occur in Mg^{2+} at considerably higher protein concentrations. However, increased Mg^{2+}-dependent activity can be obtained simply by using a longer DNA substrate (*30*). To account for the influence of these parameters on the formation of the challenge-resistant state, both the length and sequence of the DNA substrate were altered and complex formation monitored in both Mn^{2+} and Mg^{2+} solutions. In light of the data presented in Figure 8, the formation of the challenge-resistant complex was assessed at two distinct time intervals (in all cases, ssDNA prevented binding completely when added before integrase). The comparison of these data, summarized in Table 1, allows the following notable conclusions.

Table 1: Influence of DNA Length and Sequence on the Formation of Challenge-Resistant Complexes at 37 °C[a]

	$MnCl_2$				$MgCl_2$			
substrate	θ	ϕ_{10}	ϕ_{75}	−2 (%)	θ	ϕ_{10}	ϕ_{75}	−2 (%)
U5$_{21-bp}$	0.96	0.51	0.68	76 (79)	0.72	0.14	0.14	57 (79)
U5$_{42-bp}$	0.96	0.74	0.90	42 (43)	0.95	0.31	0.70	83 (87)
nsp$_{23-bp}$	0.92	0.42	0.58		0.72	0.26	0.20	
nsp$_{45-bp}$	0.94	0.77	0.86		0.97	0.26	0.69	
U5$_{42}$ (+ strand)	0.96	0.01	0.09		0.75	0.04	0.06	

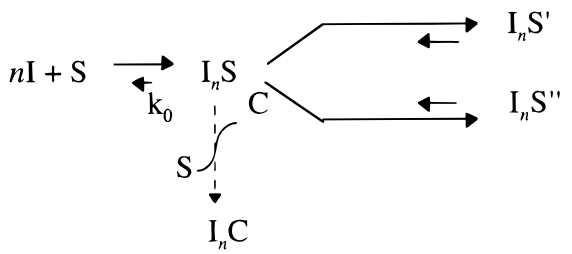
[a] Key: θ, fractional saturation determined after 10 min; ϕ_{10} and ϕ_{75}, challenge-resistant fraction at 10 and 75 min, respectively; −2, total (noncompeted) substrate cleaved to −2 product after 75 min (values in parentheses refer to value corrected for θ). Values given are mean for at least four replicates (where SD ≤ 10%).

(1) Increased challenge resistance was obtained for both cations using the longer DNA fragments. Nonspecific (nsp) substrates were incorporated just as efficiently as the viral U5 LTR in this respect. Thus, the length of the DNA substrate, rather than its sequence, appears to be the dominant factor at play. Complete resistance was not attained over the early association phase (10 min). Furthermore, at 21 °C, only 29% of the prebound complexes were formed in the resistant state (data not shown). The best combination for challenge resistance (i.e., 42-bp LTR in Mn^{2+}) led to a significant decrease in catalytic activity (42% versus 79%). Hence, in Mn^{2+}, it is difficult to conclude if this less productive complex truly reflects the properties of the pre-integration complex. (2) For all duplex substrates, challenge-resistant complexes were formed more efficiently in Mn^{2+} than in Mg^{2+} (challenge resistance was not observed with a ssDNA substrate). In Mg^{2+}, the 21-bp duplex was poorly incorporated into challenge-resistant complexes (<20%) and also proved to be a poorer substrate for 3′-OH processing. However, when corrected for the fraction of substrate bound (θ), the 3′-OH processing activity was found to be similar to that in Mn^{2+} reactions (79% in both cases). Hence, a challenge-resistant conformation does not appear to be obligatory for the efficient cleavage of the substrate DNA *in vitro*.

DISCUSSION

The prolonged stability of HIV-1 integrase complexed to viral DNA substrates has been established in several previous studies (*8*−*12*). In keeping with the stability of retroviral pre-integration complexes (*5*, *6*), once bound, integrase is likely to remain in contact with the retroviral DNA ends throughout the subsequent integration reaction. As well as providing protection from exonucleases (*11*, *31*), this attribute avoids additional problems inherent with reversible binding of the DNA termini. Dissociation would result in vast dilution in the cell and rebinding may be prevented by

Scheme 1



competition by cellular nucleic acids, particularly since selective binding has not been demonstrable for this group of retroviral proteins (*11*, *18*, *27*, *32*, *33*).

Nonetheless, in the present study nonspecific ssDNA is found to be a surprisingly effective inhibitor of HIV-1 integrase *in vitro*. We established further that ssDNA can attack the preformed HIV-1 integrase−LTR complex to displace the bound viral DNA strands directly. Since the viral DNA ends are initially stably occluded ($k_0 \sim 10^{-5}$ s^{-1} in Mn^{2+}), it is implicit that the polynucleotide exerts its effect at a second, independent site on the preassembled nucleoprotein. At very high concentrations, dissociation is accelerated ~10^5-fold, reducing the half-life of the complexes from 12.8 h to less than 1 s at 21 °C. The kinetics of the release of the otherwise tightly bound substrate fit appropriately to a noncompetitive mechanism (eqs 4−6). In this case, the observed inhibition constant, K_i, is a combined function of the reversible association of C with IN·S ($K_d = k_{-1}/k_1$) and the alternative dissociation route for S, k_c (thus $K_i = K_d + k_c/k_1$).

Evidence for a Two-State Binding Mechanism. In Mn^{2+}, a species of complex appears which is resistant to dissociation by ssDNA. During a typical *in vitro* reaction, the conversion of the stable complex to this form is incomplete and a heterogeneous mixture of challenge-sensitive and challenge-resistant complexes is obtained. Two different types of assembly are therefore implicated in the *in vitro* integration reaction.

Scheme 1 summarizes the body of evidence presented above for the two-state binding mechanism *in vitro*. The cooperative assembly of *n* integrase oligomers with each DNA molecule, S, to yield the stable bound species I_nS has been described previously (*11*). Here, the partition between the two major species is proposed to follow a sequential pathway, passing through an elementary challenge-sensitive complex I_nS, to yield the challenge-resistant state(s) I_nS' (and I_nS''). This scheme is deduced for the 21-bp LTR substrate on the following basis.

In Figure 8, we compared the kinetics of DNA-binding to that of challenge-resistant complex formation. The appearance of the challenge-resistant species followed biexponential kinetics. At present, this is best dealt with by invoking a bifurcated pathway leading to two distinct forms of challenge-resistant complexes, I_nS' and I_nS''. At 37 °C, the formation of the initial binary complex is approximately 20 and 10^3 times faster than the appearance of I_nS' and I_nS'', respectively. At 21 °C, the majority of complexes are initially formed in the stable but challenge-sensitive I_nS state (i.e., after a 5 min incubation, >90% complexes exhibit $k_0 \sim 10^{-5}$ s^{-1}, while only ~20% are ssDNA-challenge-resistant). In this case, the bulk of the challenge-resistant complexes (I_nS'') appear at a 10^4-fold slower rate than I_nS. The delayed appearance of challenge-resistant complexes cannot therefore be explained by their slow or reversible accumulation to the DNA ends. Instead, the preformed stable complex must convert from the challenge-sensitive to the challenge-resistant state without first dissociating and rebinding the viral DNA end.

The fact that challenge-resistant complexes retain the ability to integrate into exogenous DNA targets (ref 8 and 9), and our unpublished data) argues that this is not a simple aggregation phenomenon (which could nominally shield the complex from secondary DNA-binding interactions). Furthermore, the distribution between species is not dependent on the concentration of integrase (data not shown). Purified integrase may exhibit structural or conformational heterogeneity (*34*, *35*) and innate differences in their abilities to form challenge-resistant complexes cannot be ruled out. While this could explain the different rates of appearance of the two challenge-resistant forms, I_nS' and I_nS'', the differential effects of temperature, divalent cation, and DNA substrate length alter the extent of challenge resistance between <20% and >90% of the preformed stable complex. This excludes a trivial explanation for the observed partition involving two distinct preexisting forms of integrase with inflexible differences in their DNA-binding properties. Instead, the heterogeneity is more likely to be in the context of the protein−DNA complex where the rate-limiting appearance of challenge resistance is best explained at present by a direct structural rearrangement in the initial stable complex(es).

Implications to the Mechanism of Retroviral DNA Integration. Challenge resistance does not appear to play a direct role in promoting 3′-OH processing or sequence-specific interactions with the viral DNA ends (see Table 1). Nevertheless, it would seem to be a mandatory function for the pre-integration complex *in vivo*. In light of the sensitivity of the complexes to changes in the reaction conditions, the presence of other viral and/or host factors may significantly alter the kinetics of the pathway outlined above. Variations in the extent or nature of the protein−DNA interface may also be important. Furthermore, the *bona fide* 2-LTR DNA intermediate, not studied here, may assemble via a different pathway. Insufficiencies in the reconstruction of the genuine pre-integration intermediate *in vitro* may be reflected in our present results. In this respect, the ssDNA interaction may constitute a useful tool to monitor and guide the future reconstitution of such complexes.

The secondary nonspecific binding site and its normal function on the integrase nucleoprotein remain to be established. A simple but plausible hypothesis is that it equates to the host target DNA-binding site. Experimental evidence for separate sites for viral and target DNA has been proposed previously for the HIV-1 (*8*, *36*) and Moloney murine leukemia virus (MoMLV) (*29*) integrases. More recently, peptide mapping of HIV-1 integrase photo-cross-linked to an integration intermediate showed that different domains of the protein do indeed contribute to viral and target DNA interactions (*37*). Target-binding may provoke conformational changes in the stable nucleoprotein. If so, the destabilization could ensue from the anomalous response of an immature or incorrectly assembled stable complex to this event.

Finally, it is interesting to consider the potential influence of ss nucleic acids on the preassembly state of the integrase within the virion. How the integrase finds and assembles specifically and irreversibly with the retroviral dsDNA termini (and not to other potential nucleic acid targets) *in vivo* remains unknown. In line with the effect of ssDNA *in vitro*, it is conceivable that retroviral ssRNA could play an initial role in preventing the formation of inappropriate stable complexes, prior to the synthesis of the requisite dsDNA genome. The inhibitory effect is dependent on both the lattice length of the nucleic acid and a relatively high K_i *in vitro*. During reverse transcription, RNAse H actively removes the transcribed RNA strand during DNA polymerization, thereby potentiating any possible inhibitory effect.

In conclusion, our results suggest that the ssDNA-induced dissociation mechanism may have important implications at the level of the initial assembly of the higher-order nucleoprotein complex. This finding may assist in further unravelling the complex molecular events that govern retroviral DNA integration but equally may warrant further investigation with respect to novel antiviral strategies.

ACKNOWLEDGMENT

We thank Jane MacDougall for helpful comments and critical reading of the manuscript.

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BI971893J