HIV-1 reverse transcriptase and integrase enzymes physically interact and inhibit each other

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Abstract Ordered molecular interactions and structural changes must take place within the human immunodeficiency virus type 1 (HIV-1) preintegration complex at various stages for successful viral replication. We demonstrate both physical and biochemical interactions between HIV-1 reverse transcriptase and integrase enzymes. This interaction may have implications on the in vivo functions of the two enzymes within the HIV-1 replication complex. It may be one of the various molecular interactions, which facilitate efficient HIV-1 replication within the target cells.

Key words: Reverse transcription; Integration; Preintegration complex

1. Introduction

The successful replication of human immunodeficiency virus type 1 (HIV-1) requires viral genome reverse transcription to generate proviral DNA and integration into the target cell genome. These events are carried out by the viral encoded enzymes, reverse transcriptase (RT) and integrase (IN), respectively. The RT is a heterodimer of p66 and p51 subunits, which can copy both RNA and DNA templates thus producing double stranded proviral DNA [1]. The IN is a 32 kDa polypeptide that directs a defined set of DNA cleavage and joining events to insert the proviral DNA into the host genome [2]. It would be important to understand how these two viral enzymes which act sequentially during viral replication, interact to coordinate their functions within the multifunctional HIV-1 replication complexes. Both enzymes are part of the viral preintegration complex (PIC), which also contains the viral nucleic acids in association with nucleocapsid (NCp7), matrix (MA), p6 and viral protein R (vpr) proteins [3,4]. Some cellular proteins such as the HMG I (Y) and barrier to autointegration factor (BAF) are also associated with the HIV-1 PIC [5,6]. Efficient viral replication requires multiple intra- and intermolecular interactions and molecular rearrangements within this complex at various stages of replication. The exact nature of these molecular interactions in the PIC, however, is only poorly understood. For example it has been shown that reverse transcription by RT can be influenced by other viral proteins such as MA, NCp7, IN, Vif, and Tat [7–13]. Understanding how the individual proteins might interact and influence each other within the PIC might help unravel the molecular interactions underlying HIV-1 replication. In this paper we analyze HIV-1 RT and IN molecular interactions independent of other PIC components. We show that the RT and IN physically interact by coimmunoprecipitations, GST pulldown and dot blot analysis. Both enzymes can influence each other as observed in vitro with specific DNA substrates for each enzyme. Our findings suggest that RT and IN might act together within the PIC, and that this interaction might facilitate coordinated viral replication.

2. Materials and methods

The HIV-1 IN gene was PCR amplified from the pLR2P-vpr-IN [10] and subcloned into pET28a (Novagen) creating histidine tagged HIV-1 IN expression vector (pTT25). IN protein expression and purification was as described [17]. The expression and purification of histidine tagged HIV-1 RT p51 and p66 subunits and heterodimer reconstitution, were as previously described [15,16]. GST and GST tagged RT subunits were obtained as previously described [14]. Immunoprecipitations were as outlined in the legend to Fig. 1. IN was detected using rabbit raised anti-IN polyclonal antibodies HXB2[276–288] [18] and RT was detected using chicken raised polyclonal antibodies [19]. HIV-1 RT was immunoprecipitated using the anti-RT monoclonal antibody mAb21 [20]. GST pulldown assays were as outlined in the legend to Fig. 1. Dot blot analysis was performed as described [9]. The DNA substrate for IN was prepared by annealing the 21-mer HIV-1 U5 long terminal repeat (LTR) oligonucleotide (5′-ATGTGGAAAATCTCTAGCAGT-3′) to its complement and the IN assays were as described [17].

3. Results

Three approaches were used to study the physical protein–protein interactions between the HIV-1 RT and IN enzymes. In the first approach we found efficient coimmunoprecipitation of the IN with both subunits of the RT enzyme (Fig. 1A and B). These complexes were specifically immunoprecipitated in the presence of either RT p66 or p51 subunits and IN (Fig. 1A and B). As a control IN enzyme alone could not be non-specifically immunoprecipitated by the antibodies directed against the RT subunits (Fig. 1B). To exclude nucleic acid mediated interactions, these protein interaction studies were performed in the presence of a DNA intercalating agent, ethidium bromide (50 μg/ml). Next we confirmed direct protein–protein interactions between RT and IN using purified

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Abbreviations: HIV-1, human immunodeficiency virus type 1; IN, integrase; LTR, long terminal repeat; PIC, preintegration complex; RT, reverse transcriptase

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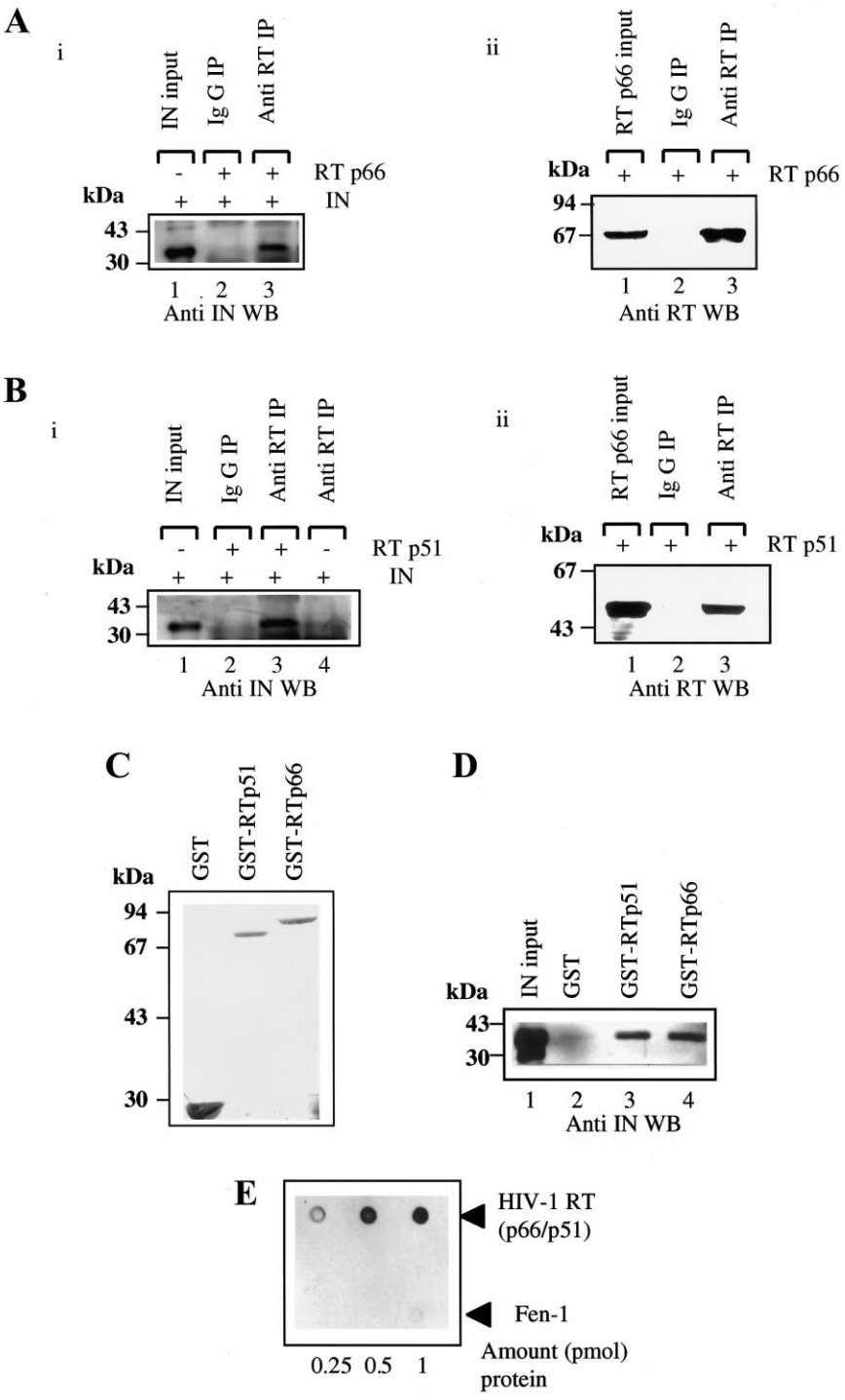

proteins in GST pulldown. As shown in Fig. 1D, specific complex formation between IN and the GST tagged HIV-1 RT subunits was detected by Western blot analysis after pulldown. Finally physical interactions between IN and the native HIV-1 RT heterodimers (p66/p51) were also analyzed by dot blot far Western. Increasing amounts of purified RT heterodimers or human flap endonuclease-1 (Fen-1)(D86A) [21], were immobilized on nitrocellulose membrane before probing with IN enzyme in solution. Specific RT–IN complexes were detected then by Western blot (Fig. 1E). No complexes could be detected between IN enzyme and the Fen-1 proteins used as a control (Fig. 1E) and additionally there was no cross-reaction between the RT and the anti-IN antibodies used for detection (data not shown). In conclusion these findings suggested direct protein–protein interactions between the HIV-1 RT heterodimer and IN enzyme indicating that the two proteins may directly interact within the HIV-1 PIC in vivo.

This prompted us to next investigate the influence of IN on the biochemical activities of HIV-1 RT. We titrated increasing amounts of the IN enzyme into HIV-1 RT DNA polymerase activity assays. There were no significant influences of IN on the RT enzyme activity in an RNA dependent DNA polymerase (RDDP) assay (data not shown). On the other hand increasing amounts of IN protein (Fig. 2A) significantly inhib-

Fig. 1. HIV-1 RT and HIV-1 IN interact. Analysis of HIV-1 RT and IN interaction by coimmunoprecipitation. Immunoprecipitation was performed by using the mouse monoclonal antibody mAb21 directed against HIV-1 RT. 50 μg of bacterial extract expressing individual RT subunits, i.e. p66 or p51 were preincubated with 1 μg of purified HIV-1 IN protein for 2 h prior to immunoprecipitation with anti-RT antibody in the presence of 50 μg/ml ethidium bromide. Protein complexes were bound on protein-G-Sepharose beads, pelleted and subsequently eluted into sodium dodecyl sulfate (SDS) loading buffer. The eluted protein samples were split into half, separated on 10% SDS–polyacrylamide gel electrophoresis (PAGE). A Western blot analysis to reveal bound proteins was performed by using the polyclonal antibodies against HIV-1 IN, HXB2[276–288] on one half of the samples and on the other half chicken raised polyclonal antibodies against HIV-1 RT used to detect immunoprecipitation of the RT subunits. A: Coimmunoprecipitation of the RT p66 and IN. (i) Detection of IN coimmunoprecipitation; lane 1, input; lane 2, mouse IgG control antibody; lane 3, anti-RT mAb21 antibody. (ii) Detection of HIV-1 RT p66 immunoprecipitation; lane 1, input; lane 2, mouse IgG; lane 3, anti-RT mAb21 antibody. B: Coimmunoprecipitation of RT p51 and IN. (i) Detection of IN coimmunoprecipitation; lane 1, input; lane 2, mouse IgG; lane 3, anti-RT mAb21 antibody; lane 4, immunoprecipitation with anti-RT mAb21 in the presence of IN alone. (ii) Detection of HIV-1 RT p51 immunoprecipitation; lane 1, input; lane 2, mouse IgG; lane 3, anti-RT mAb21 antibody. Analysis of HIV-1 RT and IN interaction by GST pulldown. C: GST fusion protein expression; lane 1, GST; lane 2, GST–HIV-1 RT p51; lane 3, GST–HIV-1 RT p66. D: RT and IN interaction in GST pulldown. Equimolar amounts of purified GST, GST–HIV-1 RT p51 and GST–HIV-1 RT p66 were bound to GS beads and incubated with 1 μg purified IN protein in the presence of 50 μg/ml ethidium bromide. Proteins were resolved on 10% SDS–PAGE and bound proteins were detected by Western blot: lane 1, input; lane 2, GST; lane 3, GST–HIV-1 RT p51; lane 4, GST–HIV-1 RT p66. Analysis of HIV-1 RT and IN interaction by dot blot. E: Increasing amounts of purified RT heterodimer (p66/p51) or hFen-1 control were immobilized on two nitrocellulose membranes. One membrane was probed with 1.5 pmol/ml IN solution while buffer only was used on the second membrane. Both membranes were analyzed by using anti-IN polyclonal antibodies by Western blot.
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ited the DNA dependent DNA polymerase activity of RT. The effect could be confirmed during processive DNA synthesis by the RT enzyme on singly primed M13 DNA template (Fig. 2B). BSA or human Fen-1(D86A), a nuclease mutant which can still bind DNA, were used as controls. They both had no significant effect on RT activity (Fig. 2A and B). As another control, the specificity of IN effect on RT enzyme was analyzed by titrating IN against equimolar amounts of bacteriophage T4 DNA polymerase (Fig. 2C). At low IN protein concentration (1:5 molar ratio) no significant inhibition of T4 DNA polymerase was detected while a slight inhibition was observed at high concentrations (1:10). Further

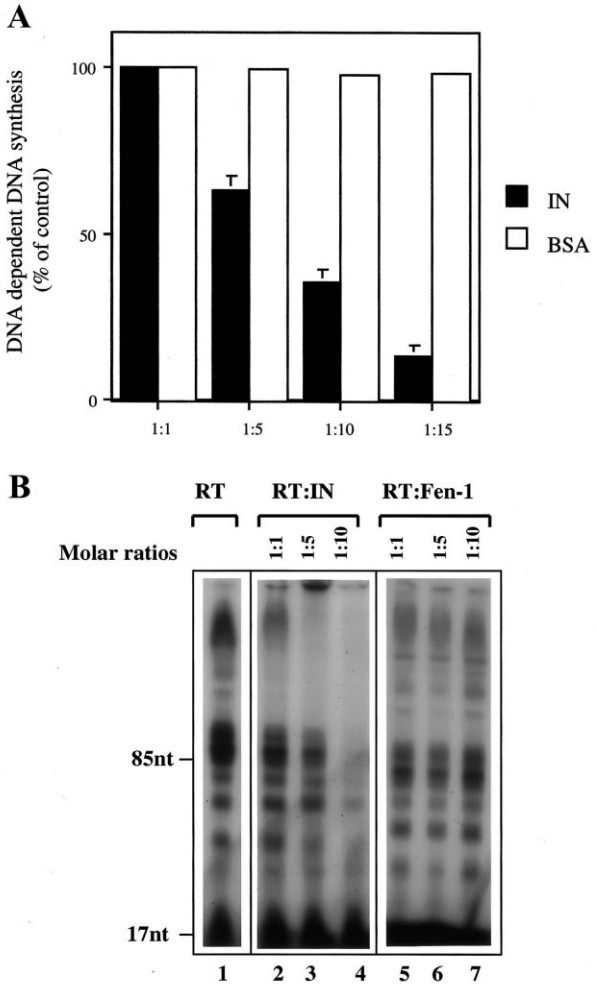

Fig. 2 (Caption of legend on next page).

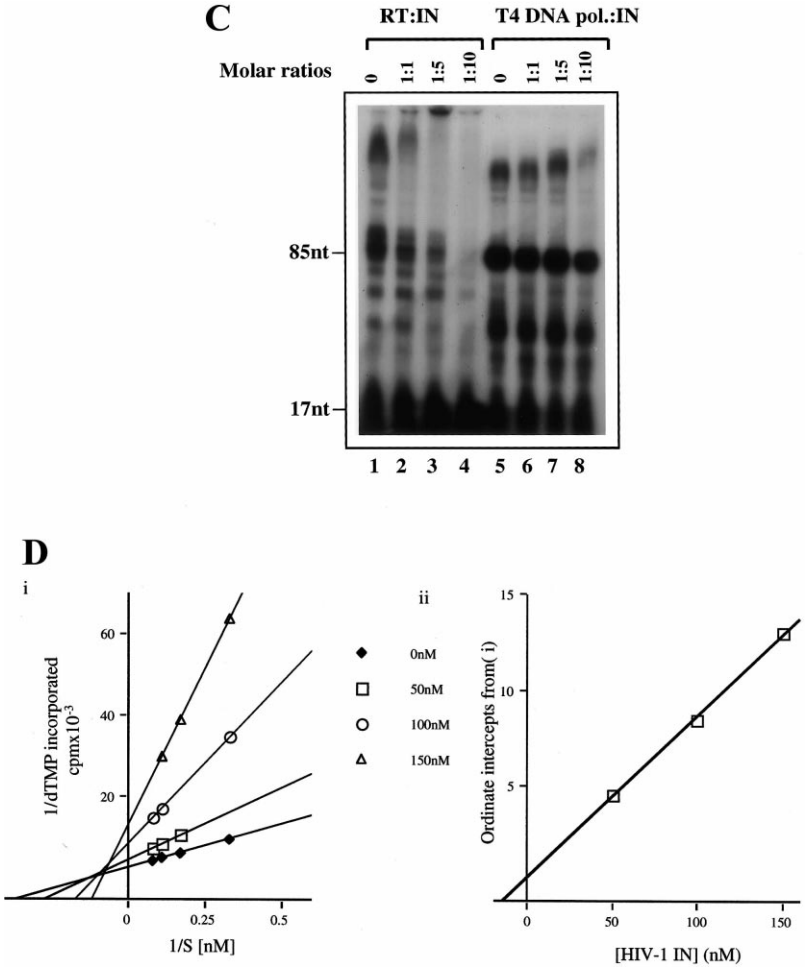

Fig. 2. IN specifically inhibits HIV-1 RT in a DNA dependent DNA polymerase assay. A: Inhibition of RT DNA polymerase activity. 0.25 pmol of HIV-1 RT were preincubated with increasing amounts of IN for 30 min at 4°C in RT buffer. 1 μg of poly-dA/oligo-dT template (18 pmol 3′-OH ends) and 40 μM [H^3]dNTPs were then added and the mixture incubated at 37°C for 30 min. The reaction was stopped by adding an excess of 10% TCA and DNA synthesis quantified by scintillation counter. As control proteins, BSA or hFen-1(D86A) (not shown) were used. Molar ratios between the IN monomers or BSA and the RT heterodimers were then plotted against average DNA dependent DNA synthesis from three independent experiments expressed as a percentage of the RT enzyme alone control. B: Product analysis on singly primed M13 DNA template under processive conditions. 0.25 pmol of RT enzyme were preincubated with IN for 30 min at 4°C. 1 μg singly primed M13 DNA template was then added and the mixture incubated for 5 min at room temperature. An excess of poly-dA/oligo-dT (3 μg) cold trap and 40 μM dNTPs were added and the mixture transferred to 37°C for 3 min. Products were separated on a 10% urea–polyacrylamide gel and exposed to an X-ray film: lane 1, RT alone; lanes 2–4, RT enzyme preincubated with IN; lanes 5–8, RT preincubated with hFen-1(D86A). C: As a control product analysis using 0.25 pmol of T4 DNA polymerase was also done after preincubation with IN: lane 1, RT alone; lanes 2–4, RT preincubated with IN; lane 5, T4 DNA polymerase alone; lanes 6–8, T4 DNA polymerase preincubated with IN. D: Kinetic analysis. RT activity was measured using 20 nM RT after preincubation with indicated IN protein concentrations and the results plotted in the Lineweaver–Burk plot (i). The K_i value was determined from a replot of the ordinate intercepts from (i) against the inhibitor (IN) concentrations (ii). Al experiments were done in triplicate.

kinetic analysis revealed a mixed type of inhibition mechanism with respect to both the DNA template (3′-OH ends) and the dNTP substrates with inhibition constants (K_i) of 66 nM and 3 nM, respectively (Fig. 2D). This suggested that the IN enzyme has a stronger affinity for the RT already complexed to the DNA template than to the RT enzyme alone. Next we tested the biochemical influence of the RT on IN activity. The HIV-1 U5 LTR oligonucleotide based assay was first used to determine the activity of purified IN protein (Fig. 3A) and to define the linear conditions of the reaction. There was significant inhibition of both 3′-end processing and strand transfer activities of IN with increasing amounts of the RT enzyme (Fig. 3B). The RT enzyme could inhibit more than 75% of IN enzyme activity even when the IN monomers were in 2-fold molar excess over the RT heterodimers (Fig. 3). We also investigated whether prior incubation of IN with its DNA substrate could overcome this HIV-1 RT mediated inhibition of IN activity. As shown in Fig. 4, an analysis of the 3′-end processing product shows that the IN enzyme bound to the DNA substrate is still accessible to RT inhibition. This suggests that DNA bound IN enzyme molecules can still interact and get inhibited by the RT under these conditions.

4. Discussion

Our current understanding of the detailed molecular structure, composition and interactions in the HIV-1 PIC is still limited. This viral derived structure provides an optimal environment for the activity of both RT and IN enzymes through various molecular interactions. The RT and IN enzymes act

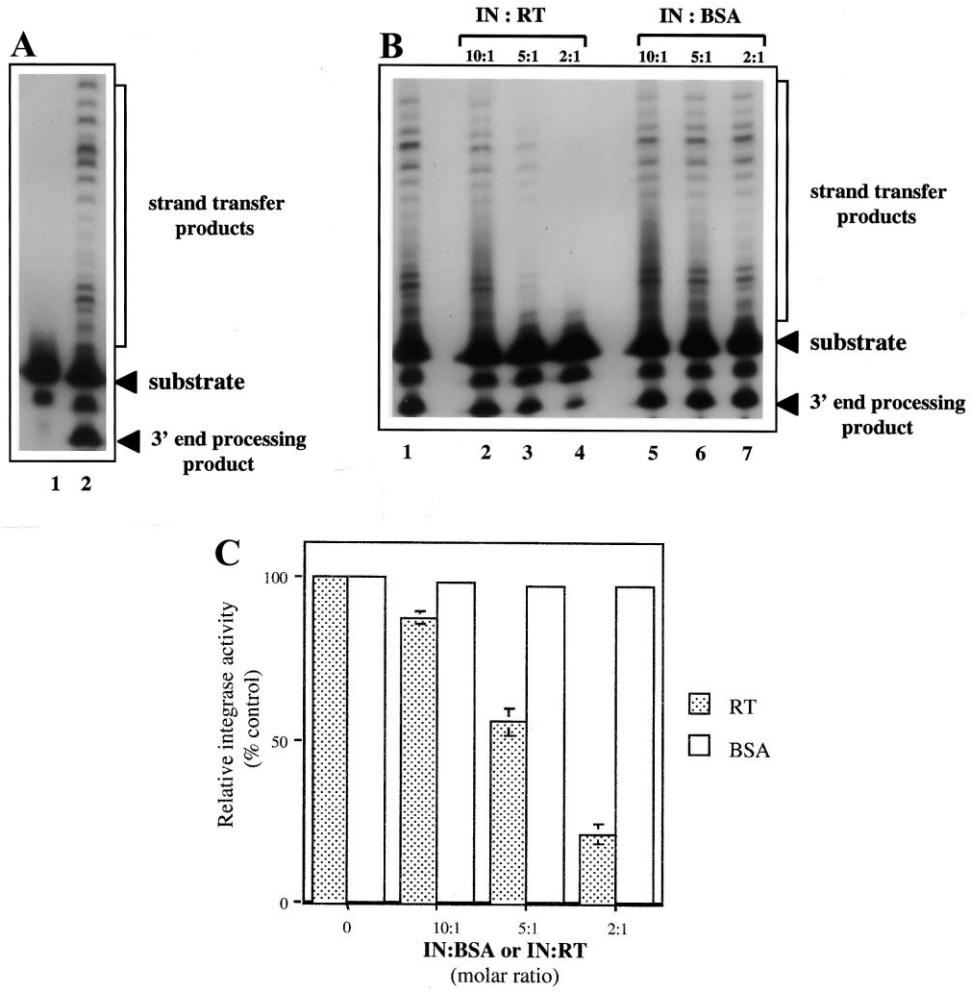

Fig. 3. HIV-1 RT specifically inhibits the IN enzyme. A: Activity of purified HIV-1 IN enzyme. 0.4 pmol of IN and 0.25 pmol of the DNA substrate were incubated for 20 min at 37°C. The reaction products were analyzed after separation on 15% urea–polyacrylamide gel: lane 1, DNA substrate alone; lane 2, IN added to DNA substrate. B: HIV-1 RT inhibits the IN activity. Increasing amounts of RT enzyme, at indicated IN:RT molar ratios, were preincubated with 0.4 pmol of IN and 0.25 pmol of the IN DNA substrate for 30 min on ice. The mixture was transferred to 37°C for 20 min and the products were separated on urea–polyacrylamide gel: lane 1, IN alone; lanes 2–4, IN preincubated with RT and DNA; lanes 5–7, IN preincubated with BSA and DNA. C: Phosphoimager quantification of 3′-end processing products. Results from three independent experiments (less than 30% variation) were quantified and their average expressed as a percentage of the activity of IN enzyme alone.

sequentially in viral replication and share a common substrate, the proviral DNA substrate being synthesized. How the two enzymes may interact and influence each other is unknown. Possible molecular interactions between RT and IN have been inferred and a possible role in the reverse transcription initiation was suggested [10]. In this paper we analyzed the putative RT/IN molecular interactions independent of the other PIC components. As summarized in Fig. 1, the IN enzyme directly interacts with both RT subunits and the RT heterodimer, suggesting that the two proteins may form complexes within the PICs also in vivo. There are a number of possible biological roles such an interaction may fulfill during viral replication. One could be the maintenance of structural stability between the two proteins and the different nucleic acid substrates (RNA and DNA) encountered during replication in the PIC. Interaction with the RT might be one of the mechanisms through which the IN enzyme is efficiently delivered to the double stranded blunt ends of proviral DNA after completion of replication by RT. A number of studies have suggested that IN specific protein–protein interactions may be essential in the early stages of viral replication including reverse transcription [10,22–25]. The exact molecular basis of how IN may contribute to efficient viral DNA synthesis is not clear. Our in vitro analysis of RT/IN protein complexes in various RT assays shows that while no influence could be detected at low IN concentration (1:1 molar ratios or less), we observed inhibition of RT DNA dependent polymerase activity with increasing IN concentration (Fig. 2). One possible explanation for this could be a competition between RT and IN enzymes for the available free 3′-OH ends or aggregation of the DNA template molecules by IN which may hinder RT access to the primer 3′-OH ends. However, this seems unlikely, since in our assay the 3′-OH ends concentration was greater than 10-fold molar excess over both the IN and RT molecules. Another possibility could be that structural constraints might be induced in the RT heterodimer upon RT/IN interaction. This may alter the optimal catalytic conformation of the RT or interactions with both the template/primer and

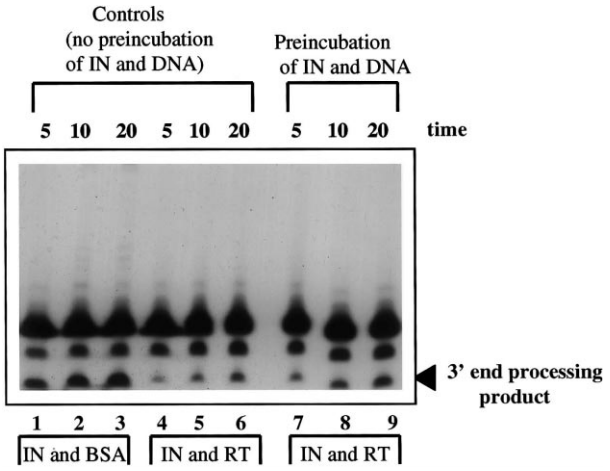

Fig. 4. Preincubation of IN with DNA has no influence on the RT inhibition of IN activity. 0.4 pmol IN enzyme was preincubated with 0.25 pmol of the DNA substrate for 30 min on ice (lanes 7–9) before adding 0.25 pmol of the HIV-1 RT heterodimer and further incubation of the mixture for 30 min on ice. As controls IN was preincubated with BSA (lanes 1–3) or RT (lanes 4–6) without prior incubation of IN and the DNA substrate alone. The reaction mixtures were transferred to 37°C and the reaction stopped after 5, 10 and 20 min, respectively.

dNTP substrates. The kinetic analysis also suggested a complex mechanism of inhibition rather than simple competition for the DNA substrate, indicating that IN bound RT more efficiently when the latter enzyme is already complexed with the DNA substrate. While in early reverse transcription IN inhibition of RT would seem not to make sense, such an influence might be important in the late stages of reverse transcription to terminate RT function, thereby preventing generation of potentially deleterious viral DNA products. Indeed our data fit along this argumentation, since the RNA dependent DNA polymerase activity was not affected by IN. The influence of the RT/IN interactions was finally investigated in IN assays (Fig. 3). The RT enzyme could influence IN activity in vitro as shown by a strong inhibition of IN activity with increasing amounts of RT. While this observation does not immediately suggest a possible biological role, it is likely that the RT molecules that are indeed part of the PIC might influence IN function or subsequent steps. Inhibition of IN by the RT might be one way of preventing premature integration or autointegration of proviral DNA by the RT bound IN molecules. In conclusion, our results suggest interactions and functional influences between RT and IN enzymes in vitro, which may have implications on molecular interactions for the two enzymes within the PIC.

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