Inhibition of the integrases of human immunodeficiency viruses type 1 and type 2 by reverse transcriptases

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We present evidence that the integrases (INs) of HIV types 1 and 2 are inhibited *in vitro* by the reverse transcriptases (RTs) of HIV-1, HIV-2 and murine leukaemia virus. Both 3′-end processing and 3′-end joining (strand transfer) activities of IN were affected by the RTs. Full inhibitions were accomplished with most RT and IN combinations tested at around equimolar RT/IN ratios. The disintegration activity of IN was also inhibited by RTs. Neither DNA synthesis nor the ribonuclease H (RNase H) domain of RT were involved in IN inhibition, since specific DNA polymerase inhibitors did not affect the level of IN inhibition, and the p51 isoform of HIV-1 RT (which lacks the RNase H domain) is as effective in inhibiting IN as the heterodimeric p66/p51 isoform. On the other hand, the catalytic activities of HIV RTs were not affected by the INs, showing that RTs can inhibit IN activities, whereas INs do not inhibit RTs. We postulate that sequences and/or three-dimensional protein structures common to RTs interact with INs and inhibit their activities. We show evidence for this hypothesis and discuss the possible sites of IN involved in this interaction.

Key words: HIV, integration, retrovirus, reverse transcription.

INTRODUCTION

All retroviruses encode two enzymes which play a key role in the early steps of the replication process of these viruses. The first enzyme in the order of catalysis is the reverse transcriptase (RT), which converts the single-stranded viral RNA into double-stranded DNA. The process of reverse transcription, which is catalysed wholly by RT, is mediated in the cytoplasm of the infected cell by the two catalytic activities of RT, the DNA polymerase function (which is capable of copying both RNA and DNA) and the ribonuclease H (RNase H) activity [1]. The second enzyme is the retroviral integrase (IN), which processes the DNA product synthesized by RT. This enzyme identifies the ends of the linear viral DNA, trims them and then accompanies this DNA to the cellular chromosome site to catalyse integration into the cellular DNA [1,2]. There have been several observations implying that there is a potential linkage between RT and IN. First, the end product of the RT-catalysed reaction is the substrate for IN. Secondly, both proteins are proteolytic products of the same polyprotein precursor, encoded by one retroviral gene, *pol*. There are cases, as in avian sarcoma leukosis virus, where IN exists as part of the large β subunit of RT in addition to it being present as a free IN protein (as a phosphoprotein of 32 kDa) [3,4]. Moreover, pre-integration complexes (PICs), the large nucleoprotein particles that are transported from the cytoplasm to the nuclei of infected cells and which are capable of carrying out integration *in vitro*, contain the viral DNA, IN, RT and other proteins [5–8]. Lastly, recombinant HIV type-1 IN and RT as well as murine leukaemia virus (MLV) RT and IN were shown to exhibit physical interactions *in vitro* [9–11].

After reverse transcription there is a substantial delay in the process of integration, depending on the time that is needed for the PICs to be transported into the nuclei of the infected cells [1]. All the potential components for integration are likely to be present in the PICs, and the viral DNA can potentially serve as both donor DNA and the undesirable acceptor or target DNA for integration. Therefore, it is very important that the viral DNA does not integrate into itself before it reaches the nuclear DNA, the only target for productive retroviral integration. Auto-integration is a suicidal process that results in destruction of the viral genome. Consequently, it is important to understand the mechanisms that regulate the integration of the retroviral genome and, thus, learn how to inhibit the integration process. Indeed, several cellular proteins have already been shown to be involved in integration [12–15], one of which might serve as a barrier to auto-integration in MLV-infected cells [16].

In the present study we explored the possibility that the viral RT itself can also serve as a regulator of integration. We present data showing that the INs of the lentivirus subfamily of retroviruses, HIV-1 and HIV-2, are inhibited by the RTs of both viruses as well as by the RT of the type-C retrovirus MLV. These studies *in vitro* with recombinant proteins highlight the possibility that RTs are involved in blocking the integration process before it is required for a productive life cycle of retroviruses. Moreover, it may lead to insights into how to inhibit retroviral replication by either inducing auto-integration of the viral DNA or inhibiting IN activity directly.

EXPERIMENTAL

Recombinant proteins: Bacterial expression and purification of the HIV INs and RTs

The HIV-1 IN coding region from the BH-10 strain of HIV-1 was cloned into plasmid pIN10, introduced into *Escherichia coli* BL21(DE3)pLysS cells, as described in [17]. DNA encoding HIV-2 IN from the Rod strain of HIV-2 was cloned into plasmid pT5m-6His, and introduced into the same *E. coli* cells. After induction by isopropyl β-D-thiogalactoside, both HIV-1 IN and HIV-2 IN, carrying N-terminal $6 \times$ His tags, were purified from the bacterial lysates on an Ni^{2+}-nitrilotriacetic acid column (Qiagen), followed by Sephacryl S-200 and heparin–Sepharose columns (both from Pharmacia) as described in [17].

Abbreviations used: RT, reverse transcriptase; IN, integrase; RNase H, ribonuclease H; MLV, murine leukaemia virus; PIC, pre-integration complex; NNRTI, non-nucleoside RT inhibitor; TBZ, thiazolobenzimidazole; LTR, long terminal repeat.

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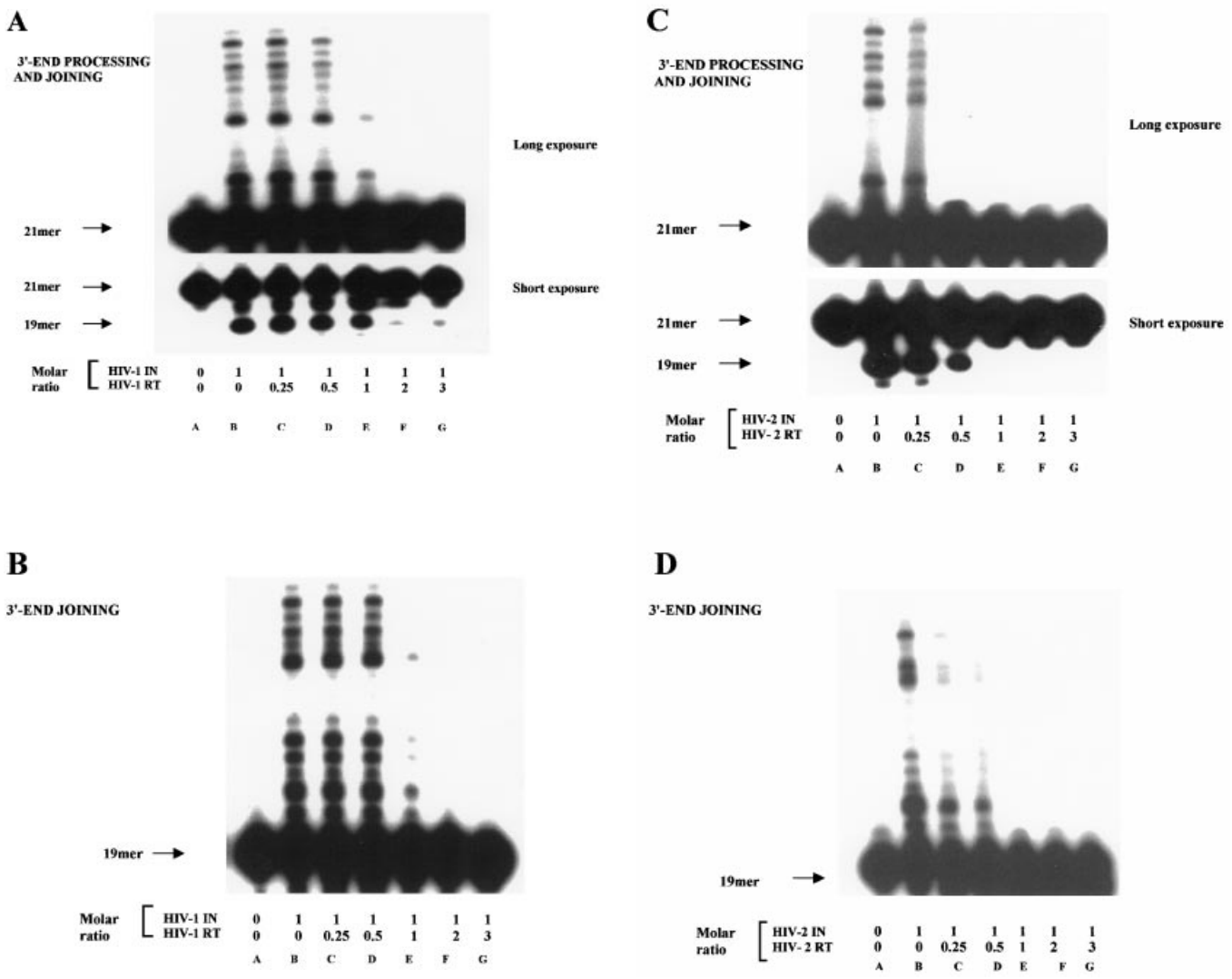

Figure 1 Effects of HIV-1 RT on the activities of HIV-1 IN and of HIV-2 RT on HIV-2 IN

Increasing amounts of the purified RT were preincubated with constant amounts of HIV INs for 5 min on ice. After adding the labelled DNA substrate, incubation proceeded at 37 °C for 30 min. The molar ratios indicated at the bottom of each panel were calculated assuming heterodimers p66/p51 of HIV-1 RT or p68/p54 of HIV-2 RT and homodimers p32/p32 of the INs. (**A** and **B**) HIV-1 RT and HIV-1 IN. (**C** and **D**) HIV-2 RT and HIV-2 IN. (**A** and **C**) Analysis of the 3′-end-processing and strand-transfer activities of IN (using oligonucleotides A and B) by urea/PAGE (as described in the Experimental section). The short exposures of the gels (1–5 h) show the 3′-end-processing products, and the strand-transfer (joining) products are shown by the long exposures (18–36 h). (**B** and **D**) Analysis of strand-transfer activity with oligonucleotides C and B (see the Experimental section).

The vectors expressing the HIV RTs were all based on our pUC12N-expressing vector, described previously [18]. This vector was modified as follows: for the p66/p51 heterodimer of HIV-1 RT we used a vector p6HRT-PROT leading to the synthesis of a heterodimer with a 6 × His tag attached to the C-terminal end of p66 [19]. The vector expressing the p68/p54 heterodimer of HIV-2 RT was constructed based on the same vector with the following modifications: (i) the HIV-1 RT coding region was replaced by DNA encoding HIV-2 RT [20] and (ii) the HIV-1 protease-encoding gene was replaced by the gene encoding HIV-2 protease. The HIV-1 p51 subunit of RT was expressed by the pUC12N p51(His) vector according to Boyer et al. [21]. All RTs were purified on Ni^{2+}-nitrilotriacetic acid agarose columns followed by CM-Sepharose as described in [22]. All RTs and INs used were highly purified, as judged from their patterns after analysis by SDS/PAGE (results not shown). Wild-type MLV RT was purchased from Amersham.

Assays for the enzymic activities of HIV INs

Oligonucleotides and 5′-end labelling

The following gel-purified oligonucleotides were used in the enzymic assays with HIV INs: A, 5′-GTGTGGAAAATCT-CTAG**CA**GT-3′; B, 5′-AC**TG**CTAGAGATTTTCCACAC-3′; C, 5′-GTGTGGAAAATCTCTAG**CA**-3′; D, 5′-**TG**CTAGTT-CTAG**CA**GGCCCTTGGGCCGGCGCTTGCGCC-3′ (bold letters indicate the conserved CA/TG dinucleotide pair). Oligonucleotides A, B and C correspond to the unique sequence (the U5) found HIV-1 long terminal repeats (LTRs) [23]. Oligo-

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nucleotide D, termed 'dumbbell', folds to form a structure mimicking the integration intermediate. The folded dumbbell substrate, which was used for assaying disintegration, has 5 bp of a viral sequence and 10 bp of a non-viral arbitrary sequence, as described earlier [24]. In order to test the 3′-end processing, and the consequent DNA-joining activities of HIV INs, we used oligonucleotide A annealed to its complementary strand, oligonucleotide B. Oligonucleotide A (50 pmol) was 5′-end-labelled using 1 unit of T4 polynucleotide kinase (Boehringer Mannheim) and 50 μCi of [γ-^{32}P]ATP (NEN), in a final volume of 50 μl of the appropriate buffer (supplied with the T4 polynucleotide kinase) for 30 min at 37 °C. Then, the samples were heat-inactivated and the labelled DNA was annealed to an equimolar amount of oligonucleotide B in 55 mM Tris/HCl (pH 7.5) and 0.27 M NaCl. To assay only the 3′-end-joining activity, we used 5′-end-labelled oligonucleotide C (which is similar to oligonucleotide A, after removal of the GT dinucleotide pair from its 3′ end) annealed to oligonucleotide B, thus creating a dinucleotide overhang at the 5′ end of oligonucleotide B. In both enzymic assays described above, the labelled 5′-end substrate was used as both the target and donor DNA [24]. For the disintegration assay 50 pmol of oligonucleotide D was 5′-end labelled, as described above. The labelled dumbbell substrate was formed by pre-annealing.

3′-End processing and joining, and disintegration assays

Reaction mixtures of 10 μl contained 0.66 pmol of the DNA substrate, reaction buffer [1 mM Hepes (pH 7.5)/54 mM NaCl/2.5 mM $MnCl_2$/50 μM EDTA/2 mM dithiothreitol/0.1 mg/ml (1 μg, 15 pmol) BSA/0.1 mM spermidine/25 mM Mops (pH 7.2)/5 % glycerol/1 % DMSO] and 250 ng of HIV-1 IN or HIV-2 IN (equivalent to 4 pmol of IN, assuming that the 32 kDa subunits of IN form dimers). HIV INs were preincubated on ice for 5 min in the presence or absence of RTs. Reactions were started on addition of the labelled DNA substrate to the reaction buffer, incubations were carried out for 30 min at 37 °C and reactions were stopped by adding 10 μl of formamide loading buffer (90 % formamide/10 mM EDTA/10 mg of Bromophenol Blue/10 mg of Xylene Cyanol). Reaction products were heat-denatured, cooled and loaded on to 6 M urea/14 % polyacrylamide denaturing gels, followed by electrophoresis (urea/PAGE). Gels were dried and subjected to autoradiography at −80 °C.

RESULTS

INs were shown to possess three distinct and related enzymic activities. The first is a 3′-end processing of the donor viral DNA. The IN recognizes specific features at the ends of the viral DNA and forms stable complexes with this DNA. The single most important sequences at the ends of the DNA are CA/TG dinucleotide pairs. The 3′-terminal pGpT dinucleotides (where p indicates phosphorylation) are removed by IN, leaving both ends of the linear viral DNA with 5′-AC overhangs [1,2]. The DNA substrate used here was a synthetic 21-mer oligonucleotide that mimics the U5 end of the HIV-1 LTR. The 5′ end of the strand, corresponding to the viral DNA 3′ end, was radiolabelled with ^{32}P. The removal of the 3′-end dinucleotide is followed by the DNA-joining process or the strand-transfer step. Another related activity of the IN *in vitro* is the reverse of the DNA-joining activity, called disintegration, which is responsible for releasing the integrated viral DNA and restoring the continuity of the target DNA strand [25]. Disintegration activity was assayed as described in [24]. Previous studies (confirmed by the results presented here) show that both HIV-1 and HIV-2 INs utilize these DNA substrates similarly [26], thereby allowing use of the same substrate species for all comparative experiments performed.

Table 1 Quantitative analyses of the inhibition of HIV INs by RTs

The gels presented in Figures 1, 2, 3 and 6 were dried and subjected to autoradiography at −70 °C. The films were scanned and the levels of IN activity calculated using the densitometric software TINA (version 2.07d; Raytest Isotopenmessgeraete GmbH). The 3′-end-processing activity was determined as a percentage of the total 5′-end labelled DNA oligonucleotides converted to 19-mer DNA. Strand-transfer (or 3′-end-joining) activity was calculated as a percentage of the total amount of labelled DNA found in DNA bands of 22 nt or more in length. All residual activities presented are relative to the activity of IN with no RT present and are expressed as percentage of the initial IN activities. ND, not determined; this refers to activities too low to be calculated reliably. The activities were determined in a reaction mixture with RT/IN molar ratios as indicated.

			IN activity (%)				
			3′-End processing			Strand transfer	
RT	IN	RT/IN molar ratio ...	1:2	1:1	2:1	1:2	1:1
HIV-1	HIV-1		97	85	2.1	96	30
HIV-2	HIV-2		52	ND	ND	42	ND
HIV-2	HIV-1		36	7.7	3.0	12	ND
HIV-1	HIV-2		81	30	4.9	44	ND
MLV	HIV-1		61	44	43	78	50
MLV	HIV-2		60	52	1.8	61	10
p51	HIV-1		45	28	ND	10.2	ND
p51	HIV-2		66	58	ND	25	ND

The effect of HIV-1 RT on HIV-1 IN

HIV-1 IN used in this study exhibits both 3′-end processing and 3′-end DNA joining (strand-transfer) activities (Figure 1). We used two sets of DNA substrates (see the Experimental section) to discriminate between the effects of RT on the two IN activities (3′-end processing and strand transfer). The 21-mer double-stranded DNA served as a substrate of IN for 3′-end removal of the dinucleotides and, consequently, for the DNA-joining activity. A short exposure of the urea/PAGE gel used for resolving the labelled oligonucleotides (Figure 1A, lower panel) shows the extent of the excision of the 3′-end dinucleotides by producing 19-mer oligonucleotides. A longer exposure reveals the formation of longer oligonucleotide products due to the strand-transfer process of these preformed 19-mer molecules (into similar 19 or 21 bp molecules that serve as target DNA). Since integration is into random joining sites, there is a ladder of longer, labelled product molecules up to about 35 nucleotides in length (Figure 1A, upper panel).

A second direct assay of the DNA-joining activity of IN was performed by using 5′-end labelled 19-mer oligonucleotides annealed to the unlabelled 21-mer oligonucleotides used in the previous assay. The formation of a ladder of oligonucleotide species is indicative of HIV-1 IN's strand-transfer activity (Figure 1B).

To assess the effects of HIV-1 RT on the activities of HIV-1 IN, we added increasing amounts of HIV-1 RT to a constant amount of IN (Figure 1). It is evident that HIV-1 RT exhibits strong inhibitory activity towards both the 3′-end endonuclease and strand-transfer activities. Quantitative analyses reveal that 3′-end processing was almost fully inhibited when the RT/IN molecular ratio was 2:1 (Table 1). The inhibition of strand

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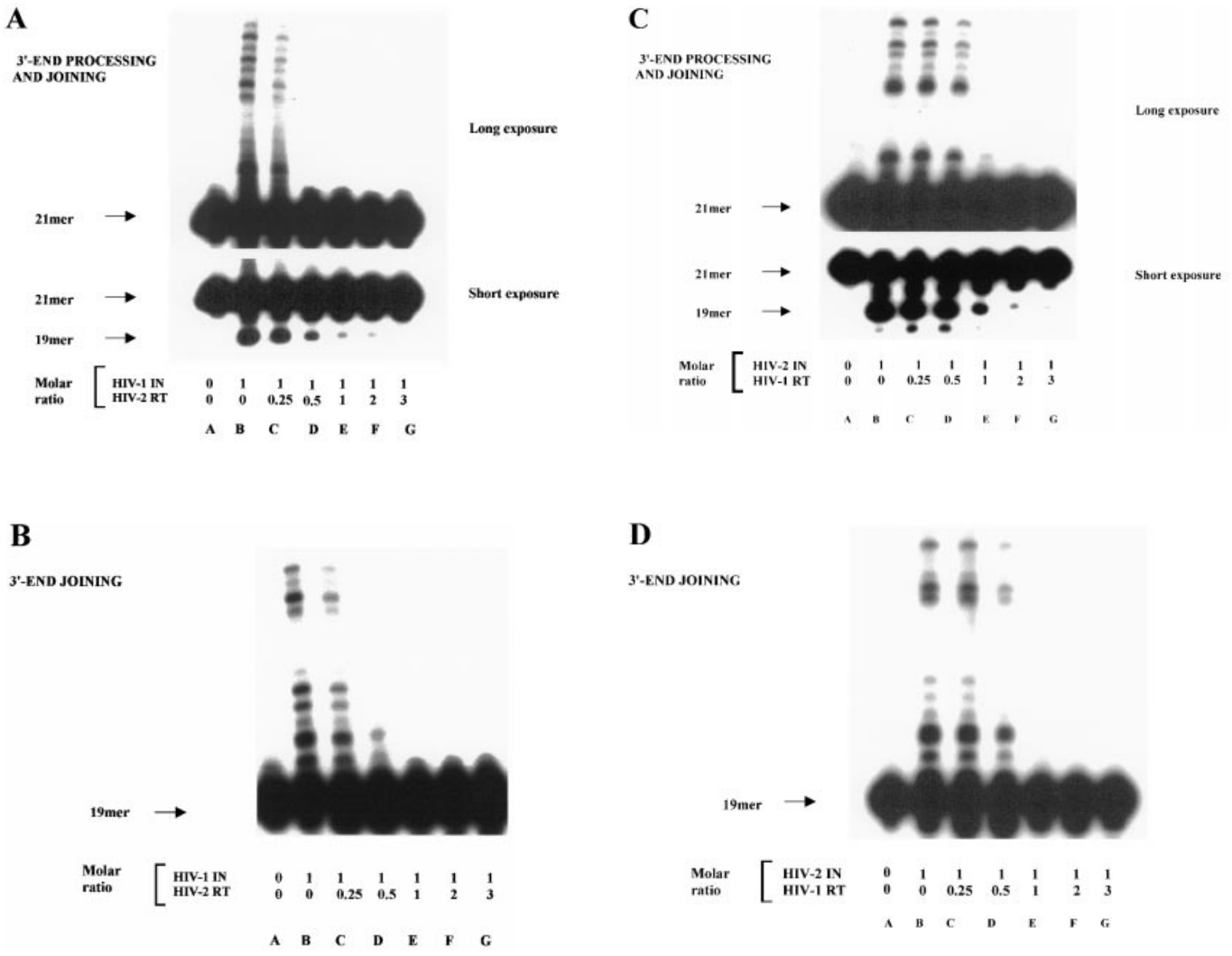

Figure 2 The heterologous effects of HIV-2 RT on HIV-1 IN and of HIV-1 RT on HIV-2 IN

The experiment was performed as described in Figure 1. (**A** and **B**) The effects of HIV-2 RT on HIV-1 IN. (**C** and **D**) The effects of HIV-1 RT on HIV-2 IN.

transfer was more efficient because substantial inhibition was observed at an equimolar ratio of RT to IN. In control assays with only RT present there was no effect on the oligonucleotides. Moreover, heat inactivation of HIV-1 RT, prior to its addition to IN in an equimolar ratio, totally abolished its effects (see below), suggesting that the observed effects depend on the native RT.

Effect of HIV-2 RT on HIV-2 IN

To further study whether the inhibition of IN by RT is restricted only to the HIV-1 enzymes, we have conducted an experiment similar to the one described above with the comparable enzymes of HIV-2 (Figures 1C and 1D). As found for HIV-1 IN, the HIV-2 enzyme shows strong 3′-end-processing and strand-transfer activities. Both activities of HIV-2 IN were inhibited by HIV-2 RT. Similar to HIV-1, strand transfer was inhibited more efficiently than 3′-end processing. That is, substantial inhibition of the former activity was observed at an RT/IN molecular ratio of 1:4 and above, whereas the latter activity was inhibited when the molecular ratios for RT/IN were 1:2 and above (Figures 1C and 1D, and Table 1). It also seems that the overall efficiency of inhibition of HIV-2 IN by HIV-2 RT was slightly higher than that of HIV-1 IN by HIV-1 RT. It is apparent that heat denaturation of HIV-2 RT abolishes its inhibitory effects (see below).

Heterologous inhibitions of HIV INs by the RTs

Despite the similarity between the RTs of HIV-1 and HIV-2, there are also distinct differences between these enzymes [18,20]. It was of interest to test whether the RT of one HIV is capable of inhibiting the IN of the other. It is evident from the results presented that each RT is capable of inhibiting the IN of the other HIV (Figure 2 and Table 1). Moreover, it seems that HIV-2 RT exerts a somewhat stronger inhibition of HIV-1 IN than does HIV-1 RT; thus there is no preference for homologous (same-strain) inhibition compared with heterologous (different-strain) inhibition of IN by RT (at least within HIVs).

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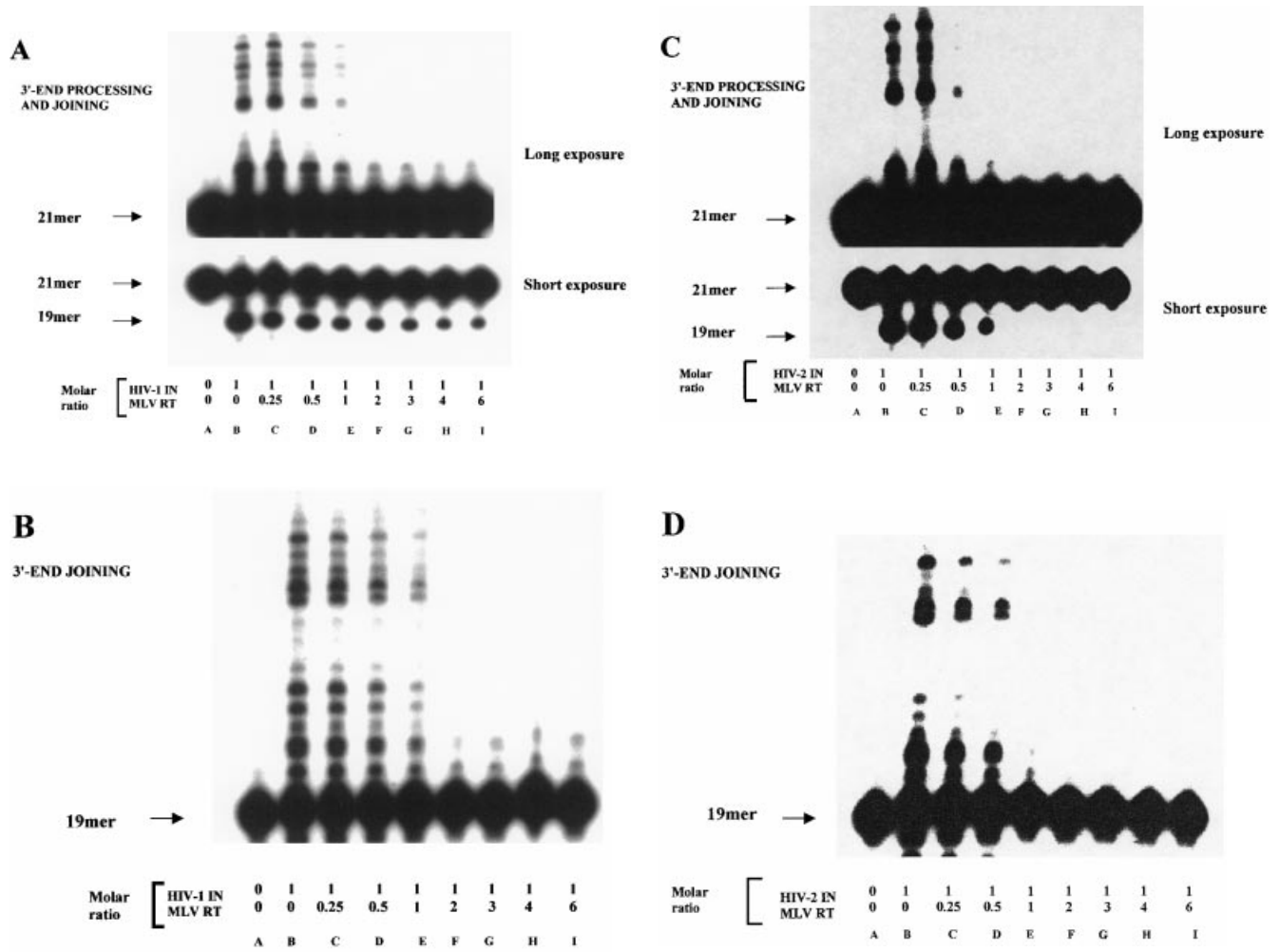

Figure 3 Effects of MLV RT on the integration activities of HIV-1 IN and HIV-2 IN

The experiment was performed as described in Figure 1, with MLV RT replacing HIV RTs. MLV RT was assumed to behave as a p70 monomer [1]. (**A** and **B**) The effects on HIV-1 IN. (**C** and **D**) The effects on HIV-2 IN.

Effects of MLV RT on the INs of HIV-1 and HIV-2

HIVs belong to the lentivirus subfamily of retroviruses. To test how general the phenomenon of inhibition of IN by RT is, we have studied an RT from MLV, which is a type-C retrovirus. The results presented in Figure 3 show that MLV RT also inhibits HIV-1 and HIV-2 INs. Quantitative analyses showed that HIV-2 IN is more sensitive to the inhibition by MLV RT than HIV-1 IN (Table 1). As found above for HIV RTs, inhibition of the strand-transfer activity of HIV-2 IN by MLV RT was slightly more efficient than the inhibition of the 3′-end-processing reaction of this IN. Heat denaturation of MLV RT prior to the addition of IN abolished the inhibition of the INs by MLV RT (see below).

Effects of heat denaturation of RTs on IN inhibition

To confirm that the observed inhibition of HIV-1 and HIV-2 INs required the presence of the native undenaturated form of RT from HIV-1, HIV-2 or MLV, we have performed the experiment shown in Figure 4. As mentioned above, it is clear that heat denaturation of the RTs abolished all detectable capacity of the RTs to inhibit IN activity.

Effect of RT inhibitors on the IN-blocking activity of HIV-1 RT

HIV-1 RT can be suppressed by a variety of highly potent inhibitors. The non-nucleoside RT inhibitors (NNRTIs) that are capable of specifically blocking the polymerase activity of HIV-1 RT in the nanomolar range are structurally diverse aromatic compounds. They block the DNA polymerase by interacting in most cases with the hydrophobic pocket proximal to the polymerase active site in the 'palm' subdomain of HIV-1 RT [27,28]. To test whether the undisturbed DNA polymerase active site of HIV-1 RT is involved in blocking the enzymic activities of HIV-1 IN, we analysed the effects of two known NNRTIs, Nevirapine (which is being used in AIDS patients to block HIV-1 replication) [29] and thiazolobenzimidazole (TBZ) [30]. The results of the experiment illustrated in Figure 5 show that neither inhibitor, at final concentrations of 40 and 100 μM for Nevirapine and TBZ, respectively, exhibited any apparent inhibitory effect on the activity of HIV-1 IN. Previously, the final concentrations of these inhibitors that lead to a 50% decrease in the final HIV-1

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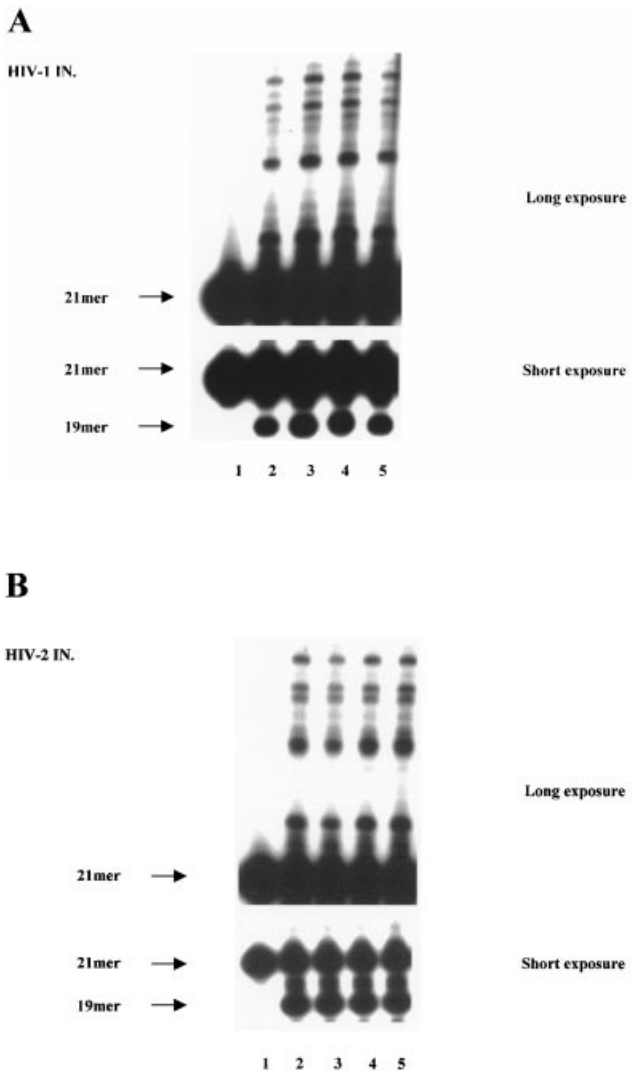

Figure 4 Effect of heat inactivation of RTs on the inhibition of INs

Each RT was heat-denatured at 100 °C for 3 min, was preincubated with HIV-1 IN or HIV-2 IN at an equimolar ratio and then subjected to the integration reaction (see the Experimental section and Figures 1–3). Lane 1, control with no HIV IN present; lanes 2–5, with HIV IN; lane 3, HIV-1 RT; lane 4, HIV-2 RT; lane 5, MLV RT. (**A**) Effect on HIV-1 IN. (**B**) Effect on HIV-2 IN.

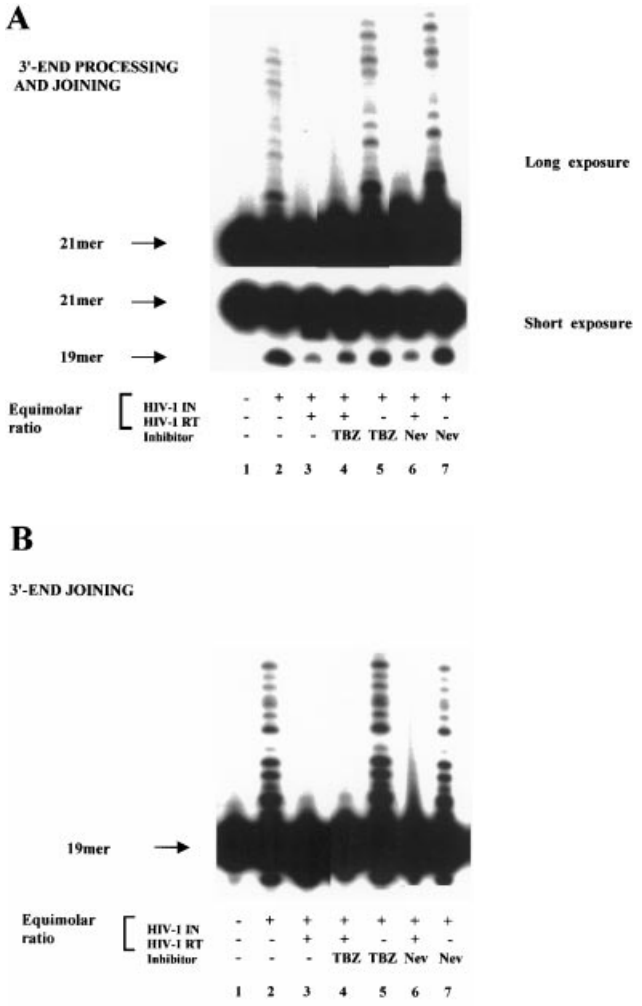

Figure 5 Influence of NNRTIs on the inhibition of HIV-1 IN, mediated by HIV-1 RT

The experiment was performed as described in Figure 1. The inhibitors TBZ and Nevirapine (Nev; 100 and 40 μM final concentrations, respectively), in a final DMSO concentration of 5%, were preincubated with HIV-1 RT at 4 °C for 5 min. HIV-1 IN was then added in an equimolar ratio to HIV-1 RT for an additional 5 min at 4 °C. The IN reactions were finally initiated by the addition of the reaction mixture, and the incubation was allowed to proceed for 30 min at 37 °C. The efficacy of inhibition of HIV-1 RT by TBZ and Nevirapine (at the same concentrations as used for in the integration assay) was tested in a DNA-polymerization assay (RNA-dependent DNA polymerase activity) and was found to be more than 95% (results not shown).

RT DNA polymerase activity were found to be 20 and 80 nM for Nevirapine and TBZ, respectively [31]. Therefore, it is clear that the experiment described here was done with a very large excess of each inhibitor, which means that these inhibitors have no significant direct effects on the activities of HIV-1 IN. It is likely, therefore, that the polymerase active site of RT is not involved in inhibiting the INs. In other experiments we have found that the presence of dNTPs in the reaction mixtures with RTs did not affect the blocking of the INs by the RTs, supporting the above conclusion (results not shown). This suggests that DNA synthesis by RT does not affect the inhibition of IN.

The effects of the p51 subunit of HIV-1 RT on the INs studied

The RTs of HIV-1 and HIV-2 are heterodimers composed of large subunits (p66 or p68 for HIV-1 or HIV-2, respectively) and smaller subunits, which are proteolytically cleaved products of the larger subunits. The viral proteases remove the C-terminal polypeptide that encompasses the whole RNase H domain of the RT [1]. The folding of the p51 subunit of HIV-1 RT in p66/p51 heterodimers is substantially different from that of the p66 subunit, despite their identical sequences (excluding the RNase H domain present only in the p66 subunit). Since, as shown above, an active polymerase domain is not a prerequisite for the IN-inhibiting activity of HIV-1 RT, we extended this study to the p51 subunit of HIV-1 RT. This protein exists mostly as a p51/p51 homodimer and has almost no DNA polymerase activity [18,32,33]. The p51 used here was found to possess only about 3% of the specific DNA polymerase activity of the heterodimer and, as expected, was devoid of any RNase H activity (results not shown).

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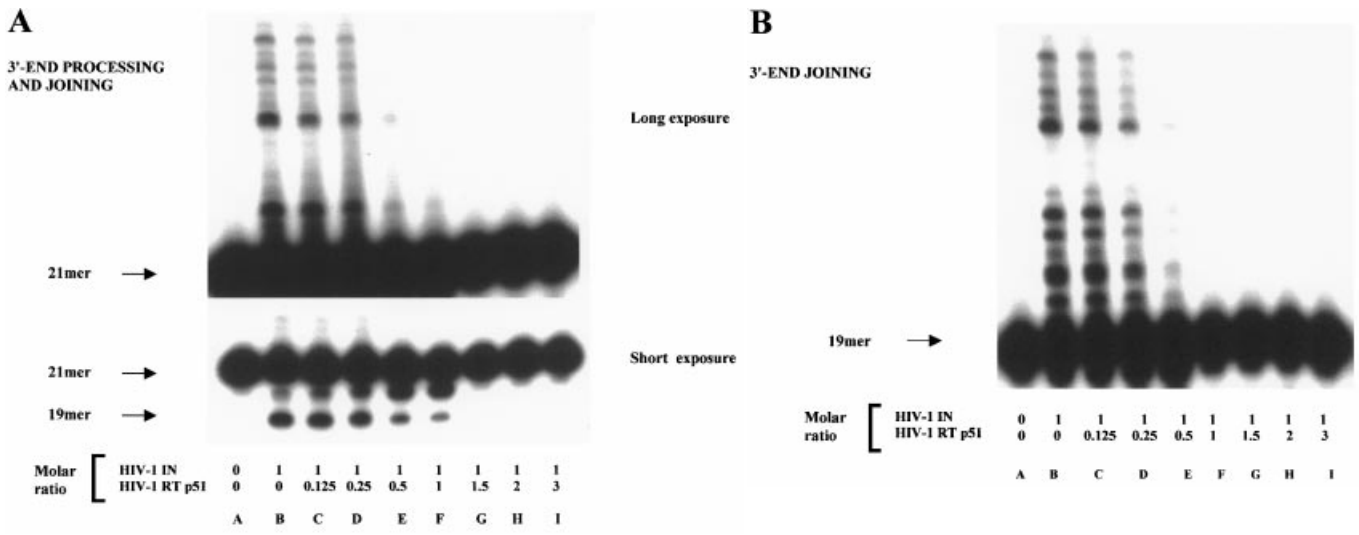

Figure 6 Effect of the HIV-1 RT p51 subunit on the integration activities of HIV-1 IN

The experiment was performed as described in Figure 1 with HIV-1 RT p51 replacing the heterodimer isoform p66/p51 of HIV-1 RT. p51 was assumed to behave as a homodimer of p51/p51 [32,33].

The results of the experiments presented in Figure 6 show that HIV-1 RT p51 is capable of inhibiting the 3′-end-processing and 3′-end-joining activities of HIV-1 IN. This inhibition reaches its maximal effect at approx. an equimolar ratio of p51/IN. In control reactions with heat-inactivated p51 there was no effect on the IN activities (results not shown). HIV-2 IN was also inhibited by HIV-1 RT p51 (results not shown). The quantitative data presented in Table 1 show that the p51 protein is generally not less efficient in inhibiting the INs than the heterodimeric HIV RTs.

Effects of the RTs on the disintegration activity of HIV-1 IN

Disintegration is the reverse reaction of the DNA-joining process that releases the viral DNA and restores the continuity of the target DNA [25]. We used the synthetic 38-mer oligonucleotide dumbbell substrate, described in the Experimental section and elsewhere [24]. Excision of the 'integrated' DNA fragment by the IN leads to formation of a 14-mer labelled linear product, as shown in Figure 7(A) (lane 2). The disintegration activity of HIV-1 IN in the presence of equimolar concentrations of the RTs from HIV-1, HIV-2 and MLV, as well as the p51 subunit of HIV-1 RT, was tested. The data presented in Figure 7 show that all RTs inhibit disintegration activity, though to various extents. In control experiments there was no effect of the RTs tested on the substrate DNA with no IN present. The experiment presented in Figure 7(B) shows that p51 of HIV-1 RT is capable of inhibiting the disintegration activity of both HIV-1 and HIV-2 INs at equimolar concentrations. Quantitative analyses of the extent of disintegration inhibition of HIV-1 IN by RT from HIV-1, HIV-2 and MLV, and the p51 isoform of HIV-1 RT (tested in two independent experiments), showed the mean inhibition of IN activity to be 85, 32, 53 and 63 % by these four RTs, respectively.

Potential effect of IN on RT

The data presented here show that INs are inhibited by RTs. Hence, it was of interest to also check the opposite, whether the catalytic activities of HIV-1 RT are affected by IN. We tested the RNA-dependent and DNA-dependent DNA polymerase as well as the RNase H activity of HIV-1 RT [34] in the presence of increasing amounts of HIV-1 IN. In all experiments there were no significant effects of IN. We also analysed the processivity of DNA synthesis and the fidelity of misinsertion and mispair extension of HIV-1 RT in the presence of IN (assayed according to Avidan and Hizi [35] and Taube et al. [36]). Again, we could not detect any significant influence of IN on RT activity (results not shown).

DISCUSSION

The infection of cells by retroviruses is established only when the viral linear two-LTR DNA is integrated successfully into the host-cell genome. Many of the biochemical steps responsible for the complex process of integration are catalysed by the virally encoded IN protein [1,2]. The machinery that carries out the integration is located in a nucleoprotein complex derived from the cores of the infectious virions [6,8]. However, to integrate the viral DNA (present within the PICs) successfully into the host nuclear DNA, the potentially self-destructive process of auto-integration must be avoided. Indeed, an 89-amino-acid cellular polypeptide has been implicated as a natural inhibitor of the auto-integration process in cells infected with MLV [16]. In the present study we suggest that the viral RT can function by itself as an inhibitor of integration, because it inhibits the enzymic activities of INs. Being part of the PIC, it is quite likely that RT can function also *in vivo* as an inhibitor or a regulator of auto-integration and/or integration processes.

The results presented in this paper show that the enzymic activities of the INs of the two related human lentiviruses, HIV-1 and HIV-2, are inhibited by the RTs of HIV-1, HIV-2 and MLV. Inhibition is accomplished at approx. equimolar ratios of RT/IN. In retroviruses, RT and IN are encoded by the same gene, *pol*, and are the cleavage products of the same polyprotein precursor. Therefore, based on the assumption that they both

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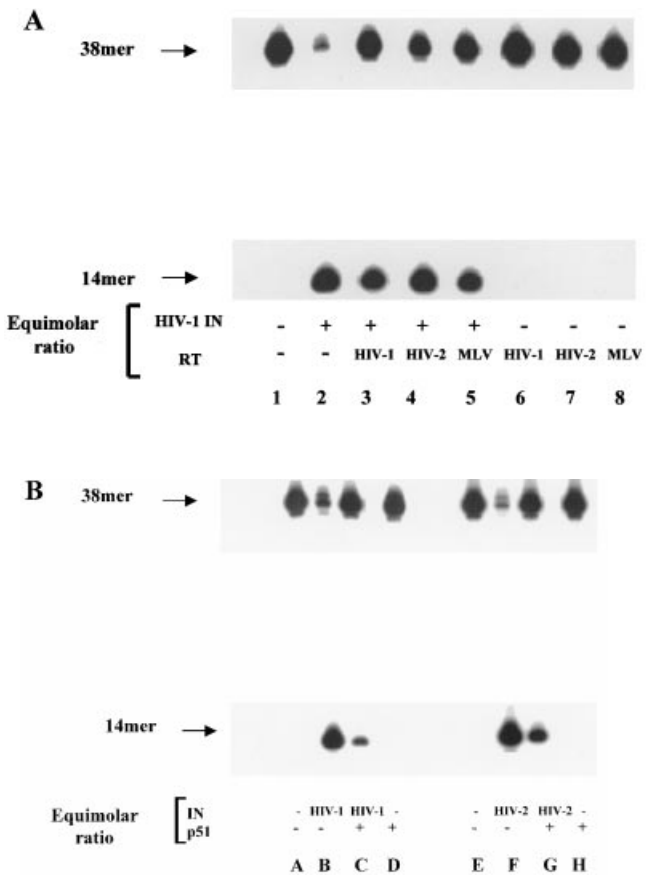

Figure 7 Effects of RTs on the disintegration activity of HIV INs

Each RT was preincubated with HIV-1 IN at an equimolar ratio and then subjected to the disintegration reaction with the 5′-end-labelled dumbell oligonucleotide D (see the Experimental section) for 30 min at 37 °C, followed by analysis by urea/PAGE. The 38-mer oligonucleotide is the substrate and the 14-mer DNA is the product. (**A**) Effects of RTs on HIV-1 IN: lanes 1 and 6–8 show the results of controls with no wild-type HIV-1 IN present. Lanes 2–5, with HIV-1 IN; lanes 3 and 6, HIV-1 RT; lanes 4 and 7, HIV-2 RT; lanes 5 and 8, MLV RT. (**B**) The effect of HIV-1 RT p51 on INs of HIV-1 and HIV-2.

have similar stabilities, the molecular ratios of RT/IN in viral cores and PICs is likely to also be approx. 1:1. This supports the biological validity of the *in vitro* results presented here. Both the 3′-end-processing and the strand-transfer steps of the INs are inhibited by the RTs tested. There was almost always a pattern in which inhibition of strand transfer by the RTs was slightly more efficient than inhibition of 3′-end processing. Such a pattern was also reported for inhibitors of these IN activities [37].

The inhibition of INs by the RTs tested in this study did not show any preference for homologous inhibition (when the RT and IN are both derived from the same retrovirus) compared with heterologous inhibition. Moreover, neither DNA synthesis by the RTs nor blocking the DNA polymerase active site by NNRTIs affected the extent of IN inhibition by RT. All DNA polymerases exhibit a 'right-hand' structure with 'finger', 'thumb' and 'palm' subdomains, with the active site located within the palm subdomain [38–41]. Since heat denaturation of RTs abolished their ability to block IN (Figure 4), it is likely that the three-dimensional structures of the RTs are important for interacting with IN and thus inhibiting its activity. The effect of the p51 subunit of HIV-1 RT (which encompasses the same subdomains) was interesting (Figures 6 and 7). This isoform of HIV-1 RT exhibits a very weak DNA polymerase activity. Therefore, taken together with the results that NNRTIs that inhibit the DNA polymerase activity of HIV-1 RT do not affect the extent of inhibition of IN by RT, it is evident that DNA polymerase activity itself is not a prerequisite for RT to inhibit IN. The three-dimensional folding of the p51 subunit in the p66/p51 heterodimers is different from that of the p66 enzymically active subunit. However, the exact structure of the p51/p51 RT form is not known; therefore, it is not clear yet whether it folds similarly to p51 in the heterodimers.

The precise mode of inhibition of the IN by the polymerase is still an open question. Based on previous data and our results, we suggest that the inhibition of IN by RT is due to interactions between the proteins and not due to competition for DNA substrates. First, there are several studies that report the presence of physical contacts between RT and IN [9–11]. This observation is quite expected, based on the topology of retroviral cores and PICs and their mutual biological interactions; both proteins originate from the same polyprotein precursors, and the DNA product of RT is the substrate for IN. Secondly, RTs also inhibit the disintegration activity of IN *in vitro*. Since this assay involves internal excision of the DNA by IN rather than interacting with the 3′-OH ends of the DNA, it is likely that the inhibition of IN is not simply due to competition for the 3′-OH ends of the donor DNA substrate. Thirdly, such competition is not likely, since we tested the inhibition of IN by RT in the presence of an excess of DNA versus enzyme at the highest technically feasible ratio (3.3 DNA molecules/enzyme molecule). This ratio is 20 times higher than in the experiments described above; yet there was no significant difference in the extent of IN inhibition by RT (results not shown). In addition, we have already argued that the inhibition takes place at RT/IN molecular ratios of approx. 1:1, supporting its biological relevance due to the equimolar stoichiometry of the two proteins in PICs. Furthermore, p51 was reported to have weak DNA-binding activity in comparison with the p66/p51 heterodimeric RT [42]. Still, p51 inhibits IN as efficiently as the heterodimers (Figures 6 and 7). It is also important to emphasize, based on the findings reported above, that IN is not capable of affecting the catalytic functions of RT. It is conceivable that RTs can modulate IN activities *in vitro*, but not vice versa.

The results presented support the hypothesis that RT is likely to inhibit IN in the cytoplasm of the retrovirus-infected cells rather than in their nuclei. The RTs tested inhibit the strand-transfer reaction more efficiently than the 3′-end-processing reaction. In infected cells the 3′-end processing by IN is performed mostly in the cytoplasm, whereas the strand-transfer reaction takes place only in the nucleus, in close proximity to the target DNA [1]. Therefore, it is more important for RT to inhibit the latter activity of IN in the cytoplasm, in order to prevent auto-integration, than to block the necessary former activity.

We have argued that the inhibition of IN is performed via direct contacts between RT and IN rather than by competition for the 3′-OH of the donor DNA. Previous studies have shown that protein interactions with IN do not necessarily inhibit IN activities. Thus the cellular proteins high-mobility group-1 and IN interactor-1 bind retroviral INs and stimulate rather than inhibit IN activities [12,15,43]. The viral matrix protein was shown to participate in the translocation of PICs into the nuclei also by interacting directly with IN [44]. On the other hand, the 89-residue cellular polypeptide, which inhibits auto-integration in MLV-infected cells, does not bind directly to MLV IN, but probably inhibits auto-integration by interacting with the viral substrate DNA [16,45]. Taken together, it might be that RTs

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constitute a new group of proteins that inhibit INs by interacting directly with them.

A recent study has shown that antibodies directed against the minimal DNA-binding domain in the C-terminal region of HIV-1 IN block RT–IN interactions and that Trp-235 of HIV-1 IN, centred in this region, is critical to the binding [10]. This may suggest that the DNA-binding domain of IN (which is located in the C-terminal region) is involved not only in the RT–IN interactions but also in the inhibition of IN by RT. It was suggested that the C-terminal domain is located in close proximity to the catalytic (core) domain of IN in the three-dimensionally folded protein [46], supporting the proposed hypothesis that RT inhibits IN by an allosteric effect via binding to the C-terminal region of IN. The interaction between RT and IN was shown in the present article to be less efficient in blocking the disintegration activity of IN than the 3′-end-processing and strand-transfer activities. This is also in line with the hypothesis presented, since the disintegration activity requires only the catalytic (core) domain of IN [47,48], whereas the other activities depend also on the C-terminal region [47,49].

In summary, the data presented here leave several questions unanswered. First, little is known about the mechanisms by which the RTs inhibit the INs, and the sequences of the RTs involved in inhibiting IN. The reported blockage of IN activities is expected to be transient. After the PICs are translocated into the nuclei of the infected cells, the inhibition should be reversed to allow integration into the host-cell nuclear DNA. We would like to know what mechanism is involved and whether other cellular proteins also participate in this process. The information obtained might be helpful in designing novel drugs that inhibit HIV IN. Integrases, by being very specific to retroviruses, are excellent targets in the search for novel anti-AIDS drugs. Alternatively, based on the speculation that RT can serve as an inhibitor of auto-integration, inducing auto-integration by interfering with the interactions between RT and IN might also help in fighting HIV infections.

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