Intramolecular Chimeras of the p51 Subunit between HIV-1 and FIV Reverse Transcriptases Suggest a Stabilizing Function for the p66 Subunit in the Heterodimeric Enzyme†

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ABSTRACT: The human immunodeficiency virus (HIV) reverse transcriptase (RT) is a heterodimeric enzyme composed of a 66 kDa (p66) and a 51 kDa (p51) subunit. Recently we showed that p51 plays an important role in the conformation of p66 within the HIV-1 RT heterodimer and hence appears to influence its catalytic activities [Amacker, M., and Hübscher, U. (1998) *J. Mol. Biol. 278*, 757−765]. This was further investigated here via construction of three intramolecular chimeras of HIV-1 and FIV RTs. The first 25 and 112 amino acids of the N terminus, respectively, as well as the last 22 amino acids of the C terminus in the p51 subunit of HIV-1 RT were exchanged with the corresponding regions of the FIV RT and combined with the wild-type HIV-1 p66. Characterization of these chimeric RT heterodimers demonstrated significant biochemical differences in (i) DNA-dependent DNA synthesis, (ii) strand displacement DNA synthesis, and (iii) RNase H activity. Our results indicate that both the N and C termini of HIV-1 RT p51 appear to be important in stabilizing the RT heterodimer for enzymatic functions.

The lentiviral reverse transcriptases (RTs)[1] of the human immunodeficiency virus type 1 (HIV-1) and the feline immunodeficiency virus (FIV) are multifunctional DNA polymerases able to copy both RNA and DNA templates for DNA synthesis (*1*). In addition, the RTs also possess RNase H activity (*2*−*4*) and the ability to perform strand displacement DNA synthesis (*5*−*7*), which are both necessary steps for a successful reverse transcription process.

The RT in HIV-1 and FIV is a heterodimer composed of a 66 kDa (p66) subunit and a 51 kDa (p51) subunit (*8*−*11*) which are both encoded by the viral *pol* gene. The p51 subunit is derived by proteolytic cleavage by the viral protease of the RNase H domain from one of the p66 subunits in the initially formed RT (p66−p66) homodimer (*9*, *12*−*14*) to form the RT (p66−p51) heterodimer. The heterodimeric form is the most stable and active form of the RT enzyme found in vivo (*8*, *12*, *15*, *16*). A fascinating feature of the HIV-1 RT heterodimer is its structural asymmetry which exists between the p66 and p51 subunits despite the fact that they are products of the same gene and identical for the first 440 amino acid residues (*17*−*19*). The polymerase domain consisting of the four subdomains (fingers, palm, thumb, and connection) has, however, a completely different overall structural arrangement. The p66 subunit forms a DNA binding cleft with the active site residues exposed in the palm subdomain, whereas p51 is closely folded with the active site residues buried by the connection subdomain. The two subunits interact mainly via their connection subdomains and also between p51 thumb and finger subdomains and the RNase H and palm subdomains of p66, respectively (*18*−*20*).

The p66 subunit encodes both the polymerase and RNase H of the RT heterodimer, whereas the polymerase domain of the p51 subunit is inactive (*21*−*25*). Several possible functions have been suggested for the p51 subunit in the RT heterodimer: (i) a role as a processivity factor for the p66 subunit (*26*), (ii) involvement in tRNA primer binding (*27*−*30*), (iii) loading of the p66 subunit onto the template primer (TP) (*31*), (iv) enhancement of strand displacement DNA synthesis (*6*, *7*), and (v) a role in the induction and maintenance of an optimal functional structural conformation of the p66 subunit (*32*). Chimeric work involving HIV-1 RT and other lentiviral RTs has so far been reported by several groups, including our own, contributing to a further understanding of the functions of the individual subunits within the HIV-1 RT heterodimer, the mapping of the catalytic sites, and the non-nucleoside inhibitor drug binding sites in HIV-1 RT. This work included HIV-1−HIV-2 (*33*−*35*), SIV−HIV-1 (*36*), HIV-1−MuLV (*37*, *38*), and HIV-1−FIV RT chimeras (*32*).

Although there are similarities in the biochemical functions of HIV-1 and FIV RT heterodimers, some differences between them exist. The two RTs are 49.5% identical and 67% similar at the amino acid level. Our recent data (*32*) indicated that the FIV RT p51−HIV-1 RT p66 chimera showed a 2.5-fold higher RNase H activity, a 50% lower strand displacement DNA synthesis activity, and resistance

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[1] Abbreviations: RT, reverse transcriptase; HIV, human immunodeficiency virus; FIV, feline immunodeficiency virus; MuLV, murine leukemia virus; SIV, simian immunodeficiency virus; TP, template primer; AZTTP, 3-azido-2,3-dideoxythymidine triphosphate; PMSF, phenylmethanesulfonyl fluoride.

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to the two RT inhibitors, 3-azido-2,3-dideoxythymidine triphosphate (AZTTP) and Nevirapine. The HIV-1 RT p51−FIV RT p66 chimera on the other hand had properties very similar to those of the natural FIV RT. As a consequence of these data, we constructed intramolecular chimeras between the p51 subunits of HIV-1 and FIV RTs. Three small defined regions of HIV-1 RT p51 were replaced with the corresponding regions from FIV RT p51. Functional RT heterodimers were reconstituted by using these intramolecular HIV-1−FIV chimeric p51 subunits and the HIV-1 RT p66 subunit. The characterization of these intramolecular chimeric RT heterodimers in comparison to the natural HIV-1 RT heterodimer (HIV-1 RT wt) suggests functions of both the N- and C-terminal regions of the p51 subunit in the maintenance of optimal structure of the p66 subunit within the HIV-1 RT heterodimer.

MATERIALS AND METHODS

Chemicals. Radioactively labeled nucleotides and AZTTP were purchased from Amersham, and deoxynucleoside triphosphates (dNTPs) were obtained from Pharmacia. All other reagents were of analytical grade and purchased from Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland). Nevirapine (BI-RG-587) was kindly provided by P. Ganong (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT).

Buffer Solutions. The following buffers were used: buffer A, which was 10 mM sodium phosphate, 10 mM imidazole (final pH of 7.2), 0.5 M NaCl, and 0.4 mM PMSF; buffer B, which was 10 mM sodium phosphate, 0.5 M imidazole (final pH of 5.5), 0.5 M NaCl, and 0.4 mM PMSF; buffer C, which was 50 mM Tris-HCl (final pH of 7.0), 1 mM EDTA, 1 mM DTT, 0.4 mM PMSF, and 5% (v/v) glycerol; buffer D, which was 50 mM Tris-HCl (final pH of 8.8), 1 M NaCl, 1 mM EDTA, 1 mM DTT, 0.4 mM PMSF, and 5% (v/v) glycerol; and buffer E, which was 50 mM Tris-HCl (final pH of 7.5), 50% (v/v) formamide, 20 mM EDTA, 0.03% (w/v) xylene cyanol, and 0.03% (w/v) bromphenol blue.

Enzymes. Calf thymus DNA polymerase α was isolated as described previously (*39*). Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, and T4 DNA polymerase were from Boehringer Mannheim or Gibco BRL. *Pfu* DNA polymerase and *Taq* DNA polymerase were from Stratagene and Perkin-Elmer, respectively.

Nucleic Acid Substrates. Poly(rA)$_{400-700}$ and oligo(dT)$_{15-18}$ were from Pharmacia. Poly(rA)/oligo(dT) (10:1 base ratio) and single-stranded primed M13 DNA were prepared as described previously (*6*, *32*). The gapped circular DNA template was based on the Bluescript SK(+) plasmid containing a defined gap of 26 nucleotides and prepared as described previously (*7*). Oligonucleotides were purchased from Microsynth (Balgach, Switzerland). The plasmid pRT-ENDO/PROT used for the production of wild-type HIV-1 RT was kindly provided by J. Mous (F. Hoffmann-La Roche, Basel, Switzerland). Plasmids pRT581 and pRT100A, carrying the HIV-1 RT p51 and p66 genes, respectively, as well as the empty vector pRT 538 were kindly provided by C. McHenry (University of Colorado Health Sciences Center, Denver, CO). Plasmids pMA134 and pMA135 used for the expression of His-tagged HIV-1 RT p51 and p66, respectively, and the plasmid pMA131 expressing the His-tagged FIV p51 have been described in detail recently (*32*).

Oligonucleotide Primers. The following PCR primers were used: MA2, 5′-CTGACGGCGAATTCTCGAGTTACCATGTTTCTGCTCCTGG-3′; TT1, 5′-CGGCTCGTATAATGTGTGG-3′; TT2, 5′-ATTGACAAACTCCCACTCAG-3′; and TT4, 5′-GCTGCTTTGAATATAAAGAGAGCG-3′

Construction of Intramolecular Chimeric HIV-1 and FIV RT p51 Genes. (i) C1 and C2 Chimeras. The first intramolecular chimera named C1 contains a chimeric p51 subunit in which the first 25 amino acids (Pro^{1}−Pro^{25}) of wild-type HIV-1 RT had been replaced by the first 28 amino acids (Ile^{1}−Asp^{28}) of FIV RT. To construct this chimera, expression plasmids pMA 131 (FIV) and pMA 134 (HIV-1) were digested using *Msc*I and appropriate fragments were ligated to form plasmid pTT1. The second intramolecular chimera, named C2, contains a chimeric p51 subunit in which the first 112 amino acids (Pro^{1}−Gly^{112}) of wild-type HIV-1 RT had been replaced by the first 121 amino acids (Ile^{1}−Pro^{121}) of FIV RT. For this, plasmids pMA 131 and pMA 134 were digested with *Nsi*I and appropriate fragments were ligated to form plasmid pTT2.

(ii) C3 Chimera. The HIV-1 RT p51 sequence (pMA 134) was amplified by PCR using TT1 (5′-primer) and TT2 (3′-primer) primers to generate a 1332 bp PCR product with an *Eco*RI site at the 5′-end. This encodes the first 417 amino acids of HIV-1 RT p51 (Ile^{1}−Asn^{417}). Similarly, the carboxy terminus of the FIV RT p51 sequence (pMA 131) encoding the last 22 amino acids was amplified by using PCR primers TT4 (5′-primer) and MA2 (3′-primer) to generate a 94 bp fragment with an *Xho*I restriction site at its 3′-end. The products from each PCR were gel purified, and 3′- and 5′-overhangs were filled up by T4 DNA polymerase, phosphorylated, and finally restricted by *Eco*RI and *Xho*I. The pRT 538 plasmid vector was restricted with *Eco*RI and *Sal*I and gel purified. The prepared PCR products from above and the vector were ligated together to form a hybrid HIV-1−FIV RT p51 gene sequence within the plasmid vector which was named pTT3. The last 22 amino acids (Thr^{418}−Phe^{440}) of the authentic HIV-1 RT p51 had been replaced by the last 23 amino acids (Ala^{419}−Trp^{442}) of FIV RT p51. This chimera was named C3.

All three chimeric p51 vectors contained the sequence for a polyhistidine tag in front of the p51 gene for purification and were all completely sequenced to confirm their chimeric status.

Expression and Purification of RT Heterodimers. Plasmids pRT100A, pMA135, pTT1, pTT2, and pTT3 were transformed into *Escherichia coli* strain JM109 (DE3). Cells were grown at 30 °C in 1 L of Luria Broth to an A_{600} of 0.6. Isopropyl thiogalactoside was added to a final concentration of 1 mM and growth continued for 4 h. Cells were harvested by centrifugation and resuspended in 10 mL of buffer A. Cells expressing the HIV-1 RT p66 subunits were then mixed with cells expressing the appropriate p51 subunit (HIV-1 RT p51 wt or intramolecular chimeric p51) prior to French press treatment (also see the Results). The mixture was centrifuged for 30 min at 30000*g* and the lysate supernatant loaded on a fast protein liquid chromatography HiTrap chelating column (Pharmacia) complexed with Co^{2+} ions and equilibrated in buffer A. The RT heterodimers were eluted from the column with a double gradient of imidazole and pH (10

to 500 mM imidazole and pH 7.2 to 5.5) in buffer B, followed by a desalting step (Hitrap desalting column, Pharmacia) by using buffer C. For the final purification, the mixtures were loaded onto a Mono S column (Pharmacia) equilibrated in buffer C, and the RT heterodimers eluted with a double gradient of NaCl and pH (0 to 1 M NaCl and pH 7.0 to 8.8) in buffer D. Fractions containing a 1:1 p51:p66 ratio were pooled, dialyzed against buffer C, and stored in aliquots in liquid nitrogen until further use. Each RT pool had a purity of ≥90%. The RT heterodimers were named on the basis of the type of the p51 they had, i.e., HIV-1 RT wt, C1, C2, and C3 (see above).

Assay for DNA Polymerases. The RNA-dependent DNA polymerase activity of RT was determined in a final volume of 25 μL containing 50 mM Tris-HCl (pH 7.5), 80 mM KCl, 7.5 mM $MgCl_2$, 1 mM DTT, 20 μM [^{3}H]dTTP (500 cpm/pmol), 0.5 μg of poly(rA)/oligo(dT)$_{15-18}$ (10:1 base ratio), 0.2 mg/mL BSA, and the enzyme to be tested. Incubation was carried out for 15 min at 37 °C. The DNA-dependent DNA polymerase activity was determined under the same conditions but in the presence of 1 μg of single-primed M13 DNA template and 40 μM dATP, dTTP, dCTP, and dGTP. Divalent cation optimization was performed under identical conditions with varying concentrations of $MgCl_2$. DNA polymerase α was tested with activated calf thymus DNA as described by Weiser et al. (*40*). One unit of DNA polymerase activity corresponds to the incorporation of 1 nmol of dTMP into acid-precipitable material by using poly(rA)/oligo(dT)$_{15-18}$ (10:1 base ratio) as a template in 60 min at 37 °C.

Assay for Strand Displacement DNA Synthesis. To measure the degree of strand displacement DNA synthesis (*6*, *7*), the following components were used in a final volume of 25 μL: 50 mM Tris-HCl (pH 7.5), 7.5 mM $MgCl_2$, 80 mM KCl, 0.2 mg/mL BSA, 1 mM DTT, 30 μM dNTPs, 1 μCi [α-^{32}P]dTTP (3000 Ci/mmol), 75 ng of the circular, gapped DNA substrate, and equal amounts of RT as indicated. The gapped DNA substrate was first pulse labeled for 10 min at 37 °C by incubating with [α-^{32}P]dTTP and dATP before adding the remainder of the dNTPs and further incubation for 30 min. Samples were heated at 65 °C for 15 min to inactivate the enzymes, and the DNA was then digested after addition of *Hin*dIII (1 unit) at 37 °C for 30 min. The reaction was stopped by addition of 5 μL of buffer E, and the DNA products were separated on a 7.5% polyacrylamide gel, containing 7 M urea. Quantification of DNA products was performed on a PhosphorImager (Molecular Dynamics) as described previously (*32*).

Assay for RNase H. The RNase H activity of RT was determined in a final volume of 25 μL containing 40 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 0.08 mg/mL BSA, 3% (v/v) glycerol, 0.02% (v/v) Nonidet P40, 40 ng of [^{3}H]poly(rA)/poly(dT) (500 cpm/pmol), and the enzyme fractions to be tested. Incubation was carried out for 30 min at 37 °C. After nucleic acid precipitation, the supernatant was mixed with 1 mL of scintillation fluid and measured in a scintillation counter.

Assay for Product Analysis. For product analysis, single-stranded M13 DNA (mp18) was primed with a radioactively labeled 17mer primer (5′-GTAAAACGACGGCCAGT-3′). The following components were used in a final volume of 15 μL: 0.5 μg of primed M13 DNA template, 50 mM Tris-HCl (pH 7.5), 80 mM KCl, 7.5 mM $MgCl_2$, 1 mM DTT, 0.02 mg/mL BSA, and 0.25 unit of different types of RT heterodimers to be tested. The mixture was incubated for 10 min at room temperature to allow the RT to bind substrate, followed by addition of 1 μg of poly(rA)/oligo(dT) cold trap and the dNTPs at 40 μM each and by incubation for 30 min at 37 °C. The reaction was stopped by adding 15 μL of buffer E; the samples were boiled for 3 min before being loaded and separated on a 6% polyacrylamide gel containing 7 M urea.

Determination of Kinetic Constants. The kinetic constants were determined by using the RNA-dependent DNA polymerase assay under conditions described above in the presence of various concentrations of [^{3}H]dTTP and ddTTP. Linear least-squares analysis was used to generate the double-reciprocal plots and the replots of K_i (*41*).

Immunoblotting and Antibodies. The RTs were detected with polyclonal antibodies raised in chickens. The details for HIV-1 RT are described in ref *42* and for FIV RT in ref *32*.

RESULTS

Construction of Intramolecular Chimeric HIV-1−FIV RT Heterodimers. Three intramolecular chimeric RT p51 subunits were constructed, two in the p51 N terminus where the first 25 amino acids (Ile^1−Pro^{25}) and 112 amino acids (Ile^1−Pro^{112}) of the HIV-1 RT p51 subunit were replaced by the corresponding regions from the FIV RT p51 subunit. The third intramolecular chimera involved the HIV-1 RT p51 C terminus, where the last 23 amino acids (Thr^{418}−Phe^{440}) were also similarly replaced (see Materials and Methods and Figure 1 for details). The regions exchanged in two of the intramolecular chimeras (C2 and C3) also included parts of HIV-1 RT p51 involved in physical interaction with the p66 subunit in the RT heterodimer. In C2, the replaced region extends from the N terminus to the β6 loop and in C3 it extends from the β21 loop to the C terminus of HIV-1 RT p51. The choice of these regions was based on the current models of the crystallized structure of the HIV-1 RT heterodimer (*18*, *20*, *43*, *44*). Each of the individual chimeric HIV-1 or FIV RT p51 subunits was cloned with a hexahistidine tag at the 5′-end of the gene.

Purification of Wild-Type HIV-1 RT and Intramolecular Chimeric HIV-1−FIV RT Heterodimers. Individual RT p51 subunits (chimeric HIV-1−FIV p51 and wild-type HIV-1 p51) were transformed separately into *E. coli*, and each subunit was expressed as described in Materials and Methods. Bacterial cells expressing the appropriate p51 subunit with a His tag and HIV-1 RT p66 subunit without a tag were mixed together prior to bacterial cell lysis, and the reconstituted RT heterodimers were then first purified on a metal chelate affinity column. Fractions containing the heterodimer were pooled and desalted before loading onto an ion exchange column for final purification. To confirm the native heterodimeric form, purified RTs were loaded onto analytical gel filtration columns (HPLC) as described earlier (*32*). A comparison to homodimeric p51 and homodimeric p66 showed that all the constructs that were made were heterodimeric (data not shown). However, a general problem with this approach and with other RT heterodimer reconstitution approaches in vitro is that heterodimer formation

A

C1

HIV-1 RT p51 1 PISPIETVPV KLKPGMDGPK VKQWPLTEEK IKALVEICTE MEKEGKISKI 50

FIV RT p51 1 ISDKIPVVKV KMKDPNKGPQ IKQWPLTNEK IEALTEIVER LEKEGKVKRA 50

C2

HIV-1 RT p51 1 PISPIETVPV KLKPGMDGPK VKQWPLTEEK IKALVEICTE MEKEGKISKI 50

FIV RT p51 1 ISDKIPVVKV KMKDPNKGPQ IKQWPLTNEK IEALTEIVER LEKEGKVKRA 50

51 GPENPYNTPV FAIKKKDSTK WRKLVDFREL NKRTQDFWEV QLGIPHPAGL 100

51 DSNNPWNTPV FAIKKKSGKW RMLIDFRELN KLTEKGAEVQ LGLPHPAGLQ 100

101 KKKKSVTVLD VGDAYFSVPL DEDFRKYTAF TIPSINNETP GIRYQYNVLP 151

101 IKKQVTVLDI GDAYFTIPLD PDYAPYTAFT LPRKNNAGPG RRFVWCSLPQ 151

C3

HIV-1 RT p51 401 WWTEYWQATW IPEWEFVNTP PLVKLWYQLE KEPIVGAETF 440

FIV RT p51 401 NLINSPYLKA PPPEVEYIHA ALNIKRALSM IKDAPIPGAE TW 442

B

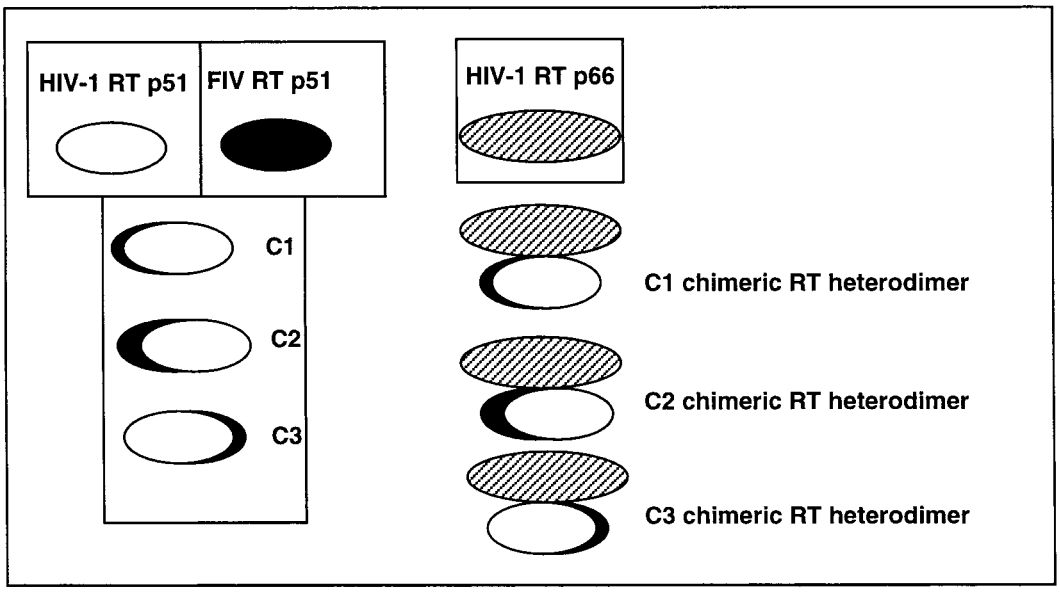

FIGURE 1: Construction of the intramolecular chimeric RT heterodimers. (A) Primary amino acid sequence comparison of the HIV-1 and FIV RT p51 subunits showing regions in HIV-1 RT (bold) which were replaced with the corresponding region in FIV RT (bold and underlined). (B) Schematic representation of intramolecular chimeric HIV-1−FIV RT p51 subunits which were constructed (C1−C3) and their reconstitution with HIV-1 RT p66 into HIV-1−FIV intramolecular chimeric RT heterodimers (C1−C3 chimeric RT heterodimers).

probably does not represent the exact folding procedure occurring in vivo even when performed in crude extracts. All heterodimers were separated on a SDS−polyacrylamide gel stained with Coomassie blue (Figure 2) and the subunit provenance confirmed by Western blot analysis (data not shown). In all experiments, the chimeric RT heterodimers were compared to the recombinant HIV-1 RT wt.

Intramolecular RT Chimeras Retain DNA Polymerase and RNase H Activities. First RNA- and DNA-dependent DNA polymerase and RNase H activities of the intramolecular RT heterodimers were determined. As documented in Table 1, all three intramolecular RT chimeras retained the three activities of the HIV-1 RT wt. All three chimeric RT heterodimers displayed a reduction in both RNA and DNA polymerase and RNase H activities when compared to the natural HIV-1 RT heterodimer. The C3 RT chimera displayed a very low specific DNA-dependent DNA polymerase activity on primed M13 and had the lowest specific activity on both templates compared to the other RT heterodimers. All subsequent characterizations of the four RT heterodimers were carried out using an equal amount of units of each enzyme as defined on poly(rA)/oligo(dT). The optimal

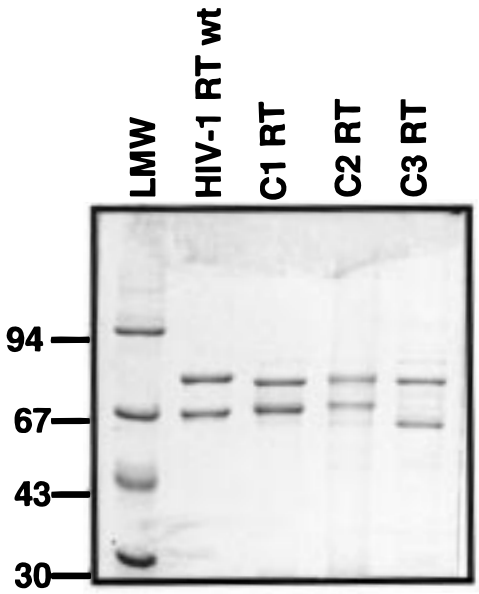

FIGURE 2: Purified intramolecular chimeric HIV-1−FIV RT heterodimers. A Coomassie blue-stained 10% SDS−polyacrylamide gel of purified RT heterodimers is shown. One microgram of each enzyme was loaded: lane 1, low-molecular weight markers; lane 2, natural HIV-1 RT heterodimer; lane 3, C1 chimeric RT heterodimer; lane 4, C2 chimeric RT heterodimer; and lane 5, C3 chimeric RT heterodimer.

Table 1: Specific Activities of Intramolecular Chimeric RT Heterodimers[a]

	polymerase activity					
	poly(rA)/oligo(dT)		primed M13 DNA		RNase H activity	
RT heterodimer	units/mg	% of control	units/mg	% of control	units/mg	% of control
HIV-1 wt	59000	100	505	100	1300	100
C1	14600	25	257	51	837	64
C2	4291	7	142	28	625	48
C3	1040	2	12	2	376	29
H66−H66	830	1	11	1	108	8

[a] Specific activities (units per milligram) were determined as described in Materials and Methods.

magnesium divalent cation requirements on both templates for each RT heterodimer were very similar for the natural HIV-1 RT and the C1 RT and C2 RT intramolecular chimeras (5−7.5 mM). The C3 intramolecular RT chimera on the other hand required a slightly higher magnesium level (10 mM) on both templates as compared to the other RT heterodimers. Next the interaction of the three chimeric RT heterodimers with the nucleoside analogue dideoxythymidine triphosphate (ddTTP) was compared to that of natural HIV-1 RT by determination of the K_i in the presence of varying amounts of ddTTP. The data indicated that the C1 RT chimera had a 14-fold higher K_i than the control enzyme (HIV-1 RT wt), while it was only slightly lower for C2 and C3 RT chimeras (Table 2).

The DNA Polymerase Activity Is Affected Much More Than the RNase H Activity in the C3 RT Chimera As Compared to Those of the C1 and C2 RT Chimeras. The RNase H activity is an essential component of the RT heterodimer for RNA template degradation, RNA primer removal to enable strand transfer, and precise determination of the initiation points for plus strand DNA synthesis during the reverse transcription process (*45*). The C terminus of the

Table 2: K_i for dTTP of the Chimeric RT Heterodimers[a]

RT heterodimer	ddTTP K_i (nM)
HIV-1 wt	28 (±4)
C1	383 (±26)
C2	12 (±2)
C3	20 (±3)

[a] The K_i values were determined as described in Materials and Methods.

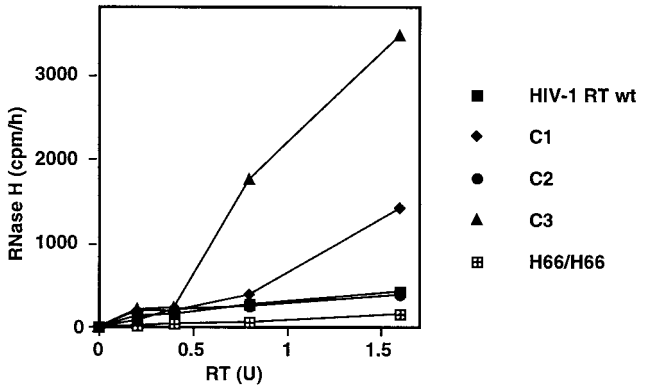

FIGURE 3: Comparison of the RNase H activities of the RT heterodimers. Equal amounts in RT units as determined on poly(rA)/oligo(dT) of each RT heterodimer were used. The average RNase H activity was plotted for each RT heterodimer. The HIV-1 p66−p66 RT homodimer was also included as a control. Between 0.4 and 1.6 units (DNA polymerase units), the C3 chimeric RT heterodimer displays an RNase H activity 6-fold higher than that of the natural HIV-1 RT heterodimer. The other two chimeric RT heterodimers did not show significant differences with respect to the HIV-1 RT wt in this range.

HIV-1 RT p51 subunit plays an important role in the maintenance of an active RNase H conformation and the stability of the RT−DNA complex (*46*). We noted that in all the three intramolecular chimeras the reduction in relative RNase H activity was smaller compared to the reduction in DNA polymerase activities (Table 1). Particularly in the C3 RT chimera was RNase H activity reduced by 60%, whereas the polymerase activity was reduced by 98% for HIV-1 RT wt. These findings suggested that the RNase H activity was less affected than the DNA polymerase activity by these chimeric alterations introduced into the p51 subunit. This was further confirmed by comparing equal amounts of the three RT heterodimers in terms of polymerase activity based on poly(rA)/oligo(dT) and by determining the retained RNase H activity. The C3 RT had a 6-fold higher RNase H activity compared to an equal amount (in units) of HIV-1 RT wt, whereas the other two intramolecular RT chimeras were within the range of the wild-type enzyme under these conditions (Figure 3). As expected, the HIV-1 RT p66−p66 homodimer showed low RNase H activity.

The C3 Intramolecular RT Chimera Has a Reduced Processivity, while C1 and C2 Synthesize Longer Products. Next we compared the DNA polymerase activity on the single-primed M13 DNA template given the reductions in DNA polymerase activity already observed for the chimeric RT heterodimers. Product analysis experiments whose results are depicted in Figure 4A show that the C3 RT chimera makes mostly short products and very few longer ones compared to HIV-1 RT wt and the C1 RT and C2 RT chimeras (Figure 4A, compare lane 7 to lanes 4−6). The C3 RT also displayed additional pausing sites compared to

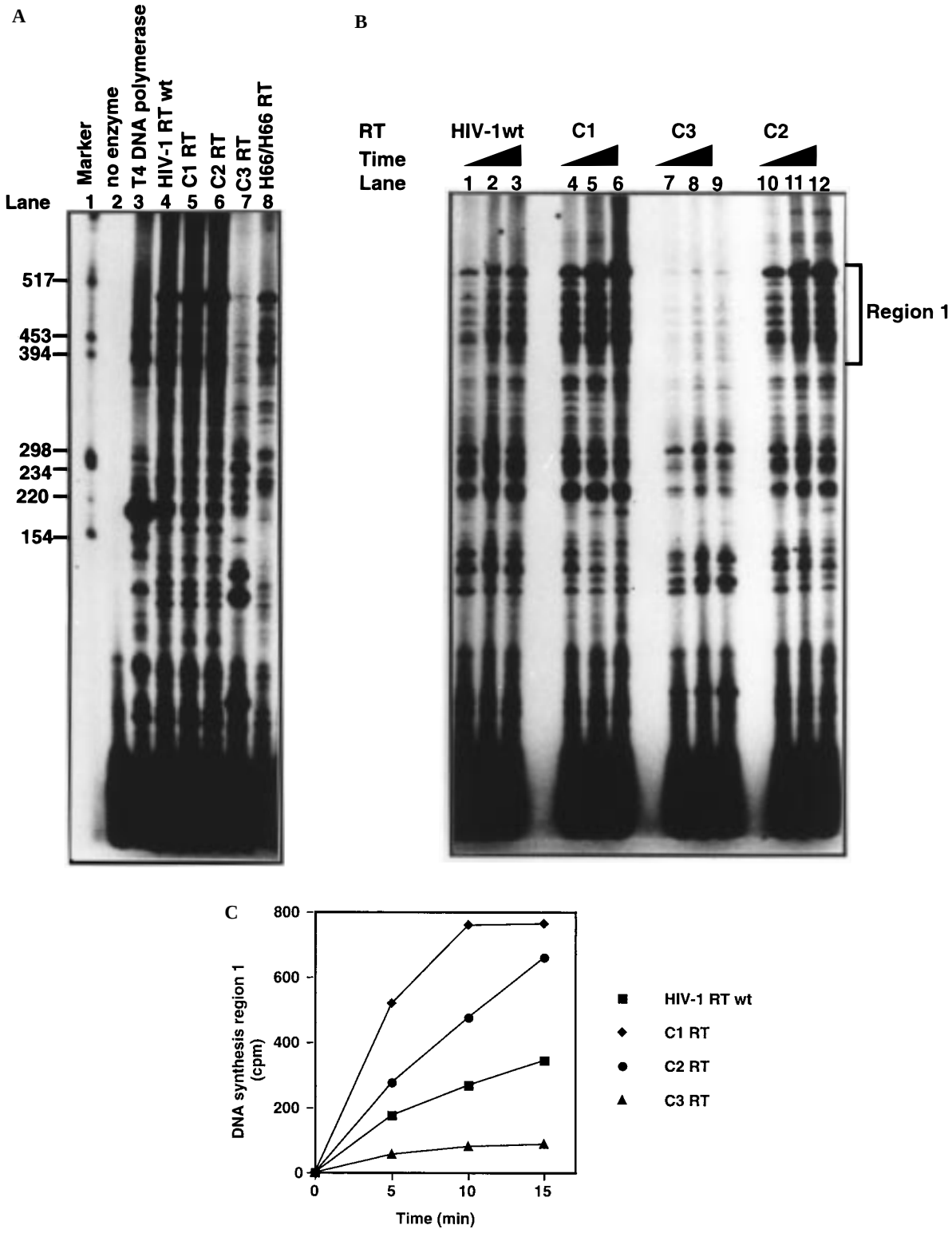

FIGURE 4: Product analysis of intramolecular chimeric RT heterodimers on single-primed M13 DNA. Equal amounts of each RT heterodimer (0.25 unit) were incubated with 0.5 μg of primed labeled M13 DNA template in the presence of 1 μg of unlabeled poly(rA)/oligo(dT) as described in Materials and Methods. Products were separated on a 6% urea−polyacrylamide gel. (A) Product analysis. The reaction mixtures were incubated for 30 min as described in Materials and Methods: lane 1, DNA molecular weight marker; lane 2, no enzyme; lane 3, T4 DNA polymerase; lane 4, HIV-1 RT wt; lane 5, C1 chimeric RT; lane 6, C2 chimeric RT; lane 7, C3 chimeric RT; and lane 8, HIV-1 RT p66−p66. (B) Kinetics of product analysis. The reaction mixtures were incubated for 5, 10, and 15 min as described in Materials and Methods: lanes 1−3, HIV-1 RT wt; lanes 4−6, C1 chimeric RT; lanes 7−9, C3 chimeric RT; and lanes 10−12, C2 chimeric RT. (C) PhosphorImager quantification of the data shown in panel B. Region 1 was quantified and plotted against time for each RT heterodimer (region 1 corresponds to DNA synthesis products ranging from about 300 to 517 bp; see also panel A for reference).

the other RT heterodimers. These results were further analyzed by performing kinetic experiments (Figure 4B). The C1 RT and C2 RT chimeras synthesized longer products on a primed M13 DNA template compared to HIV-1 RT wt. This is indicated by an accumulation of more longer products compared to those with the HIV-1 RT wt. These differences

were more pronounced after DNA synthesis for only 5 min (Figure 4B, compare lanes 1, 4, and 10). Finally, quantitation of these kinetic product analysis results (Figure 4C) confirmed the differences of the chimeric RT heterodimers observed in the autoradiogram in Figure 4B.

The C2 and C3 Intramolecular RT Chimeras Synthesize Shorter Strand Displacement DNA Synthesis Products Than the Natural HIV-1 RT Heterodimer. Strand displacement DNA synthesis of the RT enzymes is required in later stages during retroviral replication for complete replication of the viral genome. This property is influenced by the p51 subunit in both HIV-1 RT (*6*) and FIV RT (*7*). We therefore tested if any of the three regions of HIV-1 RT p51 have an influence on the strand displacement DNA synthesis activity of the HIV-1 RT heterodimer. The results showed that all three chimeric RT heterodimers retain strand displacement DNA synthesis activity (Figure 5A). However, despite some similarity in the DNA synthesis and product pattern, the C2 RT and C3 RT heterodimers appear to have difficulties in strand displacement and synthesis over an early pause site in the template, thus leading to an accumulation of short strand displacement products. This suggests a contribution by some regions in both the N and C termini of HIV-1 RT p51 toward an optimal strand displacement activity conformation of the HIV-1 RT heterodimer. A 50% reduction in the extent of relative strand displacement DNA synthesis by C3 RT was observed, while C1 RT and C2 RT showed an only 15% reduction compared to HIV-1 RT wt (Figure 5B). As expected, the HIV-1 RT p66−p66 homodimer has reduced strand displacement DNA synthesis activity and processivity as compared to the RT heterodimers (*6*).

The Intramolecular Chimeric RT Heterodimers Behave Normally in the Presence of the Nucleoside Inhibitor AZTTP and the Non-Nucleoside Inhibitor Nevirapine. Our earlier work with interspecies HIV-1−FIV RT chimeras indicated a possible role for the HIV-1 p51 in RT sensitivity to nucleoside and non-nucleoside RT inhibitors (*32*). Furthermore, it has also been shown that some particular p51 specific mutations confer resistance to certain RT inhibitors, such as the E138K mutation that confers Nevirapine and TSAO resistance (*47*−*49*) and the I135T mutation which has been suggested to be responsible for conferring dideoxyinosine (ddI) resistance (*50*). As shown for both RT inhibitors (Figure 6), the RT heterodimers display sensitivities similar to that of the HIV-1 RT wt to both AZTTP and Nevirapine. Moreover, the K_i values for both RT inhibitors were not significantly different from that for HIV-1 RT wt (data not shown).

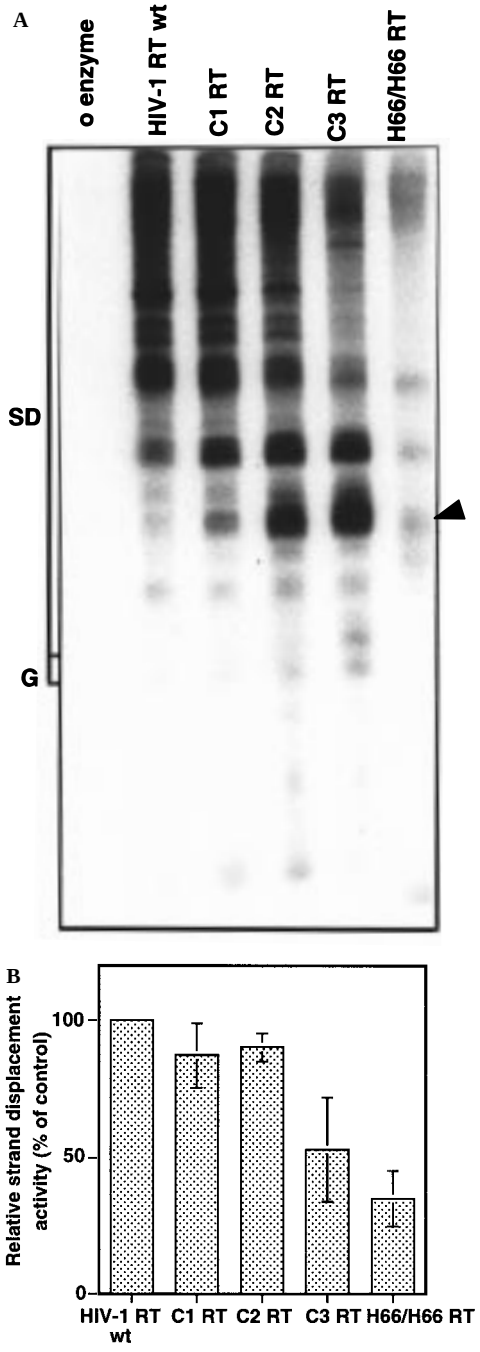

FIGURE 5: Strand displacement DNA synthesis by the RT heterodimers. (A) One-half of a unit of each heterodimer was used for the strand displacement DNA synthesis assay as described in Materials and Methods. After the reaction, the samples were separated by 7.5% urea−polyacrylamide gel electrophoresis. G indicates the position of gap filling as determined using DNA polymerase α (not shown); therefore, all products migrating higher than this band are derived from strand displacement DNA synthesis (SD): lane 1, no enzyme; lane 2, HIV-1 RT (wt); lane 3, C1 RT; lane 4, C2 RT; lane 5, C3 RT; and lane 6, p66−p66 HIV-1 RT. The arrow indicates the early pause site where C2 RT and C3 RT had difficulties in strand displacement DNA synthesis. (B) Strand displacement reactions were performed in triplicate, quantified by the PhosphorImager, averaged, and plotted as relative strand displacement activity of each form of RT in comparison to that of HIV-1 RT wt. Note that the C3 chimeric RT heterodimer displayed a significant decrease in the overall amount of strand displacement DNA synthesis activity as compared to the C1 and C2 chimeric RT heterodimers.

DISCUSSION

The functional role of the p51 subunit within the HIV-1 RT heterodimer is not clearly established. Recent findings suggested a possible role of the p51 subunit in the induction and maintenance of an optimal structure of the p66 subunit within the HIV-1 RT heterodimer, thereby influencing the catalytic activities of the HIV-1 RT heterodimer encoded by the p66 subunit (*32*). In a recent work, a similar role of HIV-1 RT p51 in p66 DNA polymerase active site structure induction has also been suggested (*31*). To study the HIV-1 RT p51 role further and to gain insight into the p66 structure-determining regions of the HIV-1 RT p51 subunit, intra-

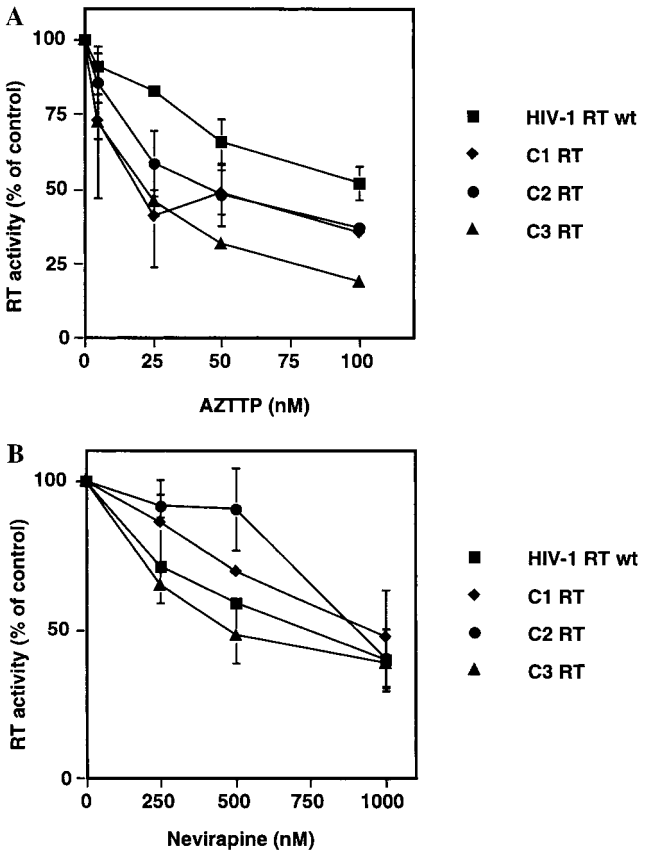

FIGURE 6: Effect of AZTTP and Nevirapine on the intramolecular chimeric RT heterodimers. The three intramolecular RT heterodimers and the HIV-1 RT wt were incubated with increasing concentrations of AZTTP (A) or Nevirapine (B). Residual RNA-dependent DNA polymerase activity was determined and expressed as a percentage of the control polymerase activity in the absence of the drug.

molecular chimeric RT p51 subunits between HIV-1 and FIV RT enzymes were constructed and functionally tested. On the basis of the crystallized structures of HIV-1 RT (*18*, *20*, *43*, *44*) and the primary amino acid sequences of HIV-1 and FIV RT p51 subunits, three defined regions in HIV-1 RT p51 were replaced with the corresponding regions from FIV RT p51. The chimeric p51 subunits when reconstituted with HIV-1 RT p66 resulted in functional RT heterodimers (Table 1). The three intramolecular chimeric HIV-1−FIV RT heterodimers called C1−C3 RTs displayed biochemical differences with respect to the wild-type HIV-1 RT heterodimer in the following three activities.

(i) DNA-Dependent DNA Synthesis. There was a reduction in DNA polymerase activity in all three intramolecular chimeric RT heterodimers compared to that in natural HIV-1 RT (Table 1), suggesting an influence of the p51 subunit on maintenance of the optimal DNA polymerase activity within the natural heterodimer. The C-terminal (Thr^{418}−Phe^{440}) C3 RT chimera seems to be the most severely affected, since it displayed the lowest RNA- and DNA-dependent DNA polymerase activities and a reduced product length on primed M13 DNA. There was additional pausing on M13 DNA with the C3 RT chimera as compared to the other two chimeric RT heterodimers (C1 and C2) and the wild-type HIV-1 RT. The C1 and C2 RT chimeras had a DNA synthesis pattern similar to that of HIV-1 RT wt but synthesized longer products on the primed M13 DNA template.

(ii) RNase H. There was also a reduction in RNase H activity in all three intramolecular RT chimeras, but the magnitude of reduction was not the same as that for DNA polymerase activity. This difference in magnitude of reduction in the two activities was most apparent with the C-terminal C3 RT chimera, upon comparison of RNase H activity between equal amounts of the RTs based on their polymerase activity as defined on poly(rA)/oligo(dT). The same amount of C3 RT activity displayed a 6-fold higher RNase H activity as compared to an equal amount of natural HIV-1 RT (Figure 3), whereas the C1 and C2 RT chimeras were within the same range as the HIV-1 RT wt.

(iii) Strand Displacement DNA Synthesis. The N-terminal (Ile^{1}−Pro^{112}) C2 and the C-terminal C3 RT chimeras had some difficulties in strand displacement DNA synthesis with frequent pausing at an early pause site in the template, thus accumulating mainly short strand displacement products. Moreover, the C3 RT had a 50% reduction in the overall amount of strand displacement DNA synthesis. These findings suggest significant contributions of both the N and C termini of the HIV-1 RT p51 subunit to the structural and functional conformation of the p66 subunit and hence activity of the HIV-1 RT heterodimer. The p51 and p66 subunits interact mainly via their connection domains and also between the p51 fingers and thumb domains and the p66 palm and RNase H domains, respectively (*18*, *20*). We speculate that it is through these interactions that the p51 subunit may be able to maintain optimal functional conformations within the p66 subunit in the HIV-1 RT heterodimer. There seem to be significant contributions coming from regions in both the N and C termini of HIV-1 RT p51 to the maintenance of optimal conformation in both the DNA polymerase and RNase H catalytic sites of the p66 subunit. The replacement of regions in these termini of HIV-1 RT p51 with the corresponding regions from FIV RT p51 may have led to the adoption of some different conformations in the intramolecular chimeric p51 subunits. This in turn may have led to the induction and adoption of different conformations in the p66 subunit as compared to the conformation in the wild-type HIV-1 RT, the result of such conformational alterations being the mentioned reductions in both DNA polymerase and RNase H activities. The most severe effects were observed when a region in the HIV-1 RT p51 C terminus was replaced. The C-terminal C3 RT chimera retained only 2% of the DNA polymerase and 30% of the RNase H activity compared to HIV-1 RT wt. This might be expected since the exchanged region in the C terminus of the p51 subunit is within the connection domain which constitutes the major part of the physical interaction surfaces between p51 and p66 in the HIV-1 RT heterodimer (*18*−*20*, *43*, *44*). Several groups have shown a contribution of this region of p51 to modulation of RNase H activity (*27*, *46*), RT heterodimer reconstitution and retention of DNA polymerase and RNase H activities (*27*), and tRNA primer binding (*18*, *27*, *29*). Our findings with the C3 RT in this study confirm the involvement of the HIV-1 RT p51 C terminus in the modulation of both RNase H and DNA polymerase activities. The extensive interactions between the RNase H domain of p66 and the p51 thumb subdomain contribute to a greater stability of the RT heterodimer (*18*, *51*−*53*). Alterations in the structure of this part of the HIV-1 RT p51 subunit in the C3 RT will also likely result in a

conformational change being induced in the p66 structure. K_i determinations for these RT heterodimers also show some changes in the interaction between the chimeric RT enzymes and the nucleoside analogue ddTTP. The C2 and C3 RTs did not display significant alterations in their interaction with ddTTP with only slight reductions in their K_i values, whereas the C1 RT chimera displayed a K_i 14 times higher than that HIV-1 RT wt. This points toward induction of a different conformation at the polymerase active site in the C1 RT chimera leading to an increased sensitivity to ddTTP.

The DNA polymerase and RNase H domains although distinct in their domains are nevertheless still functionally interdependent as shown for example by some amino acid altering mutations such as substitutions or insertions in either of the two domains or connection domain which affected both activities of the RT heterodimer (*54*−*58*). Similarly, structural alterations in the HIV-1 RT p51 subunit structure induced conformational changes in the p66 subunit, altering both the RNase H and the DNA polymerase functions of the RT heterodimer. The C3 RT chimera had a clearly reduced product length on primed M13 DNA. We assume that an altered structural conformational led to a decrease in the strength of the association of the enzyme and the TP leading to an increased tendency of the C3 RT to fall off more frequently after DNA synthesis for only a short distance. An alternative possibility would be the occurrence of some alterations in dimer formation leading to geometrical conformational changes at both active sites which could alter substrate binding of the RT or alter TP positioning at both active sites of the enzyme. In this case, the DNA polymerase active site may be poor in substrate binding, whereas in the RNase H active site, substrate interaction was not affected to the same extent.

Interestingly, both replacements of the regions in the N terminus of the HIV-1 RT p51 subunit resulted in longer DNA products on primed M13 DNA. This might be due to changes in conformation introduced via interactions between the loops in the palm (p66) and tips of the fingers (p51) subdomains, resulting in a DNA polymerase active site conformation with a firmer grip of the TP in the C1 and C2 RT chimeras. Finally, alterations in RNase H activities in these RT chimeras (C1 and C2) could also possibly be due to accompanying changes in TP positioning.

The HIV-1 RT p51 subunit can enhance the strand displacement DNA synthesis activity of the p66 subunit within the RT heterodimer (*6*). The putative strand displacement region has not yet been identified but might include the active site with the YMDD motif (*7*) and possibly the non-nucleoside inhibitor binding site of the HIV-1 RT heterodimer.[2] The results on strand displacement DNA synthesis show that both the N and C termini of the p51 HIV-1 RT subunit contribute to strand displacement DNA synthesis. The finger subdomain of the HIV-1 RT p51 subunit, the $\beta7/\beta8$ loop, forms part of the non-nucleoside inhibitor binding pocket (*47*), and from recent work, this region of HIV-1 RT p51 has been suggested also to be responsible for inducing the opening of the DNA polymerase cleft in the p66 subunit within the RT heterodimer, thereby enabling the RT to be loaded onto the template primer (*31*).

[2] M. Amacker and U. Hübscher, unpublished data.

The three regions of the p51 subunit tested in this work did not seem to have significant conformational or direct influence on the interaction of the HIV-1 RT with both nucloeside (AZTTP) and non-nucleoside (Nevirapine) RT inhibitors.

In conclusion, our presented data as well as those recently published (*32*) suggest that there are overall structural differences between the HIV-1 and FIV RT p51 subunits. By exchanging regions of HIV-1 RT p51 with FIV RT p51, we likely introduced structural alterations into the HIV-1 RT p51 subunit. Upon heterodimer reconstitution by the intramolecular chimeric p51 subunit with the HIV-1 RT p66 subunit, a change in structural conformation is thus possibly induced in the p66 subunit and hence the overall conformation of the HIV-1 RT heterodimer changed. These p51-induced conformational changes might be responsible for the differences in characteristics exhibited between the chimeric RT heterodimers and the HIV-1 RT wt heterodimer.

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