Functional Characterization of Chimeric Reverse Transcriptases with Polypeptide Subunits of Highly Divergent HIV-1 Group M and O Strains*

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Human immunodeficiency virus (HIV)-1 strains have been divided into three groups: main (M), outlier (O), and non-M non-O (N). Biochemical analyses of HIV-1 reverse transcriptase (RT) have been performed predominantly with enzymes derived from HIV-1 group M subtype B laboratory strains. This study was designed to optimize the expression and to characterize the enzymatic properties of HIV-1 group O RTs as well as chimeric RTs composed of group M and O p66 and p51 subunits. The DNA-dependent DNA polymerase activity on a short heteropolymeric template-primer was similar with all enzymes, i.e. the HIV-1 group O and M and chimeric RTs. Our data revealed that the 51-kDa subunit in the chimeric heterodimer p66MB/p51O confers increased heterodimer stability and partial resistance to non-nucleoside RT inhibitors. Chimeric RTs (p66MB/p51 and p66/p51MB) were unable to initiate reverse transcription from tRNA3m3T using HIV-1 group O or group M subtype B RNA templates. In contrast, HIV-1 group O and M RTs supported (−)-strand DNA synthesis from tRNA3m3T hybridized to any of their corresponding HIV-1 RNA templates. HIV-2 RT could not initiate reverse transcription on tRNA3m3T-prime HIV-1 genomic RNA. These findings suggest that the initiation event is conserved between HIV-1 groups, but not HIV types.

Synthesis of double-stranded DNA from the retroviral RNA genome is an essential step for replication of human immunodeficiency virus (HIV)1-1. This step is catalyzed by the viral reverse transcriptase (RT), a heterodimer composed of a 66-kDa (p66) and a 51-kDa (p51) subunit. Subdomains within each subunit are termed “fingers,” “palm,” “thumb,” and “connection” based on x-ray crystal structure and standard subdomain classification in various polymerases (1, 2). The heterodimer is generated after the viral protease cleaves one of the RNase H domains of an asymmetric p66/p66 homodimer (3). The DNA-binding cleft and polymerase active-site residues are exposed in the “open” conformation of p66. On the other hand, the p51 subunit is closely folded, with its catalytic residues occupying an internal position in the molecule (1, 4–6). In addition to providing structural support to the p66 subunit, p51 appears to stabilize the heterodimer (16). However, the molecular determinants of heterodimer stability are not known. Single-amino acid substitutions in HIV-1 RT, such as W229A, G231A, L234A, and L289K, have been shown to inhibit heterodimer association (17–19). These substitutions mediate their effects through the 66-kDa subunit, but were not found at the dimer interface of p66 and p51, suggesting an indirect conformational effect on dimer stability. Further confirmation of the role of Leu-234 in dimerization was obtained using the yeast two-hybrid system (20). Using this approach, it appears that the fingers and palm subdomains of p66 are dispensable for p51 interaction; DTT, diithiothreitol; TSAO, 2-[2′,5′-bis-(tert-butylidemethylsilyl)-β-D-ribofuranosyl]-3′-spiro-5′-(4′-amino-1′,2′-oxathiole-2′,2′-dioxide); TSAO-T, 1′-[2′,5′-bis-(tert-butylidemethylsilyl)-β-D-ribofuranosyl]thymine]-3′-spiro-5′-(4′-amino-1′,2′-oxathiole-2′,2′-dioxide); TSAO-mT, 1′-[2′,5′-bis-(tert-butylidemethylsilyl)-β-D-ribofuranosyl]N′-methylthymine]-3′-spiro-5′-(4′-amino-1′,2′-oxathiole-2′,2′-dioxide); U5, unique 5′; pbs, primer-binding site; FIV, feline immunodeficiency virus; TIBO, tetrahydroimidazo[4,5-1j][1,4]benzodiazepin-2-thione.
interaction. Deletion of 25 or 26 amino acids at the C terminus of p51 prevented dimerization, as demonstrated in reconstitution experiments with wild-type p66 and using the two-hybrid system (8, 20).

Phylogenetic analysis of HIV-1 sequences obtained throughout the world has identified three genetically distinct groups designated as major (M), outlier (O), and non-M non-O (N). Nucleotide sequence diversity between these groups can be as high as 35% in the envelope-coding region. This is considerably more than the 10–20% sequence diversity separating HIV-1 group M subtypes (A–J), but comparable to the 40–50% nucleotide sequence diversity separating HIV-1 and HIV-2. With the exception of some sporadic infections in Western Europe and the United States, the prevalence of the highly divergent, rare HIV-1 group O is greatest in central Africa (i.e. Cameroon, Gabon, and Nigeria) (reviewed in Ref. 21). In a preliminary study, we reported on the expression and characterization of an HIV-1 group O RT (ESP1 isolate) (22). ESP1 RT shares 79% amino acid sequence identity with the RT of group M:subtype B strain BH10. The two enzymes show very similar kinetics of DNA polymerization and RNase H activity, and both are equally sensitive to nucleoside analog inhibitors such as dideoxymidine triphosphate and 3'-azido-2',3'-dideoxythymidine triphosphate. ESP1 RT is resistant to NNRTIs, in agreement with other reports showing that most of HIV-1 group O isolates are naturally resistant to NNRTIs (i.e. nevirapine, delavirdine, and the TIBO derivative R82913) (23). The presence of Cys-181 in the group O RT-coding region, instead of the Tyr-181 found in sensitive group M:subtype B (M:B) strains, appears to confer NNRTI resistance (22, 23). Unfortunately, detailed studies on the enzymatic activity of HIV-1 ESP1 and related M:B/O RT chimeras was hampered by the poor level of HIV-1 ESP1 group O subunit expression, reconstitution, and purification. In this study, we initially screened for optimum *Escherichia coli* expression of the group O p66 subunit using 66 group O RT variants derived from quasi-species of six different isolates (24). The p66 subunits of group O p66 are wild-type group M:subtype B (p66M:B) RTs were then reconstituted and purified with either p51O or p51M:B subunits to compare DNA polymerase activity, initiation of (−)-strand DNA synthesis, NNRTI sensitivity, and heterodimer stability. In general, chimeric heterodimers (i.e. p66M:B/p51O and p66M:p51M:B or p66O/p51M:B) were indistinguishable from the previously characterized HIV-1 ESP1 O clones (expressing p66) were grown overnight at 37 °C in Luria broth medium containing 50 μg/ml ampicillin. Approximately 150 μl of grown cultures were used to inoculate 3 ml of freshly prepared medium. Isopropyl-β-D-thiogalactopyranoside (0.8 mM) was added when the culture reached an absorbance of 0.8–1.0 at 600 nm. Following a 1-h incubation, cells were harvested, and pellets were washed with 400 μl of 25 mM Tris-HCl (pH 8.0) containing 10 mM EDTA and 50 mM glucose and then resuspended in 400 μl of lysis buffer (10 mM Tris-HCl (pH 8.0) containing 0.3–0.5 M EDTA. Samples were incubated for 30 min at 37 °C, and reactions were stopped by adding 50 μl of 0.5 M EDTA. After acid-insoluble precipitation (25), samples were spotted on a Bio-Dyne A transfer membrane (Millipore Corp.), and the amount of incorporated 32P[GTP] was quantitated by phosphorimaging with a Fuji BAS 1500 scanner. The RT activity of each RT variant-producing clone was determined by subtracting the average RT activity of the backbone clones (bacteria containing the expression vector and pTrcHisB) plasmid.

Bacterial clones (*E. coli* DH5α) containing the plasmid pTrcHisB group O clones were transformed with the pRT6 group O plasmid. The pRT6 plasmid was created by using p66 group O RT variant sequences amplified by the polymerase chain reaction using oligo(dG)12–18 and [γ-32P]dGTP as substrates. Numbers indicate different clones obtained from three patients infected with HIV-1 group O. ESP1 represents the clone previously used for expression and purification of HIV-1 group O RT (22). C represents a control using bacteria bearing the plasmid pTrcHisB without the RT-coding sequence. B, SDS-polyacrylamide gel electrophoresis of purified RTs. C, Western blot analysis using the mouse monoclonal antibody 7C4, which recognizes a sequential epitope present in HIV-1 group M:subtype B RTs (34). The positions of the p66 and p51 subunits are shown on the right. B and C: lane 1, p66M:B/p51O; lane 2, p66M:p51M:B; lane 3, p66O/p51O; lane 4, p66O/p51M:B. Protein size markers (lane M) are indicated on the left on the SDS-polyacrylamide gel.

**EXPERIMENTAL PROCEDURES**

**Cloning and Expression of HIV-1 Group O RT Variants**—Sixty-six bacterial clones expressing RT variants were available from a previous study and were obtained from three untreated HIV-1 group O-infected individuals living in Spain (24). Genomic regions encoding the 66-kDa subunit of the RT were PCR-amplified from patient samples, cut with the NcoI and EcoRI restriction enzymes, and then cloned into the pRT6 or pTrcHisB (Invitrogen) expression vector (22, 24). The pRT6 clones were used for large-scale purification of p66, whereas constructs made with pTrcHisB were used for screening the expression levels of RT variants. The RT clones were derived from blood samples obtained from patient ESP1 in January 1996 (clones 1–4 and 21–24), April 1996 (clones 14–20), and August 1996 (clones 25–28 and 55–66; from patient ESP2 in January 1996 (clones 10–13 and 36–54) and May 1996 (clones 5–9); and from patient ESP3 in January 1996 (clones 29–35) (24). To obtain an expression vector for p51 with the histidine tag for reconstitution and purification, the coding region of the 51-kDa subunit of HIV-1 RT was cloned in the expression vector pTrcHisB. Briefly, primers RTO7 and RTO8 (22) were used to PCR-amplify the p51-coding region from the pRT6-derived plasmid (see above) and to introduce the NheI and EcoRI endonuclease restriction sites for subsequent cloning of the p51 region into the pTrcHisB plasmid.

Bacterial clones (*E. coli* DH5α) transformed with the pTrcHisB group O clones (expressing p66) were grown overnight at 37 °C in Luria broth medium containing 50 μg/ml ampicillin. Approximately 150 μl of grown cultures were used to inoculate 3 ml of freshly prepared medium. Isopropyl-β-D-thiogalactopyranoside (0.8 mM) was added when the culture reached an absorbance of 0.8–1.0 at 600 nm. Following a 1-h incubation, cells were harvested, and pellets were washed with 400 μl of 25 mM Tris-HCl (pH 8.0) containing 10 mM EDTA and 50 mM glucose and then resuspended in 400 μl of lysis buffer (10 mM Tris-HCl (pH 8.0) containing 0.01 M EDTA, 5 mM dithiothreitol (DTT), and 5% Triton X-100). Aliquots of this solution were used for SDS-polyacrylamide gel electrophoresis and for the determination of RT activity.

**Analysis of RT Activity in Crude Bacterial Lysates**—DNA polymerization assays were carried out by mixing 20 μl of the obtained lysate and 80 μl of 12.5 mM Tris-HCl (pH 8.0) containing 25 mM NaCl, 12.5 mM MgCl2, 2 mM DTT, 0.5 μg/ml polystyrene oligo(dG)12–18, and 10 μM [α-32P]dGTP. The specific activity of the nucleoside triphosphate used in the assays was 0.3–0.5 μCi/μmol. Samples were incubated for 30 min at 37 °C, and reactions were stopped by adding 50 μl of 0.5 M EDTA. After acid-insoluble precipitation (25), samples were spotted on a Bio-Dyne A transfer membrane (Millipore Corp.), and the amount of incorporated [32P]GTP was quantitated by phosphorimaging with a Fuji BAS 1500 scanner. The RT activity of each RT variant-producing clone was determined by subtracting the average RT activity of the background clones (bacteria containing the expression vector without the HIV-1 RT-coding region). The polymerase activity of each clone was then compared with that of the previously characterized HIV-1 ESP1 group O RT clone (22).
Characterization of Chimeric HIV-1 Reverse Transcriptases

TABLE I

| Enzymes | Km (nM) | km (nM) | kcat/Km (µM⁻¹·s⁻¹) |
|---------|---------|---------|----------------------|
| p66²M/p51³O RT | 2.61 ± 0.09 | 144.2 ± 21.3 | 301.7 |
| p66²M/p51¹O RT | 1.62 ± 0.07 | 98.0 ± 21.6 | 275.5 |
| p66²M/p51²B RT | 1.12 ± 0.04 | 174.6 ± 23.5 | 106.9 |
| p66²M/p51¹B RT | 2.54 ± 0.08 | 170.9 ± 22.9 | 247.7 |

Expression and Purification of HIV-1 RT—Purification of HIV-1 RT variants was carried out after independent expression of each subunit, i.e., p66 and p51 (26). The 51-kDa subunit contained an extension of 14 amino acid residues at its N terminus, which included six consecutive histidine residues to facilitate purification by metal chelate affinity chromatography. The purification procedure starts by harvesting the cells from cultures expressing p66 together with those from cultures expressing p51. This process allows for the reconstitution of chimeric heterodimers, i.e., the 66-kDa subunit of the BH10 clone (a subtype B HIV-1) with the 51-kDa subunit of an HIV-1 group O isolate, or vice versa. Purity of enzymes was assessed by SDS-polyacrylamide gel electrophoresis. RT concentrations were determined using the Bio-Rad protein assay. The specific activity of the purified enzyme was estimated in DNA polymerase assays carried out in 50 µl of 50 mM Tris-HCl (pH 8.0) containing 20 mM NaCl, 10 mM MgCl₂, 8 mM DTT, 3 µM dUTP, 5 µM dTTTP, and 0.5 µM poly(rA)/oligo(dT)₁₂₋₁₈ (22, 27). One activity unit is the amount of enzyme that incorporates 1 nmol of [3H]dTMP into acid-insoluble products in 10 min at 37 °C. HIV-2 heterodimeric RT was kindly provided by Drs. Roger Goody and Brigitta Wöhrl (Max-Planck-Institut für Molekulare Physiologie, Dortmund, Germany).

Kinetic Studies—Single-nucleotide incorporation assays were performed with template-primer D2-47/PG5-25 as previously described (27, 28). Briefly, primer PG5-25 (5′-CCGATATCCTGATGATCCGGTACTATACT-3′) was 5′-end-labeled with γ-[32P]ATP and T4 polynucleotide kinase (Promega) and then annealed to template D2-47 (5′-GGATATCCTGATGATCCGGTACTATACT-3′) in a 150 mM NaCl and 150 mM magnesium acetate at a molar ratio of 1:1. Assays were carried out at 37 °C in 35 µl of 50 mM HEPES (pH 7.6), 150 mM NaCl, 15 mM magnesium acetate, 130 mM potassium acetate, 1 mM DTT, and 5% polyethylene glycol 6000. The template-primer concentration was 30 nM, and the active enzyme concentration was 3–5 nM. After a 10-min incubation of enzyme with template-primer, reactions were initiated by the addition of dTTP at various concentrations and then stopped after 20 s at 37 °C by adding 8 µl of 10 mM EDTA in loading buffer containing 90% formamide. The products were analyzed on a 20% polyacrylamide gel and quantitated by phosphorimaging (25). Elongation measurements were fitted to the Michaelis-Menten equation to determine the corresponding kcat and Km values.

Inhibitors and RT Inhibition Assays—The synthesis of 5′-bis-O-(tert-butylmethyl)thymidine (5′-biO-TMP) and 3′-3′-bis-O-(tert-butylmethyl)thymidine (5′-biO-TMP) has been previously described (29–31). Nevirapine was obtained from Boehringer Ingelheim. RT inhibition assays were carried out with poly(A)•oligo(dT)₁₂₋₁₈ or D2-47/PG5-25 under the conditions described for kinetic studies. In assays using poly(A)•oligo(dT)₁₂₋₁₈ incorporation of radioactive dTMP was measured in the presence of increasing amounts of nevirapine. In single-nucleotide extension assays, the RT was preincubated with the inhibitor at 37 °C for 10 min before the addition of the D2-47/PG5-25 template-primer. The inhibition constants (Ki) for both assays and with each RT were determined from the kinetic parameters obtained using various concentrations of NNRTI. Nevirapine as well as T30A derivatives was serially diluted from an original 10 mM stock in dimethyl sulfoxide, but dimethyl sulfoxide concentrations in all RT assays remained below 2%.

Stability of the RT Heterodimer in the Presence of Urea—Denaturation isotherms were obtained by preincubating RT with different concentrations of urea (up to 4 M) for 10 min at 37 °C in 10 µl of 50 mM HEPES (pH 7.0) containing 15 mM NaCl, 15 mM magnesium aspartate, 130 mM potassium acetate, 1 mM DTT, and 5% polyethylene glycol 6000. The polymerase reaction was initiated by adding the D2-47/PG5-25 template-primer and dTTP in a 10-µl solution of 100 mM HEPES (pH 7.0), 30 mM NaCl, 30 mM magnesium aspartate, 130 mM potassium acetate, 1 mM DTT, and 5% polyethylene glycol 6000. The final concentrations of template-primer and dTTP in these assays were 30 nM and 50 µM, respectively. After incubating the samples for 30 s at 37 °C, reactions were stopped with EDTA/formamide, and products were analyzed as described above. The obtained amount of primer extension products was plotted versus the urea concentration, and the data were fitted to a logistic curve using the program CurveExpert Version 1.34 (Microsoft Corp.) to determine the concentration of urea at the midpoint of the denaturation isotherm.

Cloning of the HIV-1 Group O Primer-binding Site RNA Expression Vector—Proviral DNA was extracted directly from lysed peripheral blood mononuclear cells (24). A 409-base pair long terminal repeat fragment spanning the repeat unique region (U5), and primer-binding site (pbs) regions was PCR-amplified using a set of nested oligonucleotides primers. Briefly, primers 5′-3′-CCTAATTTTGTCATCCATCC-3′ and 5′-3′-AATTTAATTATTATATGTCATC-3′ were used in the first external amplification. Products (5 µl) of the external amplification were reamplified as previously described (24) using nested primers 5′-3′-CCTAATTTTGTCATCCATCC-3′ and 3′-3′-AATTTAATTATTATATGTCATC-3′, which include BglII and XhoI endonuclease restriction sites (underlined), respectively. Nested PCR products were purified (QIAquick PCR purification kit, QIAGEN Inc.), digested with BglII and XhoI, and then cloned into the pSP72 vector (Promega), cut with the same enzymes. The long terminal repeat clone used in the experiments described below was sequenced using the ABI Prism BigDye terminator cycle sequencing ready reaction kit (Perkin-Elmer Life Sciences).

RNA-dependent DNA Synthesis of (−) Strand Strong-stop DNA—RNA-dependent DNA polymerase activity of wild-type and chimeric RTs was measured using either a DNA or a RNA primer annealed to HIV-1 group O or group M subtype B RNA templates containing the pbs, U5, and repeat regions (HIV-1, pbs RNA and HIV-1-M RNA, respectively). In addition to these RNA templates, primers were also

Fig. 2. Inhibition of heterodimeric RTs by nevirapine. Assays were carried out with (polyrA/oligo(dT)₁₂₋₁₈, and [³H]dTTP as substrates in the presence of increasing concentrations of the inhibitor. The amount of enzyme used in this assay was 0.3–0.6 pmol. Data obtained with p66²M/p51³B and p66²M/p51¹B RTs, respectively.

TABLE II

| Enzymes | Nevirapine [K] | TSAO-T [K] | TSAO-m³T [K] |
|---------|---------------|------------|---------------|
| p66²M/p51³B RT | 2.54 ± 0.83 | 17.2 ± 9.6 | 20.1 ± 11.9 |
| p66²M/p51¹O RT | 15.9 ± 6.6 | 71.0 ± 11.2 | 90.1 ± 13.0 |

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yields of HIV-1 group O RT, we analyzed the expression levels and RT activity of 66 group O RT variants derived from untreated HIV-1 group O-infected individuals (24). Five RT clones (designated 42, 44, 46, 49, and 54 and all derived from the January 1996 sample of patient ESP2) consistently displayed the highest level of RT activity in bacterial lysates containing the p66 subunit (Fig. 1). RNA-dependent DNA polymerase activity was determined using poly(rC)·poly(dG)12–18 and [α-32P]dGTP as substrates. All five RTs from this ESP2 sample had nearly identical amino acid sequence in the polymerase domain, and we selected clone 46 as the group O RT prototype in the biochemical studies described in this work. The GenBankTM/EBI Data Bank accession number of the ESP2-46 RT sequence is AF068947.

Although ESP2 (clone 46) RT shared 98.4% identity with the previously characterized ESP1 RT (22), the levels of RT activity in culture supernatants expressing the 66-kDa subunit of clone ESP2-46 were 10–20% higher than those of ESP1 (Fig. 1). The variations in amino acid sequence between the two group O RT clones (T196A, L228M, I276V, L372F, W398R, E415D, M435R, and N470D; amino acids in ESP1 versus ESP2-46) have not been reported as having major effects on DNA polymerase or RNase H activity. However, the increased RT activity of clone ESP2-46 RT over ESP1 RT in bacterial lysates was not related to RT expression or purification yields. The recovery of pure p66/p51O RT was quite similar for both clones. We did observe a relatively high yield (280 μg/liter) of the chimeric RT composed of the p66 subunit of ESP2-46 and the p51 subunit of the M:B laboratory strain BH10 (p66M:B/p51M:B). In contrast, 4–6-fold less of the chimeric p66M:B/p51O and natural p66M:B/p51M:B RTs was obtained from the same bacterial clones. All purified RTs were found to be at least 95% pure and to have the correct subunit composition (Fig. 1). It is important to note that all subsequent experiments were performed with natural or chimeric heterodimers containing subunits from the group O ESP2-46 clone or the group M subtype BH10 clone.

Functional Characterization of Chimeric RTs and Sensitivity to Non-nucleoside Inhibitors—All four recombinant heterodimers were active in RNA- and DNA-dependent DNA polymerase assays. The specific activity of each enzyme was determined using poly(rA)·poly(dT)12–18 and dTTP as substrates. The heterodimers p66M:B/p51O, p66M:B/p51M:B, and p66M:B/p51O showed 50, 107, and 80% of the specific activity observed with p66M:B/p51M:B (estimated at 1700 units/mg). It is important to note for subsequent experiments that this assay for specific activity measures both distributive and processive synthesis. The kinetic parameters for the incorporation of dTTP on a heteropolymeric DNA-DNA template-primer are shown in Table I. Values of $k_{cat}$ and $K_m$ were similar for all four enzymes, with $<3$-fold differences in their catalytic efficiencies.

Inhibition assays revealed that the recombinant p66M:B/p51O RT and the chimeric heterodimer p66M:B/p51M:B were resistant to nevirapine even at the highest concentration (250 μM), whereas the p66M:B/p51M:B enzyme was inhibited by nevirapine (IC50 = 3.0 μM). However, the chimeric heterodimer p66M:B/p51O was partially resistant to this inhibitor (IC50 = 19.7 μM) (Fig. 2). This resistance was observed regardless if poly(rA)·poly(dT)12–18 and dTTP or heteropolymeric template-primer and dNTPs were employed as substrates. Contribution of the group O p51 subunit to NNRTI resistance in the p66M:B/p51O chimera was also observed in single-nucleotide incorporation assays using the 47/25-mer template-primer (Table II). Inhibition constants for nevirapine and the TSAO derivatives TSAO-T and TSAO-m3T were 4–6 times higher for the p66M:B/p51O chimera than for p66M:B/p51M:B.

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Characterization of Chimeric HIV-1 Reverse Transcriptases

Fig. 4. Oligonucleotide DNA- and tRNA\textsubscript{3Lys}-primed HIV-1 (–) strand strong-stop DNA synthesis using group M and O and chimeric HIV-1 RTs. A \textsuperscript{32}P-5'-end-labeled 18-nucleotide DNA primer or an internally \textsuperscript{32}P-labeled unmodified 76-nucleotide tRNA\textsubscript{3Lys} primer was annealed to each of three HIV-1 RNA templates, i.e. HIV-1\textsubscript{M:B} PBS RNA (I), HIV-1\textsubscript{M:B} PBS-A RNA (II), and HIV-1\textsubscript{O} PBS RNA (III). The (–) strand strong-stop DNA products and the template-primer substrates are depicted in A. Autoradiograms showing (–) strand DNA synthesis catalyzed by HIV-1 group M RT (p66\textsuperscript{M:B}/p51\textsuperscript{M:B}), HIV-1 group O RT (p66\textsuperscript{O}/p51\textsuperscript{O}), and chimeric subunit RTs (p66\textsuperscript{M:B}/p51\textsuperscript{O} and p66\textsuperscript{O}/p51\textsuperscript{M:B}) from either a DNA or tRNA primer are shown in B and C, respectively. The amounts of (–) strand DNA products in B and C were quantitated using a PhosphorImager and are represented in D (panels I and II). The histogram in panel III shows, for each enzyme, the ratio of (–) strand DNA synthesis from the tRNA\textsubscript{3Lys} primer over the DNA primer. nt, nucleotides.

An 18-nucleotide DNA primer or unmodified tRNA\textsubscript{3Lys} was heat-annealed to RNA templates containing PBS, U5, and repeat regions of HIV-1 group M and O variants (see "Experimental Procedures" and Fig. 4A). The (–) strand DNA synthesis catalyzed by HIV-1 group M RT (p66\textsuperscript{M:B}/p51\textsuperscript{M:B}), HIV-1 group O RT (p66\textsuperscript{O}/p51\textsuperscript{O}), and chimeric subunit RTs (p66\textsuperscript{M:B}/p51\textsuperscript{O} and p66\textsuperscript{O}/p51\textsuperscript{M:B}) from either a DNA or tRNA primer are shown in B and C, respectively. The amounts of (–) strand DNA products in B and C were quantitated using a PhosphorImager and are represented in D (panels I and II). The histogram in panel III shows, for each enzyme, the ratio of (–) strand DNA synthesis from the tRNA\textsubscript{3Lys} primer over the DNA primer. nt, nucleotides.

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DISCUSSION

Most information on anti-retroviral drug efficacy has been obtained from infections or in vitro studies with HIV-1 group M:subtype B, the prevalent subtype in countries with access to anti-retroviral drugs. Recent studies show a fortuitous but understandable anti-retroviral inhibition of other group M, non-clade B HIV-1 isolates that currently circulate throughout the developing world (23, 24, 39). However, HIV-1 group O and HIV-2 isolates display natural resistance to nevirapine and other NNRTIs (22, 23, 40, 41). Nevertheless, both HIV-1 group

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O and HIV-2 can infect humans and cause AIDS, although the prevalence of those viruses is low and mostly restricted to West Africa. Structural analyses of the NNRTI-binding site of HIV-1 RT and genotypic analyses of nevirapine-resistant M:B isolates suggest that residues such as Cys-181 in the p66 subunit are likely to be responsible for the intrinsic HIV-1 group O resistance to NNRTIs. However, the contribution of the p51 subunit to NNRTI resistance by group O isolates has not been measured. In our study, HIV-1 group O RT heterodimers were resistant to nevirapine, TSAO-T, and TSAO-mT. Unlike nevirapine, TSAO derivatives have been shown to destabilize the p66/p51 HIV-1 RT heterodimer (16) through binding to regions in the p51 subunit involved in RT dimerization. Chimeric heterodimers consisting of the p66 subunit of HIV-1 subtype B (clone BH10) and the p51 subunit of group O (ESP2 clone 46) were partially resistant to nevirapine and TSAO derivatives. However, increases in inhibition constants were similar when the chimeric p66/p51 RT was used in the assay in the place of the homologous p66/p51 RT. This result implies that differences in amino acid sequence between the ESP2 and BH10 RTs may also encode NNRTI resistance through the ESP2 p51 subunit rather than solely through the ESP2 p66 subunit. The substitution of Lys for Glu-138 confers genotypic resistance to TSAO derivatives in group M:subtype B isolates (45). However, phenotypic resistance is restricted to the catalytically inactive p51 subunit in the heterodimer (46). The RT-coding region of HIV-1 group O does not encode Lys-138 or any other mutations conferring NNRTI resistance to the p51 subunit. Currently, this partial resistance to nevirapine and TSAO derivatives mediated by the group O p51 subunit in the p66/p51O heterodimer cannot be attributed to any specific amino acid residue.

Stability of the heterodimeric RTs was determined by comparing the DNA polymerase activities of enzymes pretreated with increasing concentrations of urea. Previous studies revealed that homologous HIV-2 RT heterodimers are more stable than those of HIV-1 RT upon exposure to organic solvents (14, 47). However, the contribution of each subunit to stability was not measured. In our study, HIV-1 group O RT heterodimers were significantly more stable than M:B RT heterodimers. Furthermore, our analysis of chimeric RTs predicts that the molecular determinants of greater heterodimer stability lie within the p51 subunit rather than in the catalytically active p66 subunit. The role of p51 in heterodimer stability is supported by (i) studies showing reduced stability of heterodimers containing p51 with C-terminal deletions (8, 20) and (ii) structural analyses showing a greater contribution of p51 to the dimer interface via an interaction between the p51 subunit (i.e. fingers, connection, and thumb subdomains) with a more...
Fig. 7. Structural analysis of contact surfaces between the p66 and p51 subunits of HIV-1 subtype B RT. A, the contribution of each residue of p66 or p51 to the contact surface between both subunits of the p66/p51 heterodimer was determined by measuring the loss of accessibility of each subunit upon its interaction with the other, using the program WHAT IF (33). The represented values correspond to the average obtained from 26 crystal structures of HIV-1 RT since unliganded RTs and RTs complexed with inhibitors or double-stranded DNA showed similar patterns of surface accessibility. B, shown is the sequence variation at positions of p66 and p51 with the highest accessibility. The residues indicated are those with an accessibility score higher than 10. Asterisks indicate amino acids with a score above 20. C, presented is a ribbon representation of the HIV-1 RT heterodimer showing amino acids clusters involved in p66/p51 interactions. Residues of p66 (white ribbon) and p51 (yellow ribbon) are shown in cyan and magenta, respectively, using a space-filling representation. The positions indicated correspond to residues with accessibility scores above 10. Coordinates have been obtained from Protein Data Bank code 1HMV (42).
limited region in the p66 subunit (i.e. palm subdomain, connection subdomain, and RNase H domain) (Fig. 7). The partial resistance to NNRTIs shown by the chimeric p66\textsubscript{M:B}/p51\textsubscript{O} RT suggests that intersubunit interactions favor a p51\textsuperscript{1}-induced conformation over a p66\textsubscript{M:B}-induced conformation in this chimeric heterodimer. The P51 subunit of HIV-1 group O RT may also cause a significant perturbation in the NNRTI-binding site of p66\textsubscript{M:B}/p51\textsubscript{O} RT, probably by altering interactions between the fingers subdomain of P51 and the palm subdomain of P66.

We have previously shown that initiation of reverse transcription in HIV-1 group M/subtype B can be significantly impinged by single-amino acid substitutions (48). Although natural group M/subtype B and group O RTs share only 79% sequence identity (22), both enzymes are able to catalyze (−)-strand DNA synthesis from a short oligonucleotide DNA and tRNA\textsubscript{3G}\textsuperscript{A}\textsubscript{A} annealed to either an HIV-1 group O or M:B-derived RNA template. In contrast, other lentiviral RTs, including HIV-2 and simian immunodeficiency virus heterodimeric RTs, cannot efficiently catalyze (−)-strand DNA synthesis from tRNA\textsubscript{3G}\textsuperscript{A}\textsubscript{A} annealed to HIV-1 RNA (37). Three lentiviral non-HIV-1 RTs can, however, (i) catalyze (−)-strand DNA synthesis from short oligonucleotide RNA or DNA primers and (ii) initiate reverse transcription from tRNA\textsubscript{3G}\textsuperscript{A}\textsubscript{A} annealed to FIT or equine infectious anemia virus RNA (37). The A-rich loop in the HIV-1 pbs RNA interacts with the U-rich tRNA\textsubscript{3G}\textsuperscript{A}\textsubscript{A} anti-codon loop, leading to a bimolecular complex that may block other non-HIV-1 lentiviruses from utilizing primer tRNA\textsubscript{3G}\textsuperscript{A}\textsubscript{A} annealed to HIV-1 pbs RNA (35, 36). A disruption of this tRNA loop–HIV-1 RNA loop complex by deleting the A-rich region in the HIV-1 genomic RNA (HIV-1\textsubscript{3G,4,6}} pbs\textsubscript{A} RNA) also permits tRNA\textsubscript{3G}\textsuperscript{A}\textsubscript{A} utilization by other lentiviral RTs. In this study, HIV-2 RT could initiate (−)-strand DNA synthesis only when tRNA\textsubscript{3G}\textsuperscript{A}\textsubscript{A} was annealed to an HIV-1 RNA template lacking an A-rich sequence upstream of the pbs. Computer predictions of HIV-1 group M/subtype B, HIV-1 group O, and HIV-2 pbs RNA secondary structures suggest that the A-rich sequence may be accessible to the tRNA anti-codon loop in both HIV-1 group M and O RNAs, but not in HIV-2 RNA. In addition, Arts et al. (48) demonstrated that three basic residues (i.e., Lys

Acknowledgments—We thank Joe Chiba for a generous gift of monoclonal antibody 7C4 and Esteban Domingo for support.

REFERENCES
1. Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1992) Science 256, 1780–1789
2. Joyce, C. M., and Steitz, T. A. (1995) J. Bacteriol. 177, 6321–6329
3. Di Marco Veronese, F., Copeland, T. D., DeVico, A. L., Rahman, R., Oroszlan, S., Gallo, R. C., and Sarranghadharan, M. G. (1986) Science 231, 1289–1291
4. Huang, H., Chopra, R., Verdine, G. L., and Le Grice, S. F. J. (1998) Science 282, 1669–1675
5. Jacobs-Molina, A., Ding, J., Nanni, R. G., Clark, A. D., Jr., Lu, X., Tantillo, C., Williams, R. L., Kramer, G., Ferris, A. L., Clark, P., Hunt, A., Hughes, S. H., and Arnold, E. (1997) J. Virol. 71, 4805–4812
6. Wang, J., Smerdon, S. J., Jager, J., Kohlstaedt, L. A., Rice, P. A., Friedman, J. M., and Steitz, T. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7242–7246
7. Harris, D., Lee, R., Miroz, H. S., Pandey, P. K., and Pandey, V. N. (1998) Biochemistry 37, 5903–5908
8. Jacobs, P. S., Wohlr, B. M., Howard, K. J., and Le Grice, S. F. J. (1993) J. Biol. Chem. 269, 13080–13083
9. Mishima, Y., and Steitz, J. A. (1995) EMBO J. 14, 2679–2687
10. Arts, E. J., Ghosh, M., Jacques, P. S., Ehrens, M., and Le Grice, S. F. J. (1996) J. Biol. Chem. 271, 10645–10661
11. Dufour, E., El Dinari-Dahil, G., Boumène, F., Fournier, M., Nevisky, G., Tarragon, L., Litvak, S., and Androsta, M.-L. (1998) Eur. J. Biochem. 251, 487–495
12. Huang, S. C., Smith, J. R., and Mosen, L. K. (1992) Biochemistry 31, 884–992
13. Hottiger, M., Podust, V. N., Thimmig, R. L., McHenry, C., and Hübser, U. (1994) J. Biol. Chem. 269, 886–991
14. Divita, G., Restle, T., and Goody, R. S. (1993) FEBS Lett. 324, 153–158
15. Divita, G., Restle, T., Goody, R. S., Chernmann, J.-C., and Baillon, J. G. (1994) J. Biol. Chem. 269, 10380–10383
16. Sluis-Cremer, N., Dmitrienko, G. I., Balzarini, J., Camarasa, M.-J., and Pari, M. A. (2000) Biochemistry 39, 1427–1433
17. Goel, R., Beard, W. A., Kumar, A., Casas-Finet, J. R., Strub, M.-P., Stahl, S. J., Lewis, M. S., Bebenek, K., Becerra, S. P., Kuncel, T. A., and Wilson, S. H. (1993) Biochemistry 32, 13012–13018
18. Ghosh, M., Jacques, P. S., Rodgers, D. W., Ottman, M., Darlix, J.-C., and Le Grice, S. F. J. (1996) Biochemistry 35, 8553–8562
19. Wohlr, B. M., Krebs, B., Thrall, S. H., Le Grice, S. F. J., Scheidig, A. J., and Goody, R. S. (1997) J. Biol. Chem. 272, 17581–17587
20. Tachdjian, G., Aronson, H.-E. G., and Goff, S. P. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6334–6339
21. Quiñones-Mateu, M. E., Ball, S. C., and Arts, E. J. (2000) AIDS Rev. 2, 190–202
22. Quiñones-Mateu, M. E., Soriano, V., Domingo, E., and Menéndez-Arias, L. (1997) Virology 236, 364–373
23. Descamps, D., Collin, G., Letourneur, F., Apetrei, C., Damond, F., Lousseurt-Ajak, I., Simon, F., Saragosti, S., and Brun-Vézinet, F. (1997) J. Virol. 71, 8893–8908
24. Quiñones-Mateu, M. E., Albright, J. L., Mas, A., Soriano, V., and Arts, E. J. (1998) J. Virol. 72, 9002–9015
25. Martín-Hernández, A. M., Gutierrez-Rivas, M., Domingo, E., and Menéndez-Arias, L. (1997) Nucleic Acids Res. 25, 1383–1389
26. Martín-Hernández, A. M., Domingo, E., and Menéndez-Arias, L. (1996) EMBO J. 15, 4434–4442
27. Menéndez-Arias, L. (1998) Biochemistry 37, 16636–16644
28. Mas, A., Parera, M., Briones, C., Soriano, V., Martínez, M. A., Domingo, E., and Menéndez-Arias, L. (2000) EMBO J. 19, 5752–5761
29. Camarasa, M. J., Pérez-Pérez, M. J., San-Félix, A., Balzarini, J., and De Clercq, E. (1992) J. Med. Chem. 35, 2721–2727
30. Pérez-Pérez, M. J., San-Félix, A., Camarasa, M. J., Balzarini, J., and De Clercq, E. (1993) Tetrahedron Lett. 33, 3029–3032
31. Pérez-Pérez, M. J., San-Félix, A., Balzarini, J., De Clercq, E., and Camarasa, M. J. (1992) J. Med. Chem. 35, 2988–2995
32. Arts, E. J., Li, X., Gu, Z., Kleiman, L., Parniak, M. A., and Wainberg, M. A. (1994) J. Biol. Chem. 269, 16571–16577
33. Vriend, G. (1990) J. Mol. Graph. 8, 52–56
34. Chiba, J., Yamaguchi, A., Suzuki, Y., Nakano, M., Zhu, W., Ohba, H., Saito, A., Shinagawa, H., Yamakawa, Y., Kobayashi, T., and Kurata, T. (1996) J. Gen. Virol. 77, 2921–2929
35. Isel, C., Marquet, R., Keith, G., Ehresmann, C., and Ehresmann, B. (1993) J. Biochem. 268, 25269–25272
36. Isel, C., Ehresmann, C., Keith, G., Ehresmann, B., and Marquet, R. (1995) J. Mol. Biol. 247, 236–250
37. Arts, E. J., Stetor, S. R., Li, X., Rausch, J. W., Howard, K. J., Ehresmann, B., North, T. W., Wührl, E. M., Goody, R. S., Wainberg, M. A., and Le Grice, S. F. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10063–10068
38. Zuker, M., Mathews, D. H., and Turner, D. H. (1999) in RNA Biochemistry and Bio/Technology (Barciszewski, J., and Clark, B. F. C., eds) NATO ASI Series Vol. 70, pp. 11–43, Kluwer Academic Publishers, Norwell, MA
39. Palmer, S., Alaeus, A., Albert, J., and Cox, S. (1998) AIDS Res. Hum. Retroviruses 14, 157–162
40. Howard, K. J., Frank, K. B., Sim, I. S., and Le Grice, S. F. J. (1991) J. Biol. Chem. 266, 23003–23009
41. Loya, S., Bakhanaashvili, M., Tal, R., Hughes, S. H., Boyer, P. L., and Hizi, A. (1994) AIDS Res. Hum. Retroviruses 10, 939–946
42. Rodgers, D. W., Gamblin, S. J., Harris, B. A., Ray, S., Culp, J. S., Hellmig, B., Woolf, D. J., Debouck, C., and Harrison, S. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1222–1226
43. Muller, B., Restle, T., Kühnel, H., and Goody, R. S. (1991) J. Biol. Chem. 266, 14705–14713
44. Amacker, M., and Hübscher, U. (1995) J. Mol. Biol. 278, 757–765
45. Balzarini, J., Karlsson, A., Vandamme, A.-M., Pérez-Pérez, M. J., Zhang, H., Vrang, L., Obergo, B., Backbro, K., Unge, T., San-Félix, A., Velazquez, S., Camarasa, M. J., and De Clercq, E. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6952–6956
46. Jonckheere, H., Taymans, J.-M., Balzarini, J., Velázquez, S., Camarasa, M. J., Desmyter, J., De Clercq, E., and Amacker, M. (1994) J. Biol. Chem. 269, 25255–25259
47. Divita, G., Rittinger, K., Restle, T., Immendorfer, U., and Goody, R. S. (1995) Biochemistry 34, 16337–16346
48. Arts, E. J., Miller, J. T., Ehresmann, B., and Le Grice, S. F. J. (1998) J. Biol. Chem. 273, 14523–14532
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J. Biol. Chem. 2001, 276:27470-27479.
doi: 10.1074/jbc.M104342200 originally published online May 15, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M104342200

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