Structure/Function Studies of HIV-1[1] Reverse Transcriptase: Dimerization-Defective Mutant L289K[†]

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ABSTRACT: Virion-derived HIV-1 reverse transcriptase (RT) has subunits of molecular mass 66 and 51 kDa (p66 and p51, respectively) in an ~1:1 ratio. Since enzyme activity appears to depend on dimerization of these subunits, identification of critical regions of primary sequence required for proper dimerization could lead to potential targets for antiviral therapy. A central region of primary sequence contains a leucine hepta-repeat motif from leucine 282 to leucine 310 that has been suggested to be involved in dimerization [Baillon, J. G., Nashed, N. T., Kumar, A., Wilson, S. H., & Jerina, D. M. (1991) *New Biol. 3*, 1015–1019]. A region including this hepta-repeat was recently shown to be involved in protein–protein interactions required for dimerization [Becerra, S. P., Kumar, A., Lewis, M. S., Widen, S. G., Abbotts, J., Karawya, E. M., Hughes, S. H., Shiloach, J., & Wilson, S. H. (1991) *Biochemistry 30*, 11708–11719]. To investigate the role of this repeat motif in dimerization, we performed site-directed mutagenesis of these leucine residues from position 282 to position 310. Mutations were introduced into p66 and p51 RT coding sequences, and the individually purified RT subunit polypeptides were compared with wild-type polypeptides for dimerization. Physical characterization of the purified mutant peptides was conducted by circular dichroism analysis. Binding between p66 and p51 was studied by gel filtration, ultracentrifugation, and CD analysis. L289K-p66 was unable to dimerize with itself and wild-type or L289K-p51. The leucine repeat motif in the p66 subunit appears to be critical in formation of the heterodimer. Conversely, our observation that the wild-type p66 and L289K-p51 can heterodimerize indicates that this leucine residue in p51 is probably not at a hydrophobic protein–protein interface of the heterodimer. These results for subunit dimerization with wild-type and mutant polypeptides were consistent with DNA polymerase activity being dependent on dimerization.

Human immunodeficiency virus type 1 (HIV-1) is a retrovirus widely considered to be the etiologic agent of acquired immunodeficiency syndrome (AIDS) (Barré Sinoussi et al., 1983; Popovic et al., 1984). As with all retroviruses, an essential feature of HIV-1 replication is reverse transcription of the plus-strand RNA genome into DNA, a process requiring the RNA-dependent DNA polymerase known as reverse transcriptase (Baltimore, 1970; Temin & Mizutani, 1970). Virion-derived HIV-1 RT is a heterodimer of polypeptides of molecular masses 66 and 51 kDa (p66 and p51, respectively) in a 1:1 ratio (Chandra et al., 1986). The p51 is formed by proteolysis at the C-terminal end of p66 (di Marzo Veronese et al., 1986; Lightfoote et al., 1986). Enzyme activity seems to depend on dimerization, since isolated monomers are relatively inactive whereas both heterodimer and artificially produced p66 homodimer are fully active (Restle et al., 1990; Becerra et al., 1991).

Computer-aided analysis of retroviral *pol* genes, later confirmed by mutagenesis (Larder et al., 1987; Schatz et al., 1989) and monoclonal antibody probing (Hansen et al., 1988), suggested that DNA polymerase activity is contributed by the N-terminal region while the C-terminal region is responsible for RNase H activity. The polymerase and RNase H domains are separated by a relatively poorly conserved central region called "tether" (Johnson et al., 1986), and part of this region in the case of HIV-1 RT (residues 252–428) was recently shown to be involved in hydrophobic protein–protein interactions responsible for subunit dimerization (Becerra et

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[1] Abbreviations: AIDS, acquired immunodeficiency syndrome; HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; RNase H, ribonuclease H; p66, 66-kDa RT polypeptide; p51, 51-kDa C-terminally processed p66; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; mAb, monoclonal antibody M_r, molecular weight. Amino acid substitutions are indicated by the single letter code of the wild-type residue followed by position and the single letter code for the new amino acid side chain e.g., L289K represents a lysine replacement for the wild-type leucine at position 289).

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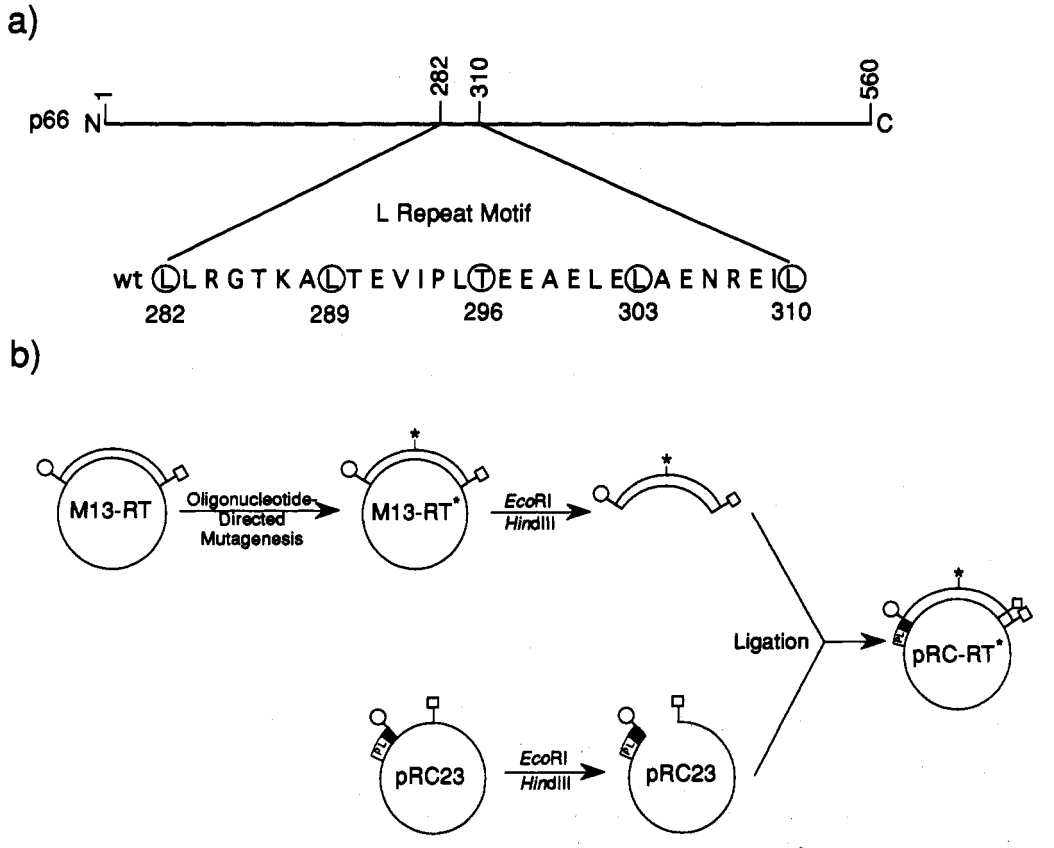

FIGURE 1: Mutagenesis of the leucine (L) repeat motif of HIV-1 RT. Part a shows the schematic representation of the localization of the leucine repeat motif in the primary sequence of HIV-1_{HXB2R} RT. Standard single letter code is used for amino acid designations. Part b shows the construction of expression vectors for HIV-1 RT mutant polypeptides. The M13-RT vector (Becerra et al., 1991) was used to mutate the reverse transcriptase sequence by oligonucleotide-directed mutagenesis at the desired position in each construct (Kunkel et al., 1991). Mutated RT sequences (RT*) in M13-RT* were isolated after *Eco*RI and *Hin*dIII digestion, followed by ligation into pRC23 plasmid at its *Eco*RI and *Hin*dIII sites to obtain pRC-RT*. The p51 expression construct was derived from pRC-RT* by deleting sequences between the *Kpn*I site at nucleotide position 1277 and the carboxy-end of RT to obtain pRC-51*. See Materials and Methods for further details. The symbols designate the following sites: ○, *Eco*RI; □, *Hin*dIII; ✱, mutation site; ◇, *Kpn*I; ■, ribosomal binding site; PL, P_L promotor.

al., 1991). On the basis of mAb mapping, Restle et al. (1992) have also indicated that a similar region (positions 230–300) is important for dimerization.

Baillon et al. (1991) examined the primary structure of RT from HIV-1 isolates in this central region and found it to contain two amino acid repeat motifs, i.e. a leucine hepta-repeat from residues 282 to 310 and a tryptophan tetra-repeat from residues 398 to 414. The leucine repeat region for HIV-1 RT differs from the classic leucine zipper α-helix motif found in the bZIP transcription factors in that it contains a threonine, instead of leucine, and proline residues in the middle of this motif. An analysis of this RT segment in a variety of HIV-1 sequences revealed that the leucine repeat motif is highly conserved and that this region has a strong probability for α-helical conformation in each case, $1.17 \leq P_\alpha \leq 1.20$ (Chou & Fasman, 1974).

To determine if the leucine repeat motif is involved in dimerization, we performed systematic site-directed mutagenesis on the leucine residues in this motif to develop a collection of mutant forms from positions L282 to L310. Physical characterization of the purified mutant peptides was conducted by circular dichroism analysis. Binding between p66 and p51 was studied by gel filtration, ultracentrifugation, and CD analysis. The effects of the leucine mutations on dimerization and enzymatic activities are described.

MATERIALS AND METHODS

Oligonucleotide-Directed Mutagenesis. To change specific residues in the HIV-1 RT coding sequence, oligonucleotide-directed mutagenesis was performed as described by Kunkel et al. (1991), using the M13-RT vector and 21-mer oligonucleotides. Four mutant RT coding sequences differing from wild-type by one amino acid were constructed: L289K (CTA to AAA), L303R (CTG to CGG), L303K (CTG to AAG), and L310K (CTA to AAA). Two mutants contained double amino acid substitutions: T296K (ACA to AAA), L303K (CTG to AAG); and L303K (CTG to AAG), L310K (CTA to AAA). To ensure that the resulting RT genes contained only the desired change(s), the entire coding sequence of each mutant was confirmed by DNA sequence analysis.

Construction of Plasmids for Expression of HIV-RT. The mutated RT-containing DNA fragments (RT*) were isolated after digestion of M13-RT* DNA with *Eco*RI and *Hin*dIII and ligated into expression vector pRC23 at its *Eco*RI and *Hin*dIII sites to obtain pRC-RT* (Figure 1b) as described before (Becerra et al., 1991). These expression plasmids contained the coding sequence for the p66 polypeptides (Pro^1–Leu^{560}). To obtain p51 expression plasmids, pRC-RT* DNAs were digested with *Kpn*I and *Xba*I, and their ends were blunt-ended and self-ligated to obtain pRC-51* plasmids as described by Becerra et al. (1991). The carboxy-end truncations involved amino acid residues 426–560 with the addition of a cysteine residue (i.e., p51 comprised residues Pro^1–Leu^{425}-Cys).

Reverse Transcriptase Assays. Enzyme activities were determined using a standard reaction mixture (20 μL) containing 50 mM Tris-HCl, pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 30 μM [α-^{32}P]dTTP and 1 μM poly(rA)-$(dT)_{16}$ (expressed as 3′-hydroxyl primer termini). Reactions were

initiated by addition of bacterial extract, incubated at 25 °C for 10 min and stopped by the addition of 10 μL of 0.5 M EDTA, pH 8. Quenched reaction mixtures were spotted onto Whatman DE-81 filter disks and dried. Unincorporated [α-^{32}P]dTTP was removed by four washes of 0.3 M ammonium formate, pH 8, followed by two washes with 95% ethanol and one wash of acetone. The dried filters were counted in 5 mL of RPI Bio-Safe II.

Assays to follow dimerization were determined with purified enzyme in a 50-μL reaction mixture as described above, except the reactions were stopped with the addition of 20 μL of 0.5 M EDTA. Additional details are given in the caption of Figure 6.

Protein Purification. Buffers used for protein purification: buffer A, 100 mM Tris-HCl, pH 7.5, and 5 mM benzamidine; buffer B, 50 mM sodium phosphate, pH 7.5, and 50 mM NaCl; buffer C, 50 mM sodium phosphate, pH 6.6, and 5% (w/v) glycerol; buffer D, buffer C without glycerol. All buffers contained 1 mM EDTA and 1 mM dithiothreitol. Cells, 100-g net weight, were suspended in 250 mL of buffer A and broken as previously described (Becerra et al., 1991). After centrifugation at 10000*g* for 60 min to remove particulate material, $(NH_4)_2SO_4$ was added to 50% saturation. The precipitate was collected and dissolved in 150 mL of buffer B and dialyzed with several changes of the same buffer. The dialysate was applied to a Whatman P11 phosphocellulose column (5 cm diameter × 10 cm) and the protein was eluted with a 50–500 mM NaCl linear gradient in buffer B. Column fractions, 2 mL, were collected and the presence of HIV-1 RT was monitored by SDS–PAGE. HIV RT containing fractions were dialyzed against buffer C and applied to a mono-S (Pharmacia) column (5 cm diameter × 10 cm) equilibrated in buffer D. A 0–500 mM NaCl linear gradient was applied, and eluted protein containing RT was filtered through a 0.22-μm filter (Millex-GV, Millipore Corp.), and the filtrate was concentrated using an Amicon-10 cell with a Diaflo ultrafiltration YM3 membrane and passed through a Superose-12 sizing column (Pharmacia) equilibrated with 50 mM sodium phosphate buffer, pH 7.5. Fractions containing RT were pooled, sterile-filtered, and stored at −70 °C.

CD Spectroscopy. Circular dichroism spectra were recorded at 25 °C on a Jasco J-710 spectropolarimeter using demountable rectangular Suprasil quartz cells (closed both ends; 50-μm path length; Uvonic Instruments, Inc.). Spectra (single accumulation, unless indicated) were acquired in the wavelength range between 250 and 180 nm at a scan speed of 20 nm min^{-1}, with a resolution of a data point every 0.1 nm and a response time of 16 s. Protein samples were in 100 mM potassium phosphate buffer, pH 7.5, containing 250 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, and 5% (w/v) glycerol. Net spectra were generated upon subtraction of buffer ellipticity; no smoothing was applied.

Dimerization Assays. (*i*) *Analytical Ultracentrifugation.* Analytical ultracentrifugation was performed using a Beckman Model E analytical ultracentrifuge equipped with a scanning absorption optical system as described before (Becerra et al., 1991).

(*ii*) *Analytical Gel Filtration.* Purified wild-type and L289K-p66 and wild-type and L289K-p51 were used for gel filtration analysis. Gel filtration was performed using a Superose-12 column on a FPLC system as described before (Becerra et al., 1991). The column was equilibrated and developed with 100 mM potassium phosphate, pH 7.5, 200 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, and 5% glycerol at 25 °C. Protein samples were incubated at 30 °C for 2 h

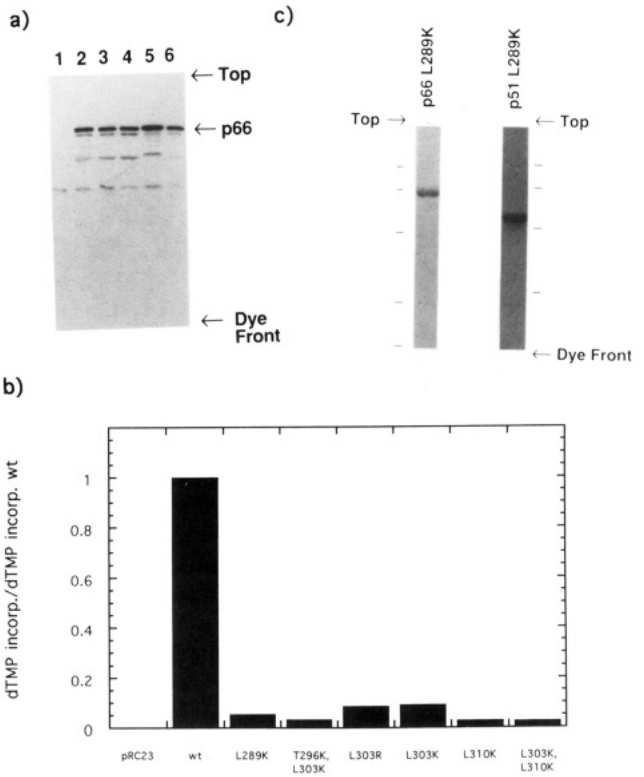

FIGURE 2: Analysis of HIV-1 mutant polypeptides. Part a shows the immunoblot analysis of crude *E. coli* extracts of mutant p66 RTs as described previously (Becerra et al., 1991). Extracts from bacteria with the following plasmids were used in lane: 1, pRC-23; 2, L289K; 3, T296K/L303K; 4, L303R; 5, L303K; 6, L310K. Analysis of the L303K, L310K double mutant extract is not shown. Part b shows the polymerase activity of wild-type (wt) and leucine repeat motif RT mutants in *E. coli* extracts. Bacterial extracts were assayed for RNA-directed DNA polymerase activity as described under Materials and Methods. Activities are normalized with wt HXB2R p66 which incorporated 135 pmol of dTMP/10 min. pRC23 represents the parent plasmid lacking the p66 coding region. Part c shows the SDS–PAGE analysis of purified L289K-p66 and L289K-p51 as described under Materials and Methods. A photograph of Coomassie Brilliant Blue stained 12.5% polyacrylamide gel is shown. Molecular weight markers and their masses were as follows: phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 42.7 kDa; carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kda. The respective positions of the markers are indicated.

before assaying for dimerization. The column was run at a flow rate of 0.4 mL/min, and 0.5-mL fractions were collected. Protein elution was followed by UV absorbance with a time constant of 1 s for the UV monitor. This column was routinely calibrated using three protein standards: IgG, BSA, and ovalbumin.

Immunoblot Analysis. Proteins were transferred from polyacrylamide gels onto nitrocellulose paper by the method of Towbin et al. (1979); the blotted paper was treated as described (Sobol et al., 1991) and reacted with a monoclonal antibody, mAb(RT) (New England Nuclear Inc., Boston, MA), against HIV RT at 1:1500 dilution for 1.5 h. The paper was incubated with biotinylated anti-mouse IgG (Cappel Laboratories) at a 1:500 dilution for 1 h and subsequently with an avidin–biotinylated horseradish peroxidase complex (Vectastain ABC Kit, Vector Laboratories) for 1 h. Color was developed by reaction with 0.3% HRP color development reagent (Bio-Rad) and 30% hydrogen peroxide.

Other Methods. Protein concentration was determined by the Bradford assay (1976) using a Bio-Rad protein assay kit (Bio-Rad Laboratories). SDS–PAGE was performed according to Laemmli (1970). Protein markers were from Bio-

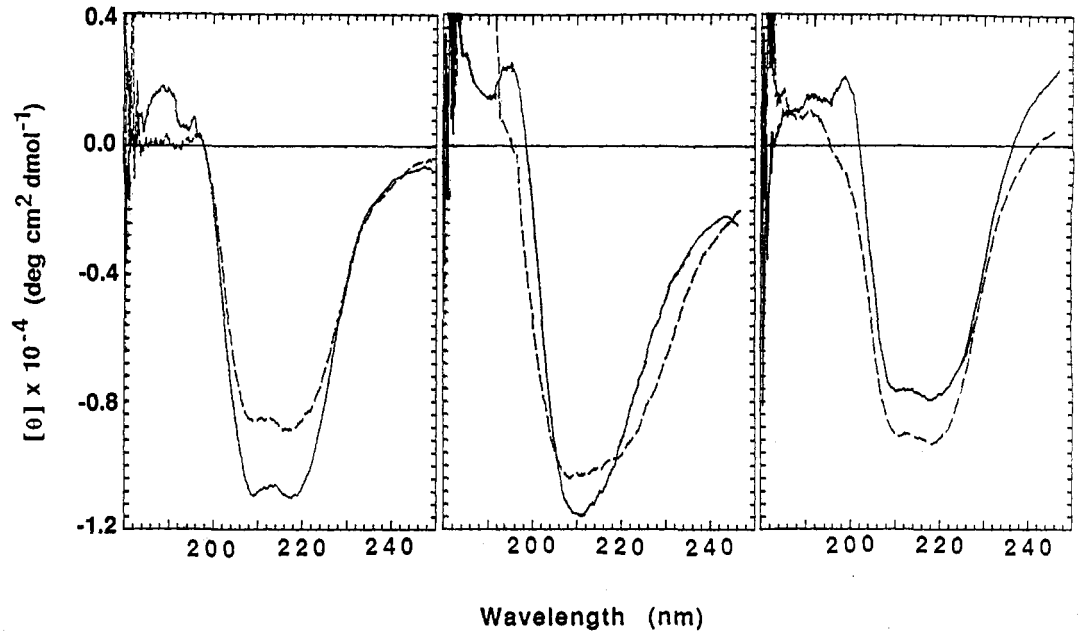

FIGURE 3: Circular dichroism spectra of wild-type and L289K mutant HIV-1 RT: left panel, spectra for wild-type (solid line) and L289K (dashed line) p51 polypeptides at 0.16 mg/mL; center panel, spectra for wild-type (solid line) and L289K (dashed line) p66 polypeptides at 0.08 mg/mL; right panel, composite spectra of wild-type p66 mixed with wild-type p51 (solid line) or L289K p51 (dashed line) at a total protein concentration of 0.16 mg/mL (i.e., 0.08 mg/mL of each polypeptide). From the dimerization association constants determined by Becerra et al. (1991), the proportion of wild-type p66 dimerizing as homo- and heterodimer would be approximately 4 and 40% in the center and right panels, respectively.

Rad. Poly(rA), $(dT)_{16}$, and dTTP were from Pharmacia. [α-^{32}P]dTTP (3000 Ci/mmol) was from New England Nuclear.

RESULTS

Six site-directed mutants were constructed by modifying L289, L303, L310, or T296 of HIV-1 RT (Figure 1). Four of the changes were single-point mutations at residues L289, L303, and L310, while the other two mutants were changes at two sites, L303 and T296 or L303 and L310. Residue 303 was changed to lysine or arginine, whereas all of the other mutants involved a change to lysine. We also developed a construct for the p51 polypeptide where L289 was changed to lysine. Since the dimerization interface is believed to be hydrophobic in character (Becerra et al., 1991), substitution of a charged residue at this interface should perturb subunit interactions (Bordo & Argos, 1991).

The mutant RT polypeptides had the same apparent molecular weight as the wild-type polypeptide and were soluble in the crude extract. Each of the mutant RT polypeptides immunoreacted with a monoclonal antibody to HIV-1 RT, mAb(RT), about as well as the wild-type polypeptide (Figure 2a). Analysis of the DNA polymerase activity of the mutant p66 polypeptides in crude extracts revealed that each had much lower activity than wild-type p66 (Figure 2b).

For more detailed study to be described below, we selected the L289K due to its better expression and purification and the observation that L289 can be UV cross-linked to the single-stranded probe $p(dT)_{15}$ (Basu et al., 1992). The L289K-p66 peptide was overexpressed and purified to homogeneity, and a construct was prepared for overexpression of L289K-p51. SDS–PAGE analysis of the two purified mutant polypeptides, p66 and p51, is shown in Figure 2c.

L289K-Mutants Are Structurally Similar to Their Wild-Type Counterparts As Determined by Circular Dichroism Spectroscopy. To study the influence of the L289K mutation on heterodimer formation, the individually purified p51 and p66 polypeptides were first characterized by circular dichroism (CD) spectroscopy. The CD spectrum of the wild-type p51 polypeptide was similar to that of the L289K-p51 mutant but suggested a small structural alteration in the mutant protein as compared with wild-type (Figure 3, left panel). Similarly, wild-type p66 exhibited a CD spectrum that resembled that of the L289K-p66 mutant peptide, but the spectrum for mutant p66 was slightly different; the mutant and wild-type p66 spectra exhibited extrema of negative ellipticity at about 210 nm, and the mutant polypeptide spectrum exhibited a moderate shoulder of red-shifted negative ellipticity (Figure 3, center panel). We conclude from these experiments that both L289K-p51 and L289K-p66 are structurally similar, but not identical, to their wild-type homologues and that L289K-p66 exhibits a greater difference from its homologue than L289K-p51.

CD analysis of a 1:1 mixture of wild-type p51 and wild-type p66 revealed a spectrum with two extrema of negative ellipticity at approximately 210 and 218 nm, respectively (Figure 3, right panel). This spectrum is very similar to that observed with purified wild-type p51. Except for a lower band of positive ellipticity at 200 nm when L289K-p51 is mixed with wild-type p66, no clear structural difference, in either wild-type or mutant p51, could be detected as a result of mixing with the wild-type p66 peptide. This suggests that the structure of the heterodimer containing wild-type p66 and either of the two p51 peptides was similar.

Mutation at Position 289 in p66, but Not in p51, Prevents Dimerization. To further characterize the dimerization capacity of L289K-p51 and wild-type p66, we conducted gel filtration analysis and ultracentrifugation analysis of appropriate mixtures of the two purified peptides. Gel filtration analysis, shown in Figure 4, revealed that when L289K-p51 was mixed with wild-type p66 (Figure 4d), mutant p51 eluted in higher M_r fractions, indicating that heterodimer formation had occurred. By contrast, all combinations involving L289K-p66 failed to reveal dimer formation (Figure 4a,b). Mutant p51 itself failed to dimerize (Figure 4c), a property similar

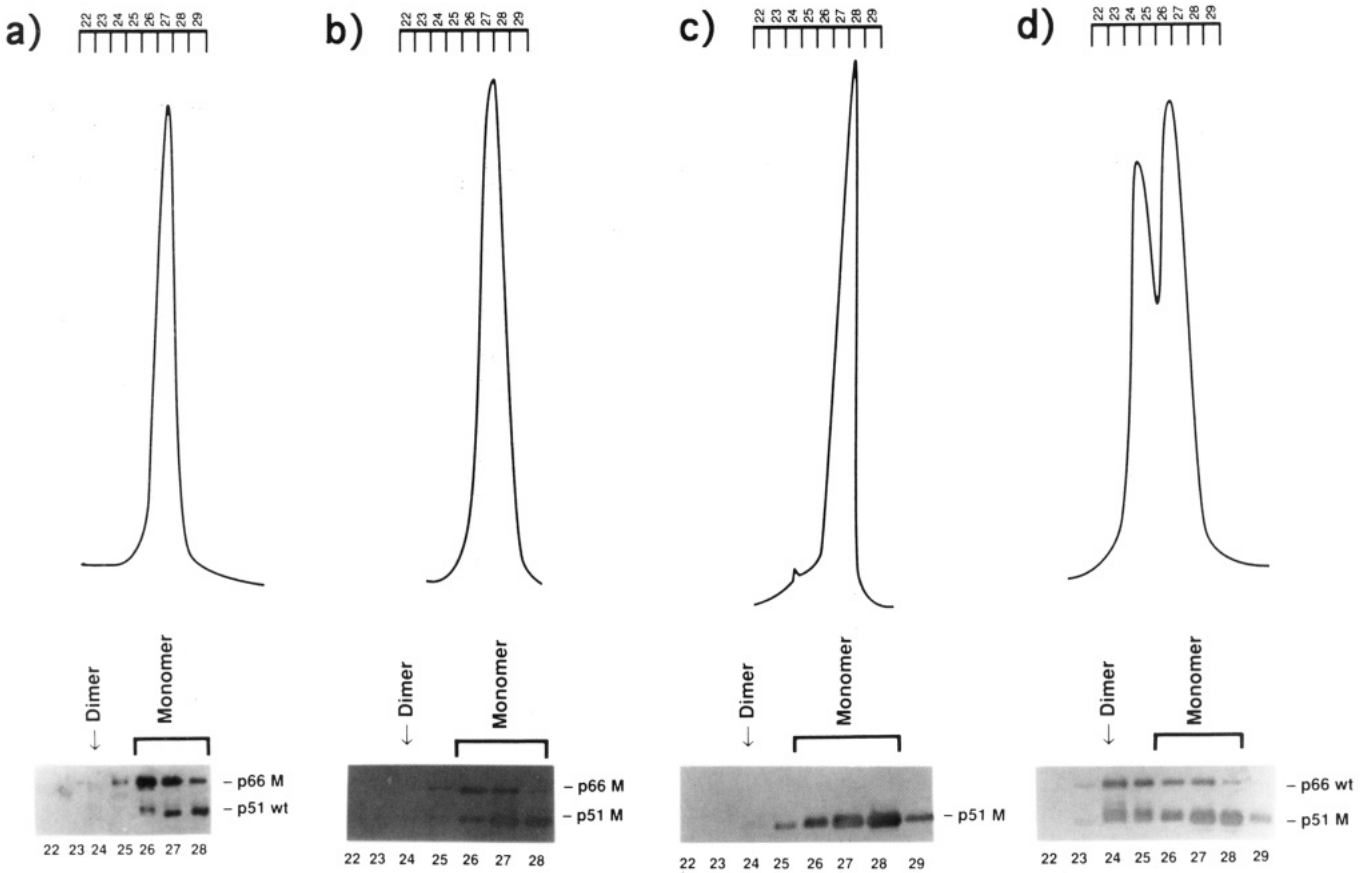

FIGURE 4: Gel filtration analysis for dimerization of wild-type and L289K polypeptides: (a) chromatographic pattern of a mixture of L289K-p66 and wild-type p51 (20 μg each); (b) chromatographic pattern of a mixture of L289K-p66 and L289K-p51 in a 1:1 ratio; (c) chromatographic pattern of L289K p51 (35 μg) by itself; (d) chromatographic pattern of a mixture of L289K p51 and wild-type p66. To determine if dimerization can occur between subunits, samples of protein mixtures were incubated at 30 °C for 2 h. Each mixture (100 μL) was layered on a FPLC Superose-12 sizing column, and 500-μL fractions were collected as outlined under Materials and Methods. In each panel, a chart recording of the 280-nm absorbance is presented along with a photograph of the immunoblot of the indicated fractions. Each fraction was analyzed on 12.5% SDS–PAGE gels, and immunoblots were probed with mAb(RT) to identify RT subunits (p66 and p51). M and wt refer to mutant and wild-type polypeptides, respectively.

to that of wild-type p51 (Becerra et al., 1991). Analysis of L289K-p66 by ultracentrifugation (Figure 5a) and gel filtration (Figure 5b) as a lone polypeptide revealed that it behaved as a homogeneous monomer, rather than a mixture of monomer and dimer, as seen earlier for wild-type p66 polypeptide (Becerra et al., 1991). These results indicate that a dimerization defect in these experiments correlates with mutation at position 289 in the p66 peptide, rather than mutation in the p51 peptide.

L289K Mutation in p51 Does Not Affect Polymerase Activity of the Heterodimer. We evaluated the question of whether L289K-p51 has the capacity to enhance DNA polymerase activity of purified p66, as is observed with wild-type p51. The results of these experiments are illustrated in Figure 6. Note that wild-type heterodimer (p66/p51) has 1.8 times greater DNA polymerase activity than the wild-type p66 homodimer when expressed as activity per p66 subunit. Similar DNA polymerase activity was observed with heterodimers of both wild-type peptides and of wild-type p66 and L289K-p51. Thus, L289K-p51 is similar to wild-type p51 in its ability to confer greater activity upon p66. In contrast, the mixture of L289K-p66 and wild-type p51 exhibited lower DNA polymerase activity, as did L289K-p66, wild-type p51, and mutant p51 polypeptides alone.

DISCUSSION

Our observation that L289K-p51 can dimerize with wild-type p66 is taken as evidence that the leucine repeat motif in the p51 moiety of the heterodimer is not buried in a protein–protein interface. The leucine to lysine change is predicted to create a structural abnormality at a hydrophobic protein–protein interface (Bordo & Argos, 1991), and since dimerization occurs with wild-type p66 and mutant p51 peptides, the location of the mutation is probably outside such an interface. On the other hand, the leucine repeat motif in the p66 moiety of the heterodimer may well be in a buried protein–protein interface at some time during dimerization. This interpretation is suggested from the lack of dimer formation with all mixtures containing L289K-p66. Bordo and Argos (1991) reported a survey of the occurrence of permitted buried and solvent-exposed site-directed changes in many proteins. They found that, in the case of buried leucine residues, no examples of permitted change to lysine occurred. Therefore, we strongly expect that the leucine to lysine change in the p66 peptide is a knockout mutation for a buried residue. These interpretations of our results are consistent with the model of an asymmetric heterodimer for the HIV-1 reverse transcriptase as recently observed with the 3.5-Å resolution crystallographic structure (Kohlstaedt et al., 1992). The precise location of L289 is not clear at the moment from the published model but is situated in a subdomain that consists of a bundle of possibly four α-helices referred to as the "thumb" subdomain. Our results clearly suggest that the L289 residue in the p51 moiety of the heterodimer is solvent-exposed, whereas in the p66 moiety of the heterodimer it is buried and cannot be substituted by lysine without the loss of dimerization and, therefore,

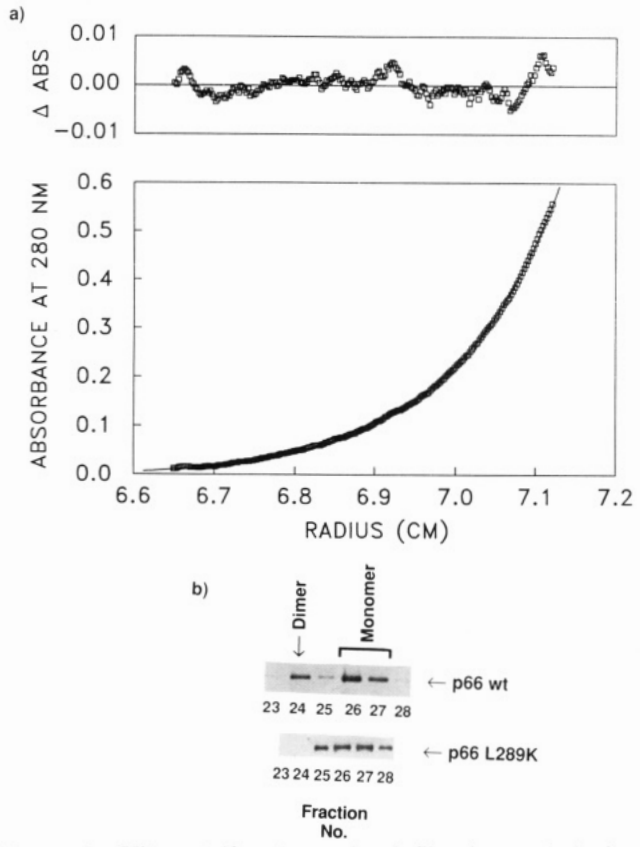

FIGURE 5: Ultracentrifugation and gel filtration analysis for homodimerization of p66. Part a shows typical results of Model E ultracentrifugation analysis of L289K-p66 as described under Materials and Methods. The data points were fit to a theoretical curve assuming L289K-p66 as a homogeneous monomer. The insert at the top is a plot of the residuals showing excellent fit of the data points to the theoretical curve. Part b illustrates the gel filtration results for dimerization using a FPLC Superose-12 column. Photographs of immunoblots of the indicated fractions are presented. In each case, 100 μL of a sample containing 35 μg of wild-type p66 or L289K p66 in 100 mM potassium phosphate, pH 7.5, 200 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, and 5% glycerol was layered on the column. Full-scale absorbance at 280 nm was 0.02, and the fraction size was 500 μL. Each fraction was analyzed by 12.5% SDS–PAGE, and immunoblots were prepared using mAb(RT).

activity. This asymmetry in amino acid function in the heterodimeric RT has been noted for D110 (Hostomsky et al., 1992) and D185-D186 (Le Grice et al., 1991), which are critical for polymerase activity in p66 but not in p51.

The results illustrated in Figure 6 indicating that mutant p51 is able to stimulate the activity of wild-type p66, about as well as wild-type p51, and that mutant p66 alone is defective in DNA polymerase activity can be interpreted in terms of dimerization. The defective DNA polymerase activity of mutant p66 could be due to its lack of dimerization capacity or an intrinsic defect in some requirement for DNA polymerase function or both. Clearly, however, addition of wild-type p51 to L289K-p66 does not restore activity to the level seen with wild-type p66 and wild-type p51. Since we have shown that mixtures with L289K-p66 do not dimerize (Figure 4), these results are consistent with the idea that the lower DNA polymerase activity is due to the inability of the individual subunits to dimerize, rather than due to some intrinsic defect in DNA polymerase capacity of L289K-p66. However, we cannot rule out this latter possibility on the basis of our present results. If the intrinsic DNA polymerase activity of L289K-p66 is not affected, then the low activity measured is due to a small amount of homodimer formation (<3%). Additionally, wild-type p51 stimulates the low polymerase activity of L289K-

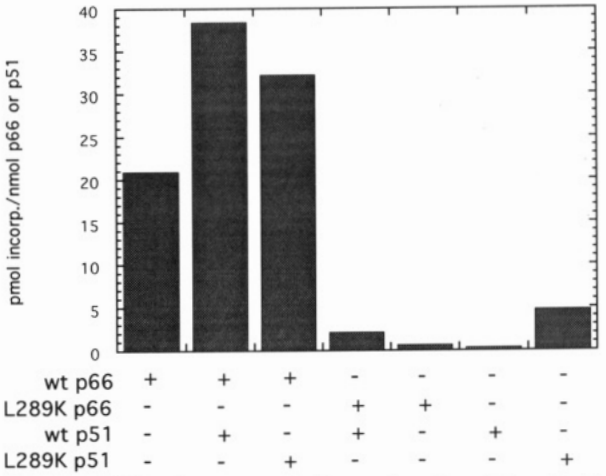

FIGURE 6: DNA polymerase activity as a function of dimerization between mutant and wild-type p66 and p51 subunits. The indicated subunits (600 nM each) were incubated at 4 °C in 50% glycerol, 50 mM Tris-HCl (pH 7.4, adjusted at 25 °C), 100 mM KCl, and 10 mM DTT. After 5 h, the stock solutions were diluted 10-fold in 10 mM Tris (pH 7.4) at 25 °C and immediately assayed in triplicate for activity as described under Materials and Methods. The standard error was less than ±8% for all conditions. When only a single subunit was present, an equivalent amount of BSA was included in the incubation mixture.

p66 about 2-fold (Figure 6), suggesting a small increase in the amount of heterodimer formed. This residual polymerase activity indicates that dimerization is dramatically altered, but not totally abolished. However, the amount of dimer present as suggested by the activity measurements would be below the limits of detection with the other assays employed. Activity measurements, by their nature, require the presence of substrates which must influence dimerization (Beard & Wilson, 1993).

The lysine substitution for leucine at position 289 of p66 may perturb ionic interactions indirectly through small structural alterations. The effect of pH or ionic strength on dimerization has not been rigorously examined. The dimerization of wild-type p66 and p51 appears to be largely hydrophobic since the amount of dimer immunoprecipitated was not reduced in the presence of 1 M NaCl (Becerra et al., 1991). However, there may be an ionic contribution which was not detected by that assay. Further characterization of dimerization of wild-type polypeptides by analytical ultracentrifugation is warranted.

The observation that L289K-p51 can stimulate the activity of wild-type p66 is consistent with the dimerization studies described above wherein L289K-p51 is able to heterodimerize with wild-type p66. Thus, the polymerase activity results for wild-type p66 and L289K-p51 are consistent with the biophysical measurements showing normal dimerization with this mixture.

Residues in the "thumb" subdomain have been implicated in template·primer interactions (Sobol et al., 1991; Basu et al., 1992; Kohlstaedt et al., 1992; Mitchell & Cooperman, 1992). Basu et al. (1992) were able to UV cross-link L289 to $p(dT)_{15}$. In addition, the dissociation rate for template·primer from a complex with L289K-p66 RT is more rapid than that with wild-type enzyme, indicating that mutation at this position to a positively charged side chain destabilizes template·primer interactions (W. A. Beard and S. H. Wilson, unpublished results). These results suggest that L289, in addition to its role in dimerization, can also interact with template·primer. Important structural changes occur upon binding of DNA to RT (Arnold et al., 1992), and

it is not clear how the structure of the "thumb" subdomain is altered upon binding. More importantly, how the "thumb" subdomain is structured in a monomeric subunit and how this subdomain plays a role in the dimerization or in the proper folding or organization of a "mature" heterodimer remain to be determined. Neutralization of dimerization of the respective subunits (p66 and p51) remains a potential target for antiviral therapy, and a better understanding of this process could lead to new targets for drug design.

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