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Subunit-selective mutagenesis indicates minimal polymerase activity in heterodimer-associated p51 HIV-1 reverse transcriptase

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We have purified and determined functional parameters of reconstituted, recombinant HIV-1 reverse transcriptase (RT) heterodimers within which either the p66 or p51 polypeptide was selectively mutated in one or both aspartic acid residues constituting the proposed polymerase active site (-Y-M-D-D-). Heterodimers containing a mutated p51 polypeptide retain almost wild type levels of both RNA-dependent DNA polymerase and ribonuclease H (RNaseH) activity. In contrast, heterodimers whose p66 polypeptide was likewise mutated exhibit wild type RNaseH activity but are deficient in RNA-dependent DNA polymerase activity. These results indicate that in heterodimer RT, the p51 component cannot compensate for active site mutations eliminating the activity of p66, indirectly implying that solely the p66 aspartic acid residues of heterodimer are crucial for catalysis.

Key words: heterodimer reconstitution/HIV-1/reverse transcriptase

Introduction

Human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT), as with other retroviruses, is translated as a component of the *gag–pol* polyprotein precursor, which is subsequently processed by the *pol*-encoded PR to yield the active form of the enzyme. Mature HIV-1 RT is a heterodimer of 66 kDa and 51 kDa polypeptides (denoted p66 and p51) which are colinear at their N-termini (Chandra *et al.*, 1986; Müller *et al.*, 1989). This form is observed in both virions (Di Marzo-Veronese *et al.*, 1986; Lightfoote *et al.*, 1986) and heterologous bacterial expression systems (Farmerie *et al.*, 1987; Hansen *et al.*, 1988; Le Grice *et al.*, 1987; Mous *et al.*, 1988). The larger subunit, p66, contains both RT and ribonuclease H (RNaseH) activities; sequence homology studies, together with mutational analyses, have indicated that the polymerase domain extends roughly over the N-terminal three quarters of the polypeptide (Johnson *et al.*, 1986; Larder *et al.*, 1987b; Hizi *et al.*, 1989; Prasad and Goff, 1989) whereas the RNaseH domain is located within a C-terminal portion, comprising ~120 amino acids (Hansen *et al.*, 1988; Tanese and Goff, 1988; Schatz *et al.*, 1989). These two domains appear to be connected by a protease-sensitive linker region (Lowe *et al.*, 1988; Müller *et al.*, 1989; Ferris *et al.*, 1990).

The p51 HIV-1 RT subunit is generated by HIV-1 protease via endoproteolytic cleavage of p66 between Phe440 and Tyr441 (Mizrahi *et al.*, 1989; Le Grice *et al*, 1989; Becerra *et al.*, 1990; Graves *et al.*, 1990). The processed p66/p51 heterodimer has been reported by several groups to display a higher specific RNA-dependent DNA polymerase activity than p66 (Lowe *et al.*, 1988; Mizrahi *et al.*, 1989; Müller *et al.*, 1989; Le Grice and Grüninger-Leitch, 1990). Whether heterodimer-associated p51 RT contributes to this increased activity remains to be established. Several independent reports have indicated that purified p51 exhibits only very low levels of polymerase activity (Hansen *et al.*, 1988; Hizi *et al.*, 1988; Starnes *et al.*, 1988; Tisdale *et al.*, 1988), although appreciable activity has been observed via (i) activated gel analyses of the viral enzyme (Lori *et al.*, 1988), (ii) deletion mutants of a recombinant RT–*trp*E fusion protein (Prasad and Goff, 1988), and (iii) recombinant p51 with a slightly altered C-terminus (Restle *et al.*, 1990).

The enhanced activity of heterodimer RT relative to its homodimeric p66 counterpart might be explained either by activation of p51 (which retains the active site -Y-M-D-D- motif) or stabilization of p66 in an active conformation. In order to clarify the role of p51 RT within the p66/p51 heterodimer, we have established a system to reconstitute heterodimeric RT from its constituent polypeptides and analyse the effects of mutations present in only **one** subunit. Reconstituted p66/p51 heterodimers, produced by our technique of 'subunit-selective mutagenesis', have been purified to >90% homogeneity and analysed for both RNA-dependent DNA polymerase and RNaseH activities. Our results indicate that wild type p51 RT fails to restore activity to a heterodimer whose 66 kDa subunit is mutated at either of the aspartic acid residues. Minimal loss of RT activity in a series of heterodimers whose p51 subunit is selectively mutated indicates that this polypeptide makes no direct contribution to the catalytic activity of the parental heterodimer.

Results

Analysis of p66 and p51 RT mutants

In a preliminary analysis, several recombinant forms of p66 and p51 HIV-1 RT were expressed in *Escherichia coli*. These polypeptides were extended at their N-termini with a small poly-histidine extension (His_6), which allows a rapid first step purification from crude bacterial extracts via metal chelate affinity chromatography on Ni^{2+}–nitrilotriacetic acid–Sepharose (Ni^{2+}–NTA–Sepharose; Hochuli *et al.*, 1987, 1988). The purification protocol employed has been used to prepare both homodimeric and heterodimeric forms

of HIV-1 RT with both polymerase and RNaseH activities indistinguishable from those of the histidine-free enzyme (Le Grice and Grüninger-Leitch, 1990)

The mutant forms of both p66 and p51 HIV-1 RT analysed in the present study have been represented schematically in Figure 1. Poly-histidine-extended p66 RT expressed from plasmid p6HRT (His-p66) is present as a mixture of monomer and homodimer (Müller *et al.*, 1989; our unpublished data). An equivalently extended form of p51 RT (His-p51), ending at Phe440 (the site of proteolytic maturation), is expressed from plasmid p6HRT51. In our hands, the resulting p51 RT molecules are predominantly monomers (cf. also Müller *et al.*, 1989; Hostomsky *et al.*, 1991). Plasmid pRT determines expression of the 66 kDa subunit lacking the poly-histidine extension. RT molecules lacking this extension fail to bind to Ni^{2+}–NTA–Sepharose (Le Grice and Grüninger-Leitch, 1990). As we will demonstrate later, this form of p66 RT is however, capable of reconstituting with His-p51 into a functionally active heterodimer. As control for these experiments, heterodimer RT containing the poly-histidine extension on the N-terminus of both p66 and p51 was prepared from the plasmid p6HRT-PROT, involving co-expression of the 66 kDa RT and 17 kDa PR domains (Le Grice and Grüninger-Leitch, 1990).

For construction of suitable polymerase active site mutants, we chose to alter the conserved aspartic acid residues within the -Y-M-D-D- motif present in several different polymerases (Argos, 1986; Johnson *et al.*, 1986). An exchange of Asp185 for His within this motif has previously been shown to abrogate the polymerase activity of HIV-1 RT (Larder *et al.*, 1987a). Therefore, it seemed likely that the conserved aspartic acid doublet, Asp185/Asp186 was an integral feature of the polymerase active site. This notion is most likely correct, since our active site mutants in which these are altered to asparagine (converting the side chain carboxyl groups to the corresponding amides) are virtually devoid of RNA-dependent DNA polymerase activity (Table I). A drastic reduction in RT activity is observed with both single amino acid substitutions (His-$p66^{ND}$/His-$p66^{ND}$ and His-$p66^{DN}$/His-$p66^{DN}$) as well as the double substitution (His-$p66^{NN}$/His-$p66^{NN}$), suggesting that both residues play a role in catalysis. These mutations selectively affect polymerase function, since all enzyme preparations display normal levels of RNaseH activity (Table I). In addition, the binding of the HIV-1 replication primer, $tRNA^{Lys,3}$ (Barat *et al.*, 1989), remains unchanged in the active site mutants (J.-L.Darlix, personal communication). Note that p51 displays very little RT activity, no matter whether assayed in the wild type or mutant form. For all RT mutants analysed in Table I, both subunits of homodimer were likewise affected by the point mutations introduced. We next attempted to reconstitute heterodimeric RT from individually mutated polypeptides and measure the relative contributions to RT activity of the p66 and p51 subunits.

Table I. RT and RNaseH activities of mutant and wild type RT forms

Enzyme	RT[a]	RNaseH[b]
His-$p66^{DD}$/His-$p51^{DD}$	1580	81.2
His-$p66^{DD}$/His-$p66^{DD}$	1091	56.1
His-$p66^{ND}$/His-$p66^{ND}$	7.1	49.3
His-$p66^{DN}$/His-$p66^{DN}$	5.1	60.6
His-$p66^{NN}$/His-$p66^{NN}$	5.9	54.5
His-$p51^{DD}$	14.7	–[c]
His-$p51^{ND}$	9.6	–
His-$p51^{DN}$	5.3	–

The specific activity of each enzyme preparation is presented.
[a]One unit of RNA-dependent DNA polymerase is defined as the amount catalysing incorporation of 1 nmol precursor dGTP into polynucleotide in 10 min at 37°C using a poly(rC)/oligo(dG) template/primer.
[b]1 unit of RNaseH activity is defined as that amount catalysing release of 1 nmol ribonucleotide from an RNA/DNA hybrid in 20 min at 37°C.
[c]Since p51 RT lacks the RNaseH domain, these assays were not performed. Values are the mean of three independent assays.

Strategy for RT reconstitution

Recently, a reconstitution system for HIV-1 RT has been described based on the expression of p66 and p51 from a single plasmid (Restle *et al.*, 1990). We decided to use an alternative system in which the reconstitution partners could be expressed in different bacterial strains and reconstituted post-translationally. This can be accomplished by introducing the poly-histidine extension into one subunit of heterodimer and purification of reconstituted enzyme via Ni^{2+}–NTA–Sepharose. Mixing RT polypeptides expressed in two different bacterial strains furthermore allows greater flexibility in testing several combinations of mutant and wild type subunits. In preliminary work, this procedure has enabled us to prepare reconstituted heterodimer HIV-1 RT within which the 51 kDa subunit is selectively deuterated, facilitating structural analysis of heterodimeric RT by NMR spectroscopy and low angle neutron solution scattering (H.Lederer *et al.*, manuscript in preparation). For these reconstitution experiments, we elected to extend selectively p51 RT with poly-histidine, since it dimerizes inefficiently (Restle *et al.*, 1990) and is therefore expected to be readily accessible to associate with a poly-histidine-free p66 dimerization partner. Earlier experiments (Le Grice and Grüninger-Leitch, 1990), have demonstrated that enzymatically proficient heterodimer RT expressed from a single plasmid can be prepared by Ni^{2+}–NTA–Sepharose when only one of the subunits carries the poly-histidine extension. Based on these observations, we assumed that reconstitution and purification might be possible by mixing lysates of the appropriate bacterial strains, possibly making use of chaperone proteins in the bacterial lysate (Ellis and Hemmingsen, 1989; Goloubinoff *et al.*, 1989). The heterodimeric forms of RT reconstituted for the present analysis are indicated in Figure 1.

Following the protocol outlined in Figure 2, we mixed pellets of bacterial strains expressing p66 and His-p51. The ratio of the two subunits was adjusted such that p66 RT was in excess over His-p51 (as estimated from the intensity of bands in a control Western blot). Combined bacterial pellets were lysed and cell debris removed by ultracentrifugation. The supernatant, containing a mixture of reconstituted heterodimers and the corresponding homodimers (or monomers) was applied directly to Ni^{2+}–NTA–Sepharose. As can be seen in Figure 3, substantial amounts of the poly-histidine-free p66 co-elute

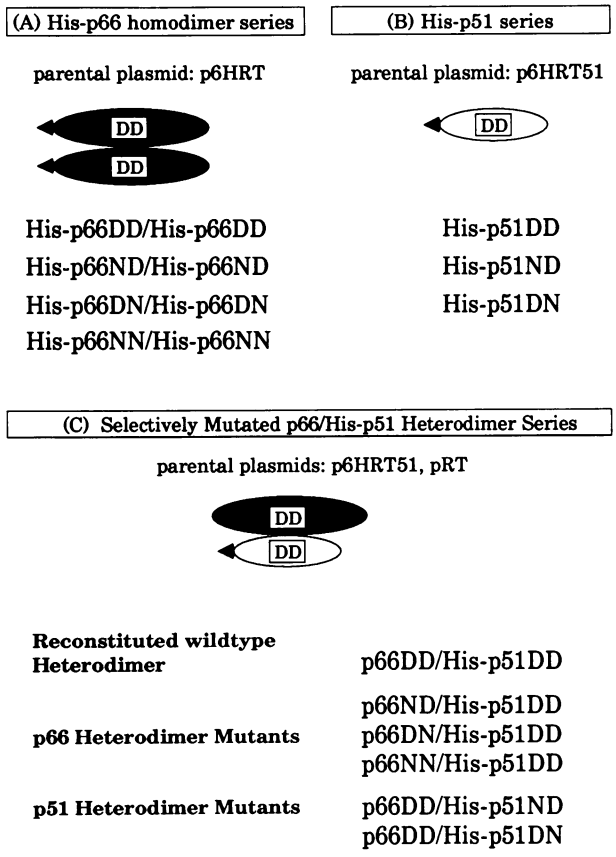

Fig. 1. Preparation of homodimer and selectively mutated heterodimer HIV-1 RT. Three RT-producing plasmids were used for this study, i.e. p6HRT (N-terminally extended p66), pRT (p66 lacking poly-histidine extension) and p6HRT51 (N-terminally extended p51). Mutations introduced into RT encoded by p6HRT and p6HRT51 yield the series of homodimer (**A**) or monomer mutants (**B**), respectively. The heterodimer mutant series (**C**) is prepared from mixed bacterial lysates of cultures containing plasmids pRT and p6HRT51. In each series, the corresponding wild type enzyme was prepared as control. DD represents the Asp185/Asp186 portion of the active site -Tyr-Met-Asp-Asp- motif of polymerizing enzymes (Larder *et al.*, 1987a). The N-terminal poly-histidine extensions are represented by solid segments.

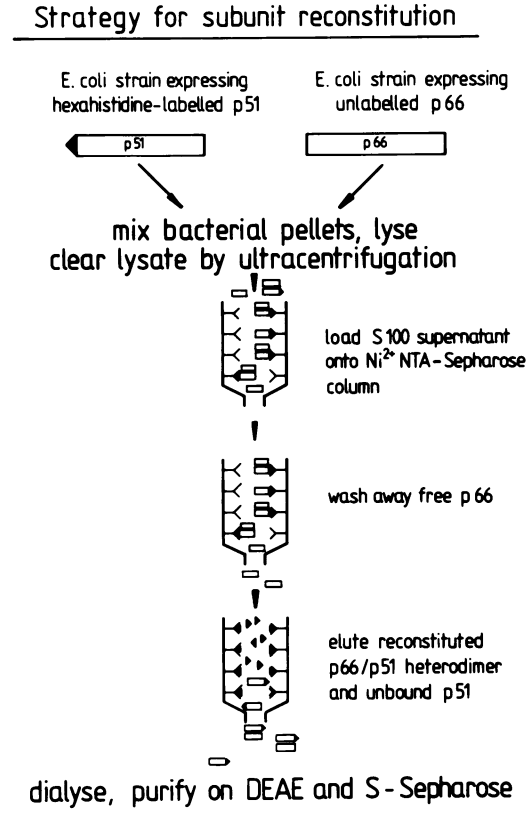

Fig. 2. Reconstitution of selectively mutated heterodimer RT. RT subunits are represented by the open boxes, of which p51 contains the N-terminal poly-histidine extension (designated s); (>-) matrix-immobilized nitrilotriacetic acid (NTA) groups with complexed Ni^{2+}. Only those RT subunits bearing the poly-histidine extension are retained on the column. This includes the monomeric form of p51, together with reconstituted p66/p51 heterodimer. Application of a gradient of imidazole results in displacement of both reconstituted heterodimer and excess p51, the latter of which is partially eliminated by DEAE and S−Sepharose ion exchange chromatography.

from Ni^{2+}−NTA−Sepharose with His-p51. This is not a consequence of residual binding of p66 to Ni^{2+}−NTA−Sepharose, but rather due to heterodimer formation, since unlabelled p66 RT does not bind to the metal chelate affinity matrix (Le Grice and Grüninger-Leitch, 1990). A considerable fraction of unbound p66 RT is found in the flow-through and first wash fractions, suggesting that the reconstitution is not fully quantitative (only 20−30% of p66 RT is bound in a heterodimer with His-p51 in most preparations). We suspect that only a subset of the p66 preparation is available for dimerization with p51 (presumably those not already engaged in p66/p66 homodimer complexes). Nevertheless, the yield of heterodimer obtained through this procedure was sufficient for subsequent analyses, and attempts were not made to increase the efficiency of heterodimer formation by supplying more p66 or prolonged incubation of the lysates.

Peak fractions containing both reconstituted heterodimer together with excess His-p51 (which is quantitatively adsorbed by Ni^{2+}−NTA−Sepharose) were pooled, dialysed and subjected to DEAE and S−Sepharose chromatography. DEAE−Sepharose removes most of the remaining impurities, while S−Sepharose partly separates monomer and homodimer His-p51 from the reconstituted heterodimer. The various combinations of selectively mutated, reconstituted heterodimers were processed in this manner, and the final preparations examined by SDS−PAGE (Figure 4). Certain mutant polypeptides migrate slightly differently than the corresponding wild type polypeptides, possibly due to the change in charge introduced by the point mutations. In some of the reconstituted RT preparations there is still a small excess of the His-p51 polypeptide relative to the p66 counterpart. Since this polypeptide makes a minimal contribution to RNA-dependent DNA polymerase activity, and is totally devoid of RNaseH activity, we assumed this minor subunit imbalance would not significantly interfere with subsequent enzymatic analyses.

Only the p66 active site -D-D- motif is crucial for RT activity

When we analysed RNA-dependent DNA polymerase and RNaseH activities of the reconstituted heterodimer preparations, all preparations containing a selectively mutated

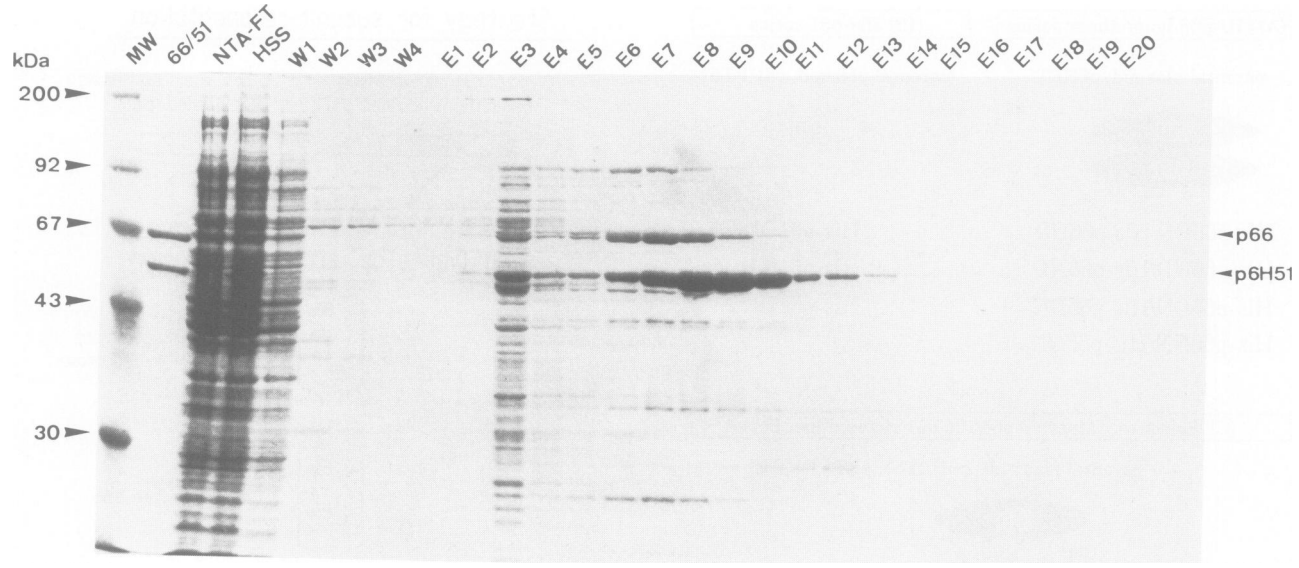

Fig. 3. Elution profile of a reconstituted heterodimer RT preparation. A stained SDS−polyacrylamide gel is illustrated, through which 5 μl aliquots of fractions eluted from the Ni^{2+}−NTA−Sepharose column (first purification step) were fractionated. MW, protein M_r markers; HSS, high speed supernatant; NTA-FT, column flow-through, i.e. proteins eluted with buffer A (pH 7.8). Fractions denoted W1−W4 were eluted by application of buffer B (pH 6.2). His-p51 and heterodimer RT elution was achieved by application of a linear gradient of 0 (E1) to 400 mM imidazole (E20) in buffer B. His-p51 RT quantitatively binds to the column, as almost no material is found in the flow-through fraction; conversely, a considerable proportion of poly-histidine-free p66 RT flows through the column. However, a portion of this polypeptide, presumably that reconstituted into heterodimer, co-elutes with His-p51 (lanes E6−E9). Fractions E6−E9 were pooled and purified further as described in Materials and methods.

p66 subunit (i.e. single Asp185→Asn, Asp186→Asn substitutions and the double Asp185/Asp186→Asn substitution) were practically devoid of activity, whereas those containing an equivalently mutated version of His-p51, complexed with a wild type p66 subunit, demonstrated wild type or only moderately reduced levels of RT activity (Table II). The control preparation of reconstituted heterodimer ($p66^{DD}$/His-$p51^{DD}$), with both subunits in wild type configuration displayed 70% of the specific activity of the heterodimer produced by PR-mediated maturation of His-p66 RT (Le Grice and Grüninger-Leitch, 1990). The observation that reconstituted heterodimer in which the p51 subunit is mutated in either Asp185 or Asp186 retains high levels of RNA-dependent DNA polymerase activity suggests that these residues are dispensable in the heterodimer-associated p51. However, the reciprocal reconstitutions (i.e. those in which p66 is mutated) display maximally 2.5% of the RNA-dependent DNA polymerase activity of the control preparation, indicating that a wild type p51 cannot compensate for mutations introduced into the active site of p66. Despite these observations, we cannot exclude the possibility that another region of p51 may contribute to catalysis.

The differences observed in specific RT activity between the reciprocal reconstitutions cannot be ascribed to differential stability of the corresponding heterodimers, since the subunits do not dissociate when subjected to a second round of metal chelate affinity chromatography, i.e. poly-histidine-free p66 still co-elutes with His-p51 following application of the imidazole gradient (data not shown). Although it is possible that minor distortions in quaternary structure of the various reconstituted heterodimers may have been induced by the mutations, it is unlikely that these should be responsible for the striking decrease in activity we have observed. Moreover, no significant differences could be detected in the RNaseH activities of the same preparations

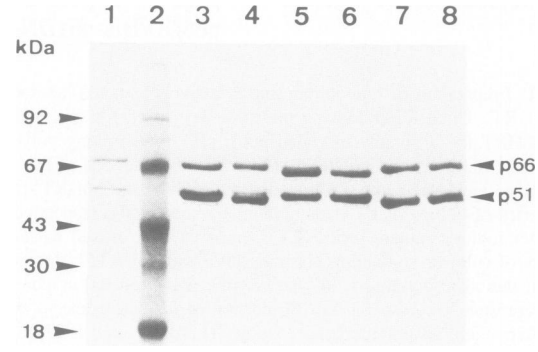

Fig. 4. Analysis of purified reconstituted heterodimers. 2 μl of the final preparations were fractionated through a 10% polyacrylamide−SDS gel and stained. The gel is overloaded in order to visualize residual trace impurities. For unknown reasons, the p66 and His-p51 bands migrate slightly differently for certain of the mutated enzymes. Lane 1, control His-p66/His-p51 RT; lane 2, protein M_r marker; lane 3, reconstituted $p66^{DD}$/His-$p51^{DD}$, i.e. wild type heterodimer; lane 4, reconstituted $p66^{ND}$/His-$p51^{DD}$; lane 5, reconstituted $p66^{DN}$/His-$p51^{DD}$; lane 6, reconstituted $p66^{NN}$/His-$p51^{DD}$; lane 7, reconstituted $p66^{DD}$/His-$p51^{ND}$; lane 8, reconstituted $p66^{DD}$/His-$p51^{DN}$.

Table II. RT and RNaseH activities of the reconstituted heterodimers

Heterodimer Mutant	RT (% of wt)	RNase H (% of wt)
$p66^{DD}$/His-$p51^{DD}$	100	100
$p66^{ND}$/His-$p51^{DD}$	2.1	65.4
$p66^{DN}$/His-$p51^{DD}$	0.7	98.6
$p66^{NN}$/His-$p51^{DD}$	2.5	91.9
$p66^{DD}$/His-$p51^{ND}$	120.9	99.9
$p66^{DD}$/His-$p51^{DN}$	54.2	100.8

Activities are expressed as percentage of wild type reconstituted RT which is taken as 100%.

(Table II). From these combined observations, the most likely explanation is that both Asp185 and Asp186 of heterodimer-associated p66 HIV-1 RT are directly involved in a catalytic step which is blocked in the mutants.

Discussion

We have presented evidence that enzymatically active HIV-1 RT heterodimers can be reconstituted from bacterial strains independently expressing the constituent polypeptides. This procedure affords a new approach to define the function of individual subunits by 'subunit-selective mutagenesis'. Differential labelling of the subunits with a small poly-histidine extension selects the desired heterodimer from the homodimer by-products. Such a strategy should be generally applicable with several other proteins similarly capable of forming a heterodimer. Given a compatible dimerization interface, it should also possible to construct 'chimeric' heterodimers consisting of subunits derived from different species. Such chimerae could facilitate the analysis of species-specific differences in parameters, for example reaction kinetics, substrate specificity or inhibition characteristics. In this respect, enzymatically active chimeric HIV-1/HIV-2 RT heterodimers have recently been prepared and analysed for their response to an inhibitor specific for the HIV-1 enzyme (K.Howard, S.Le Grice *et al.*, manuscript submitted) The degree of purity of the reconstituted heterodimers could be further enhanced if a positive selection is available for the dimerization partner (e.g. affinity purification via a monoclonal antibody coupled to a matrix).

Using this approach, we have investigated a possible enzymatic function for the 51 kDa HIV-1 RT subunit in a reconstituted heterodimer. We have established that heterodimer in which the carboxyl side chains of the active site aspartic acid residues of p66 are exchanged with the corresponding amide groups are inactive. In contrast, analogous exchanges in the 51 kDa polypeptide yields heterodimeric RT displaying full or only moderately reduced RNA-dependent DNA polymerase activity. In addition, each of these mutations is achieved with minimal perturbation of both the RNaseH and $tRNA^{Lys,3}$ binding activities, which argues strongly that, despite the introduction of point mutations, the RT subunits have been reconstituted in the correct orientation. The inability of p51 RT to complement a defective p66 in the context of heterodimer suggests that the low intrinsic polymerase activity of p51 is not enhanced following dimerization. One possible explanation of our data is that heterodimer-associated p51 RT, rather than contributing an enzymatic function, serves to modify p66 allosterically, allowing optimal expression of its polymerizing and RNaseH functions. This does not exclude possible other roles for p51, e.g. selection of the + strand primer during reverse transcription of genomic RNA (Huber and Richardson, 1990).

Our results confirm earlier observations that p51 HIV-1 RT has very low polymerizing activity (Hansen *et al.*, 1988; Hizi *et al.*, 1988; Starnes *et al.*, 1988; Tisdale *et al.*, 1988) but are in seeming conflict with a recent report demonstrating substantial levels of activity in the purified polypeptide (Restle *et al.* 1990). The latter authors argue that activities of the different RT forms (p66, p51 and p66/p51) might reflect the amount of enzyme present in dimer conformation, and that this proportion varies depending on both the storage and assay conditions. According to these authors, only p66/p51 forms a stable heterodimer, whereas p66 homodimers are less stable and p51 homodimers extremely unstable. p51 alone has been demonstrated to have appreciable amounts of RT activity when in dimeric form (in the work of Restle *et al.*, 1990, the RT activity observed in a partially dimerized p51 is linearly extrapolated to 100% dimer content). While this may explain why we do not detect appreciable activity in our p51 preparations, another discrepancy remains unsolved: why do we observe almost no RT activity in the reconstituted heterodimers containing a mutant p66 and a wild type p51, whereas the reciprocal combinations are practically unaffected? Although we lack data on the conformation of p51 in a homodimer, we would speculate that one of the p51 subunits in homodimer p51 prepared by Restle *et al.* (1990) may adopt a p66-like 'active' conformation. In the case of the heterodimer, only p66 would be in this 'active' conformation, possibly stabilized by an interaction of the C-terminal RNaseH portion with an as yet unidentified part of p51 (cf. Hostomsky *et al.*, 1991). If such interactions do indeed exist, they might also explain why the heterodimer species are more stable than the homodimer forms. With the aid of monoclonal antibodies, such interactions might be detectable.

In the present communication, we have not addressed the question of factors involved in heterodimer formation. However, by following a similar approach, it should be possible to introduce mutations within the putative 'leucine-zipper' motif present in both the HIV-1 and HIV-2 enzymes (between amino acids 281 and 310) and determine whether the mutated polypeptide is capable of interacting with its non-mutant counterpart. Since chimeric heterodimer reconstitution is possible (K.Howard, S.Le Grice *et al.*, manuscript submitted), the property of dimerization can be evaluated in the context of such chimerae. Such experiments illustrate the potential of the approach of subunit-selective mutagenesis.

Materials and methods

Plasmid constructions

The polypeptides expressed from recombinant plasmids, pRT (p66/p66), p6HRT (His-p66/His-p66, Le Grice and Grüninger-Leitch, 1990) and p6HRT51 (His-p51, Schatz *et al.*, 1990) are outlined in Figure 1. p51 RT was constructed by insertion of a translational stop codon between Phe440 and Tyr441, determined as the p66/p51 RT cleavage site recognized by HIV-1 protease (Mizrahi *et al.*, 1989; Le Grice *et al.*, 1989; Graves *et al.*, 1990; Becerra *et al.*, 1990). The poly-histidine extension on the N-termini of RT produced by plasmids p6HRT and p6HRT51 allows their isolation by Ni^{2+}–NTA–Sepharose metal chelate affinity chromatography (Hochuli *et al.*, 1987, 1988). The N-terminal extension does not affect the enzymatic properties of HIV-1 RT (Le Grice and Grüninger-Leitch, 1990).

The active site double mutant (p6HRT, NN) was constructed by oligonucleotide directed mutagenesis of a 1.7 kb *Bam*HI–*Hin*dIII RT fragment in M13 according to protocols suggested by the supplier (Amersham). Double-stranded mutated M13 DNA was cut with *Bam*HI and *Hin*dIII and the resulting 1.7 kb fragment transferred into the analogously digested parental plasmid. The integrity of the construct was verified by DNA sequencing. This mutagenesis procedure (Asp185→Asn, Asp186→Asn) introduces a *Sna*BI site as a silent mutation. Using mutant oligonucleotide primers that reconstructed half of this *Sna*BI site, and an oligonucleotide hybridizing at an upstream *Eco*RV site, 130 bp fragments were synthesized by the polymerase chain reaction (PCR) that could be cloned into *Eco*RV–*Sna*BI-cleaved p6HRT, NN, generating p6HRT, DN and p6HRT, ND. The integrity of each mutant was verified by DNA sequencing.

The *E.coli* strain M15::pDMI.1 (Certa *et al.*, 1986) was used throughout this work, permitting inducible gene expression under the control of the

lac regulatory elements. Upon introduction of the appropriate plasmid, recombinant bacteria were selected on LB-agar plates containing 25 μg/ml kanamycin and 100 μg/ml ampicillin. For induction of gene expression, cultures were grown in antibiotic-supplemented Luria-broth until A_{600nm} = 0.8, then IPTG added to a final concentration of 400 μg/ml, thereafter allowing a further 5 h growth.

Purification of reconstituted RT

Ni^{2+}–NTA–Sepharose metal chelate affinity chromatography. Pellets of His-p51 and p66 RT-producing recombinant bacteria were resuspended at 2 ml/gm wet weight in 50 mM Na_2HPO_4/NaH_2PO_4, pH 7.8 (buffer A) and homogenized as previously described (Le Grice and Grüninger-Leitch, 1990). Following high speed centrifugation (100 000 *g*, 30 min, 4°C), the supernatant was carefully decanted and applied to a 10 ml Ni^{2+}–NTA–Sepharose column. The column was washed with 40 ml of buffer B (200 mM NH_4Ac, pH 6.2, 300 mM NaCl) and RT forms eluted with a 40 ml gradient from 0 to 400 mM imidazole in buffer B. 2 ml fractions were collected and analysed for RT content by SDS–PAGE. The fractions containing both His-p51 and p66 were pooled and concentrated by overnight dialysis against 100 vol storage buffer D [50 mM Tris–HCl, pH 7.0, 25 mM NaCl, 0.5 mM DTT, 1 mM EDTA, 50% (v/v) glycerol].
DEAE–Sepharose ion exchange chromatography. The RT-containing dialysate from Ni^{2+}–NTA–Sepharose was applied to a 10 ml DEAE–Sepharose FF column (Pharmacia), previously equilibrated with buffer D containing 10% (v/v) glycerol, at a flow rate of 0.8 ml/min. Under these conditions, RT was recovered in the non-binding fraction. Without analysis, the entire material in the column flow-through was pooled and further purified.
S–Sepharose ion exchange chromatography. Flow-through material from DEAE–Sepharose was applied immediately to an S–Sepharose column similarly equilibrated in buffer D containing 10% (v/v) glycerol. Following extensive washing of the column with the same buffer, RT was eluted by applying a gradient of 0–0.6 M NaCl in buffer D containing 10% (v/v) glycerol. All material eluting in the salt gradient was analysed by SDS–PAGE, and fractions containing heterodimer with an approximate 1:1 subunit stoichiometry pooled, concentrated by dialysis against storage buffer D (i.e. containing 50% glycerol) and stored at −20°C.

Control preparations of His-p66/His-p51, His-p66 and His-p51 RT were purified by the same procedure. For analysis of RT purity, samples fractionated by SDS–PAGE were stained with Coomassie Brilliant Blue and scanned with an LKB Ultroscan laser densitometer, using successive lateral displacements, and the peaks integrated with a Hewlett Packard recording integrator. The purification scheme employed yielded RT preparations exceeding 90% purity.

Electrophoretic and immunological methods

Following induction of protein synthesis, 1 ml culture aliquots were removed at hourly intervals, centrifuged 1 min in an Eppendorf centrifuge, and the pellets resuspended in 200 μl SDS–PAGE sample buffer (Laemmli, 1970). After heating at 100°C for 10 min, 10 μl aliquots were loaded onto 10% SDS–polyacrylamide gels containing a 3.3% stacking gel. Following electrophoresis, fractionated proteins were visualized by staining for 10 min in Coomassie Brilliant Blue and destained for 2 h at 65°C. For immunological analysis, fractionated proteins were transferred to nylon membranes according to the method of Towbin *et al.* (1979), then assayed with rabbit polyclonal antibodies raised against p66 HIV-1 reverse transcriptase. Colorimetric determination of immunoreactive polypeptides was accomplished using an alkaline phosphatase-coupled second antibody (Bio-Rad).

Enzyme assays

Reverse transcriptase. RT assays were performed in 30 μl reaction mixtures containing the following: 50 mM Tris–HCl, pH 8.0, 80 mM KCl, 5 mM dithiothreitol (DTT), 6 mM $MgCl_2$, 0.05% (w/v) Triton X-100, 50 μM dGTP, 0.5–1.0 μCi [α-^{35}S]dGTP, 0.025 units poly(rC)p$(dG)_{12-18}$ (Pharmacia) and 25, 50 or 100 ng RT. After 15 min at 37°C, 7.5 μl aliquots were spotted onto DEAE filtermats (Wallace-Oy, Finland) and dried. Filtermats were washed twice in 2×SSC, once with ethanol, dried and covered with a Meltilex™ solid scintillation sheet (Wallace-Oy, Finland). After melting of the scintillation sheet, the radioactivity incorporated into polynucleotide was determined in a Beta plate counter.

We define one unit of RT activity as that amount catalysing incorporation of 1 nmol precursor dGTP into polynucleotide in 10 min under the conditions described above.
RNaseH. RNaseH reactions were performed in 100 μl reaction mixes, in a buffer of 50 mM Tris–HCl, pH 8.0, 5 mM DTT, 80 mM KCl, 6 mM $MgCl_2$ and a synthetic radiolabelled DNA/RNA hybrid [prepared by *in vitro* transcription of poly(dC) with *E.coli* RNA polymerase in the presence of [^{3}H]GTP (7 Ci/mmol, Amersham): under such conditions, the nascent RNA remains hybridized to the template DNA strand] and 50 ng RT. After 5, 10 and 20 min incubation at 37°C, duplicate 7.5 μl aliquots were removed and spotted onto DEAE filtermats. Processing and determination of radioactivity remaining in polynucleotide form were as described above.

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