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Reverse Transcriptase of Human Immunodeficiency Virus Type 1: Functionality of Subunits of the Heterodimer in DNA Synthesis

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From an in vitro analysis of the DNA-synthesizing abilities of certain specifically mutated forms of the heterodimeric reverse transcriptase of human immunodeficiency virus type 1, we can conclude that in a heterodimer, the functionality of p66 is necessary while the functionality of the p51 subunit is not needed. Conversely, p51 is not able to catalyze DNA synthesis when associated with p66, and yet when the p66 protein is absent, p51 can function. These conclusions applied to DNA synthesis on heteropolymeric RNA and DNA templates.

For a number of different retroviruses, the reverse transcriptase (RT) has been purified and characterized. As reviewed elsewhere (4), for most of the retroviruses, such as avian myeloblastosis virus and human immunodeficiency virus type 1 (HIV-1), there is clear evidence that the RT is normally active as a dimer. This dimer is usually a heterodimer of related subunits with identical amino termini. An apparent exception to the rule is the RT of Moloney murine leukemia virus, which has been claimed to function as a monomer, even though a smaller subunit can also be found inside virus particles (9).

For HIV-1, the RT is a heterodimer composed of two subunits, p51 and p66, which have identical amino termini (3, 13), with p51 lacking the carboxy-terminal RNase H domain. Since both of these subunits contain the polymerase domain, it is of interest to determine the role of the each subunit in DNA synthesis. A partial answer has already been obtained by means of certain HIV-1-specific inhibitors; apparently these bind to a specific site on p66, and this binding is associated with inhibition of RNA-directed DNA synthesis (18). In other experiments, cross-linking of template-primer and thymidine triphosphate substrates occurred exclusively to the p66 subunit of heterodimeric HIV-1 RT, suggesting that the polymerase activity resides on this subunit (1). As explained below, we applied a different strategy to test the roles of each subunit in both RNA- and DNA-directed DNA syntheses. After our study was initially submitted for publication, Le Grice et al. (12) reported the use of a similar approach, with a similar conclusion.

The key to our study was to synthesize heterodimers of HIV-1 RT in which one or the other subunit was specifically inactivated for polymerase function. To do this, we made use of the observation that mutation of amino acid 110 from aspartic acid to glutamine can completely inactivate the polymerase function (11). To obtain appropriate mixed heterodimers, we made plasmid constructs containing both p51 and p66 encoded as separate genes. This system was based on a study by Müller et al. (16) demonstrating that coexpression of p51 and p66 polypeptides in *Escherichia coli* leads to formation of a stable heterodimeric form of HIV-1 RT with no need for proteolytic processing. Unlike the plasmid used in their work (16), our expression construct contained genes for p51 and p66 subunits transcribed from a single promoter as a bicistronic mRNA (Fig. 1A). Owing to the significantly faster association of the p51 and p66 subunits and the high stability of the resulting p51-p66 heterodimer, compared with the slow formation of the much less stable p66-p66 homodimer (17), we expected the mixed heterodimer to be the predominant product of the coexpression. To ensure that every p66 would be associated with p51 before the former could undergo proteolytic processing, the p51 gene was placed upstream of the p66 gene. This tandem arrangement led to a slight molecular excess of p51 over p66 during expression (7). Free, uncomplexed p51 was then separated from the main heterodimeric product in the course of our previously described purification procedure (8). The heterodimeric character of the final enzyme preparation was confirmed by analytical gel filtration (7).

As indicated in Fig. 1B, this enabled us to mutate either subunit and, after expression in *E. coli*, purify mixed heterodimers of the desired composition. We also chose to test heterodimers in which p66 was mutated in the RNase H domain; this was done by changing active-site residues 443 and 498 from aspartic acids to asparagines, mutations reported to abolish RNase H activity completely without affecting the DNA polymerase function (15). In this way, we obtained the wild-type and three mutated forms of the HIV-1 heterodimer. As a control, we tested a fifth species that contained only wild-type p51; this protein behaved as a monomer during purification (8), although it is possible that it subsequently functioned as a dimer (17).

We tested the activity of each of these five polymerases with two primer-template complexes. For each complex, the template was a 401-nucleotide heteropolymeric sequence and the end-labeled primer was 14 nucleotides long. This primer was positioned such that the product of a primer extension assay would be 222 nucleotides long. The only difference between the two complexes was in the sugar moiety of the template strand; it was either ribose (RNA) or deoxyribose (DNA).

Reactions were carried out with comparable rate-limiting concentrations of the five different enzymes; that is, such that the amount of the product was linearly dependent upon the amount of the enzyme used. These reactions were run for 1 h at 37°C, after which the products were extracted, denatured, and submitted to electrophoresis in a urea-con-

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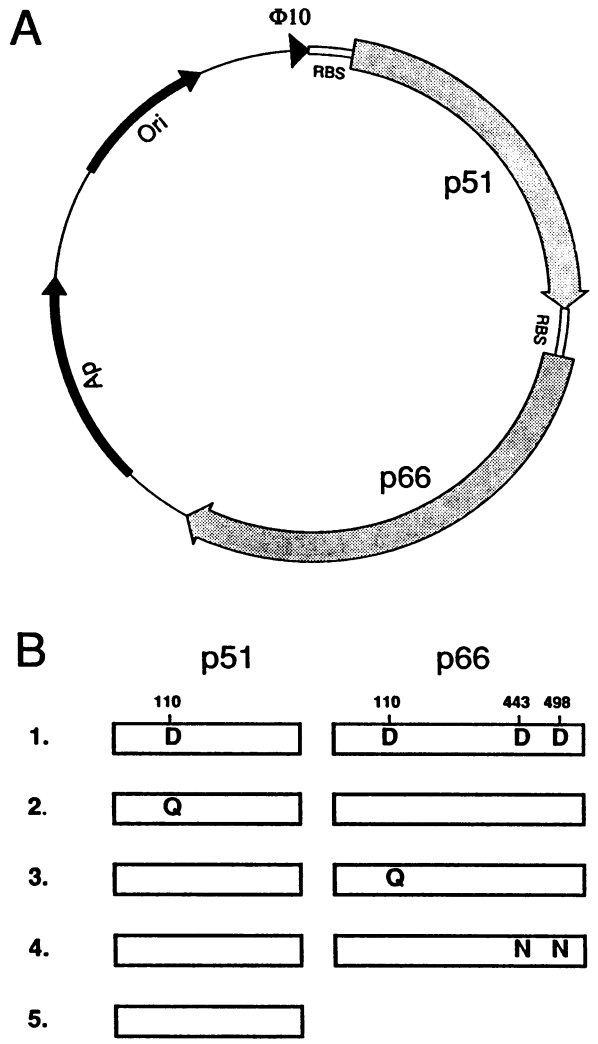

FIG. 1. Construction of various forms of HIV-1 RT. (A) Schematic representation of the typical plasmid construct used for synthesis of a mixed heterodimer of the RT. As indicated, the construct contained an origin of replication (Ori) and the ampicillin resistance gene (Ap). Induction of *E. coli* harboring such a construct was done as previously described (6). Expression was under control of the ϕ10 promoter of bacteriophage T7, leading to synthesis of a bicistronic mRNA. Each cistron had its own ribosome-binding site (RBS), as derived from the *E. coli* gene for dihydrofolate reductase (6). Translation of each subunit was initiated at an AUG codon immediately followed by Pro-1 of the HIV-1 RT sequence. Termination codons were introduced after Phe-440 for p51 and Leu-560 for p66. (B) Summary of the subunit compositions of the four different RT constructs that were synthesized in this way. Three of these have critical mutations in specific subunit domains, as described in the text. Positions of certain catalytically important aspartic acid residues (D), at the indicated residue positions, were changed, as indicated, to either glutamine (Q) or arginine (N). The fifth construct, which contains only the p51 subunit, was expressed by a different method, as previously described (8).

taining gel of 6% acrylamide. After electrophoresis, the gel was dried and quantitated directly. Typical results are shown in Fig. 2, and a quantitation of the full-length primer extension products is summarized in Table 1.

Consider first the results obtained with the wild-type RT heterodimer. It appears from Table 1 that this RT activity

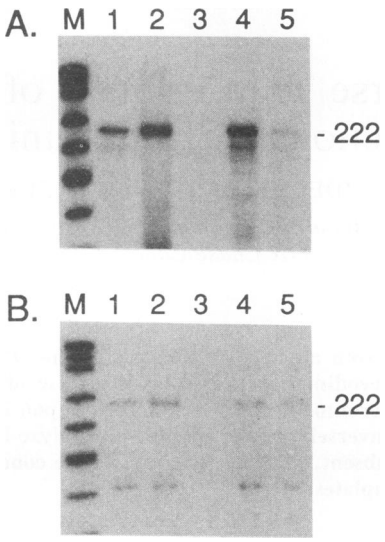

FIG. 2. Assay of the abilities of various forms of HIV-1 RT to carry out primer extension on RNA and DNA templates. The reactions were in 20 μl of buffer containing 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM dithiothreitol, and 1 mM deoxynucleoside triphosphates. To this was added 50,000 cpm of end-labeled DNA primer (10 pmol) that had been prehybridized to either a DNA or an RNA template (0.1 pmol). The 401-base RNA was synthesized in vitro from a DNA template by using T7 RNA polymerase, as recommended by the manufacturer (B.R.L.), and then gel purified. The DNA was synthesized from the same region of the above-described template but with the use of two oligonucleotide primers in an asymmetric polymerase chain reaction (14), after which the product was gel purified. Reactions were initiated by addition of the purified RT constructs, as described in Fig. 1, to a 50-μg/ml final concentration. After 1 h at 37°C, the reaction products were extracted, denatured, and submitted to electrophoresis on two gels of 6% polyacrylamide containing 6 M urea. The gels were dried and submitted to autoradiography, as shown, or direct quantitation, as presented in Table 1. Panels A and B show the results obtained with the RNA and DNA templates, respectively. The lane numbers correspond to the RTs used, as designated in Fig. 1. Lane M shows the mobilities of end-labeled fragments of a plasmid DNA standard. At the right is indicated the mobility of the full-length 222-base primer extension product.

was 2.2-fold higher with the RNA than with the DNA template. As discussed later, this might be a consequence of different levels of processivity.

When tested in parallel, the heterodimer with a mutation in p51 gave similar results. However, the heterodimer with a

TABLE 1. Quantitation of primer extension assays

RT construct[a]	Activity[b] on the following template:		RNA/DNA ratio
	RNA	DNA	
1. p51/p66	1,550	580	2.6
2. p51(D110Q)/p66	1,790	440	4.0
3. p51/p66(D110Q)	<40	<10	
4. p51/p66(D443N, D498N)	4,300	480	9.0
5. p51	620	160	3.8

[a] The RT constructs are described in Fig. 1.

[b] The assays were done as described in the legend to Fig. 2, and quantitation of radioactivity in the primer extension products was performed directly from the dried gel by using a Radioanalytic Imaging System (AMBIS, San Diego, Calif.). The data represent averages of two such experiments.

mutation in the polymerase domain of p66 showed no activity with either RNA or DNA. Activity was undetected even with 20 times more enzyme per reaction (7).

These data suggest that only p66 needs to be functional in the heterodimer for either RNA- or DNA-directed DNA synthesis. However, as shown by results obtained with only p51, in the absence of p66, the smaller subunit does exhibit polymerase activity on both nucleic acid templates. Thus, we might conclude that p51 per se is active but in the presence of p66, to make a heterodimer, the activity of p51 is somehow masked.

One possible interpretation of the observed dual mode of p51 functionality, dependent upon the presence or absence of the p66 subunit, is that p51, upon association with p66, assumes a different, nonfunctional conformation in which mutations of active-site residues become irrelevant. Of the two DNA polymerase domains present in the heterodimer, only one, that of the p66 subunit, would then be in a functional conformation.

The implied conformational asymmetry in the heterodimer would be consistent with the model proposed to explain the asymmetric processing in HIV-1 RT (5). In this model, the unequal cleavage by proteases is a consequence of a prior asymmetric arrangement of the subunits in the homodimer precursor of HIV-1 RT, leading to partial unfolding of one of the RNase H domains (2), which enables the subsequent processing to generate the p51 subunit.

In these studies, we also tested a heterodimer of the mutated RNase H domain; it functioned as well as the wild-type RT on a DNA template and about two to three times more efficiently on an RNA template relative to the RT forms with a functional RNase H. The apparent increased efficiency on an RNA template of RT mutated in RNase H might be tentatively explained by the absence of RNase H activity, which could otherwise cleave some template molecules in the region complementary to the primer before DNA synthesis could even begin.

Finally, it might be noted that in our assays, not all of the primer extensions led to full-length DNA products; there were some less-than-full-length species (Fig. 2). This was more obvious on DNA than RNA templates, a finding consistent with the previous report of Huber et al. (10), who showed that the HIV-1 RT was less processive on single-stranded DNA than on RNA templates. If the less-than-full-length species seen in Fig. 2 are valid indicators of the processivity of the enzymes, then we might conclude that of the RT mutations tested here, with the exception of the p51/p66(D110Q) mutant, there were no concomitant alterations in processivity.

As mentioned earlier, since the submission of this report, Le Grice et al. (12) have reported a similar study. Our results confirm their basic conclusion. Four main differences between the two studies need to be stressed. (i) We mutated a single aspartic acid residue at position 110, whereas they changed aspartic acids at 185 and 186. (ii) They obtained mutant heterodimeric RTs by use of a fusion protein and mixing of separate bacterial lysates; we did not use a fusion protein, and both subunits were expressed from a single plasmid, allowing heterodimers to form within the bacterial cell. (iii) They tested RT functionality with only a homopolymeric RNA template, whereas we used both a heteropolymeric RNA and an identical sequence of DNA. Our data thus exclude the possibility that within the heterodimer p51 can function to copy not RNA but DNA. (iv) Their p51, even without mutation, was nonfunctional, whereas we found that on both RNA and DNA templates p51 alone could function. This supports the hypothesis that within the heterodimer, p51 assumes an altered conformation (2). In conclusion, both studies have shown that certain specific mutations in the p51 subunit of a p51/p66 heterodimer can be tolerated while the converse, with the same mutations in p66, cannot. Nevertheless, it is important to note that we cannot say whether or not the functionality of the HIV-1 RT is, at some higher level, dependent upon formation of the heterodimer.

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