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Reconstitution *in vitro* of RNase H activity by using purified N-terminal and C-terminal domains of human immunodeficiency virus type 1 reverse transcriptase

(AIDS/retrovirus/DNA polymerase/trans-complementation)

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ABSTRACT **Two constituent protein domains of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase were expressed separately and purified to homogeneity. The N-terminal domain (p51) behaves as a monomeric protein exhibiting salt-sensitive DNA polymerase activity. The C-terminal domain (p15) on its own has no detectable RNase H activity. However, the combination of both isolated p51 and p15 *in vitro* leads to reconstitution of RNase H activity on a defined substrate. These results demonstrate that domains of HIV-1 reverse transcriptase are functionally interdependent to a much higher degree than in the case of reverse transcriptase from Moloney murine leukemia virus.**

Reverse transcription of the viral genomic RNA into the double-stranded DNA of the provirus is an essential step in retrovirus replication. This complex process is catalyzed by a virus-encoded multifunctional enzyme, reverse transcriptase (RT) (1, 2). Three distinct enzymatic activities associated with RT have been detected—namely, RNA-dependent DNA polymerase, DNA-dependent DNA polymerase, and RNase H (3–8). Each of these activities seems to be essential for replication of human immunodeficiency virus type 1 (HIV-1). Thus, RT represents an attractive target for chemotherapy of AIDS.

The RNase H activity associated with RT is required at several stages in reverse transcription of viral genomic RNA. It is involved in selective degradation of RNA in the intermediate RNA·DNA hybrid and in generation of an RNA primer for synthesis of plus-strand DNA (3, 6). The RNase H activity is also responsible for specific removal of the host tRNA primer as well as of the plus-strand primer by specific cleavage at RNA·DNA junctions (7, 8).

A computer-aided comparison of the amino acid sequences of various RTs with two *Escherichia coli* enzymes of similar function (RNase H and α subunit of RNA polymerase) led to the proposal that RNase H activity resides at the C terminus of the RT. The N terminus contains DNA polymerase activities (9). This functional organization was later confirmed for murine leukemia virus RT by mutational analysis (10–13). Both DNA polymerase and RNase H domains of Moloney murine leukemia virus (MoMuLV) RT were also expressed separately and were shown to exhibit the expected enzymatic activities independent of each other at levels basically comparable to the levels found in the wild-type enzyme (10).

RT activity isolated from HIV-1 virions is associated with two polypeptides of 66 and 51 kDa (p66 and p51), which have identical N termini but p51 is C-terminally truncated (14, 15). Studies on recombinant HIV-1 RT expressed in *E. coli* show that the subunits form a heterodimer (16, 17), which apparently arises by proteolytic processing of one of the subunits of the p66/p66 homodimer. The resulting p66/p51 heterodimer appears to be the mature and the most stable and active form of HIV-1 RT (16, 18).

Although the overall domain organization as found in MoMuLV RT seems to be valid also for RT of HIV-1, the assignment of functions to these domains is less clear. It has been observed in several laboratories (19–21), including our own, that absence of the C-terminal region of HIV-1 RT, corresponding to the putative RNase H domain, leads to a decrease of DNA polymerase activity. Mutations that affect RNase H activity are not clustered in a single domain, but rather they are distributed along the whole p66 molecule. With only a few exceptions, mutations affecting RNase H activity also have a strong negative effect on DNA polymerase function (22, 23).

All this information indicates that the two domains of HIV-1 RT, though structurally distinct, are functionally interdependent. To examine more closely the functional relationship of the N-terminal p51 and the C-terminal p15 domains, we decided to express and purify them separately. In this report, we demonstrate that p15 as an isolated domain has no detectable RNase H activity. However, the addition of the purified p51 domain to this protein leads to the reconstitution of HIV-1 RNase H activity *in vitro*.

MATERIALS AND METHODS

Enzymes. The following enzymes were used: avian myeloblastosis virus (AMV) RT (Promega), MoMuLV RT (Stratagene), *E. coli* RNase H (Pharmacia), RNase A, and α-chymotrypsin type I-S (Sigma).

Expression Clones. A schematic outline of the construction of the expression plasmids is given in the legend to Fig. 1. The *E. coli* strain AP401 (24) harboring the plasmid constructs was induced for expression by infection with phage mGP1-2, an M13 derivative, which carries a gene for T7 RNA polymerase. This expression system was provided by S. Tabor (25).

Purification of RT. Soluble lysates of induced *E. coli* containing RT activity were subjected to heparin Sepharose CL-6B chromatography in 50 mM Tris·HCl, pH 8.0/1 mM dithiothreitol. In a gradient of 0–1 M NaCl, RT eluted at 0.6 M NaCl. After dialysis, the RT sample was applied to a FPLC Mono Q column in 25 mM diethylamine buffer, pH 8.5/1 mM dithiothreitol. In a gradient of 0–250 mM NaCl, RT eluted at 170 mM NaCl. Fractions of active RT, 90% pure at this stage, were applied to a hydroxylapatite column (Bio-Gel HT) in 25 mM sodium phosphate/1 mM dithiothreitol/100 mM NaCl.

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Abbreviations: AMV, avian myeloblastosis virus; DHFR, dihydrofolate reductase; HIV-1, human immunodeficiency virus type 1; MoMuLV, Moloney murine leukemia virus; RT, reverse transcriptase.

Pure RT eluted with 200 mM sodium phosphate, pH 6.8/200 mM NaCl/1 mM dithiothreitol.

Purification of Dihydrofolate Reductase (DHFR)–p15 Fusion Protein. Fusion protein was purified from a soluble lysate of induced *E. coli* harboring plasmid pBKMH5 by affinity chromatography on methotrexate/agarose as described (26) and subsequent gel filtration on Sephacryl S-200 in 25 mM Tris·HCl, pH 8.0/1 mM dithiothreitol/25 mM NaCl (buffer A).

Isolation of p15 Domain. Purified DHFR–p15 fusion protein (BKMH5) at 2 mg/ml was processed with α-chymotrypsin (final concentration, 10 μg/ml) in buffer A on ice for 6 hr. The cleaved sample was applied to FPLC Mono S in buffer A to remove chymotryptic activity. The flow-through, containing DHFR, p15, and unprocessed fusion protein was directly applied to FPLC Mono Q in buffer A. Pure p15 protein was found in the flow-through.

N-Terminal Amino Acid Sequence Analysis. N-terminal sequences of selected fragments were determined after separation on SDS/polyacrylamide gel and electroblotting onto poly(vinylidene difluoride) membranes (27).

Enzyme Assays. RT activity was assayed by using poly(C)·oligo$(dG)_{12-18}$ at 50 μg/ml (Pharmacia) as primer template and 20 μM [^{3}H]dGTP (New England Nuclear) (10 μCi/ml; 1 Ci = 37 GBq). Reactions were performed in 50 mM Tris·HCl, pH 8.0/6 mM $MgCl_2$/75 mM KCl/5 mM dithiothreitol. The samples were processed basically as described (28) by spotting on DEAE-cellulose filters, washing in 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate), and measuring radioactivity by liquid scintillation counting.

Analysis of RNase H activity was based on the experiments described by Mizrahi and coworkers (29, 30). Descriptions of the RNA·DNA hybrid substrate preparation and of the RNase H assay are given in the legends to Figs. 5 and 6, respectively.

Analytical Gel Filtration. Superose-12 FPLC column (1 × 30 cm) was equilibrated in 50 mM Tris·HCl, pH 7.5/1 mM dithiothreitol/0, 75, or 150 mM NaCl, respectively. One hundred-microliter samples were loaded.

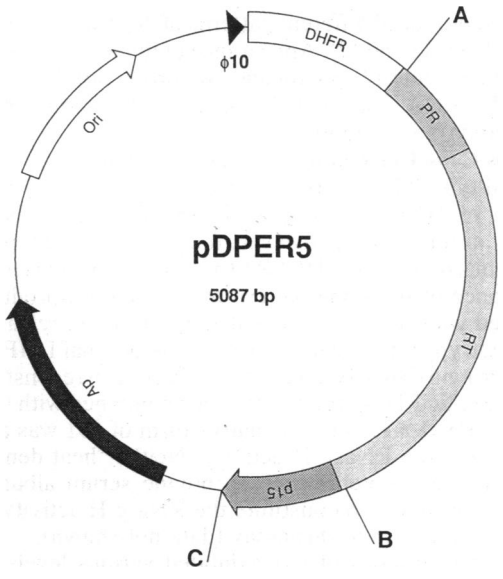

FIG. 1. Schematic representation of plasmid constructs used for expression of HIV-1 RT and its domains. The parent construct for all subsequent manipulations was pDPE3. This plasmid, used for the expression of self-processed soluble HIV-1 protease (23), encodes a DHFR–protease fusion protein under the control of the ϕ10 promoter of phage T7. The synthetic gene for HIV-1 protease (PR) was extended with the RT region derived from the clone BH-10 (31). A stop codon was introduced either after codon Leu-560 of the RT amino acid sequence (construct pDPER5, position C) for expression of wild-type RT or after codon Phe-440 (position B) for expression of the p51 form of RT (construct pDPERTE25). Deletion between positions A and B of the plasmid pDPER5 was the first step in construction of plasmid pBKMH5 used for expression of the DHFR–p15 fusion protein. The C-terminal portion of the RT gene starting from codon Tyr-427 was then fused to the DHFR gene via a synthetic linker, the sequence of which is shown in Fig. 4. bp, Base pairs.

RESULTS

Purification and Characterization of Two Forms of RT. RT was expressed in *E. coli* as part of a larger precursor polyprotein and was processed immediately during expression by HIV-1 protease, which was also a part of this precursor (cf. Fig. 1). Two forms of soluble HIV-1 RT, p66/p51 and p51, were purified to virtual homogeneity (Fig. 2, lanes 3 and 5). The p51 form of RT exhibited only ≈10% of the RNA-dependent DNA polymerase activity of the p66/p51 form in standard reaction conditions. The activity of p51 was found to be sensitive to the presence of potassium chloride as shown in Fig. 3. While the p66/p51 form of RT displayed a maximum activity in 75 mM KCl, the p51 form of RT had its highest polymerase activity in the absence of KCl (28% of the maximum activity of p66/p51). Polymerase activity of p51 decreased steadily with an increasing concentration of KCl. This salt-dependent decrease of activity was reversible.

Analytical gel filtration on Superose-12 demonstrated that the p66/p51 form of RT eluted as a protein with an apparent molecular mass of 130 kDa, which corresponds to a dimer, while p51 eluted as a protein of 50 kDa, indicating that this form of RT is monomeric. Gel filtration performed in the absence of salt (not recommended because of the unspecific interaction of proteins with matrix) showed that p51 eluted as a protein with an apparent molecular mass of 40 kDa.

Isolation of p15 Protein. Our initial attempts to express the C-terminal portion of HIV-1 RT as a single domain resulted in only low levels of this protein after induction of synthesis in *E. coli*. As p15 is synthesized during viral replication as

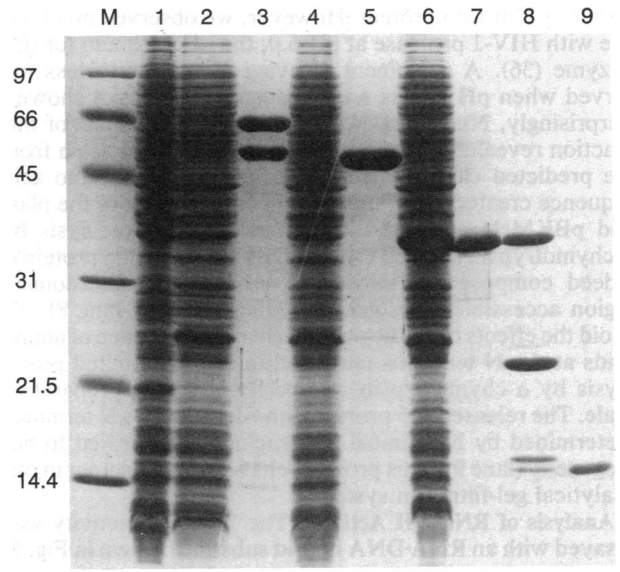

FIG. 2. Purification of HIV-1 RT and its domains. Coomasie blue stained SDS/16% polyacrylamide gel shows total bacterial lysates of uninduced (lane 1) and induced (lane 2) *E. coli* with plasmid pDPER5, and the p66/p51 form of RT (lane 3), purified from this strain; total lysate of induced *E. coli* with pDPERTE25 (lane 4) and the purified p51 domain (lane 5); total lysate of induced *E. coli* with pBKMH5 (lane 6) and purified DHFR–p15 fusion protein BKMH5 (lane 7); 20 μg of BKMH5 (2 mg/ml) after limited proteolysis with α-chymotrypsin (10 μg/ml) for 4 hr on ice (lane 8) and purified p15 domain, released from BKMH5 by α-chymotrypsin (lane 9). Standards are in lane M, with molecular mass in kDa indicated on the left.

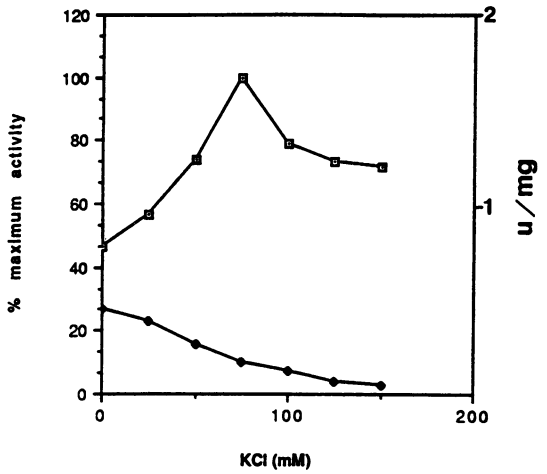

FIG. 3. Effect of KCl concentration on the RNA-dependent DNA polymerase activity of HIV-1 RT. Activity of p66/p51 (□) and p51 (■) forms of RT was assayed under standard reaction conditions, except that the final concentration of KCl was varied as indicated. Units of activity (u) are defined as μmol of incorporated dGMP per min at 25°C.

part of the gag-pol polyprotein, we tried to mimic this aspect of the *in vivo* situation by expressing the C-terminal domain of HIV-1 RT fused to a stable *E. coli* protein. The DHFR–p15 fusion encoded in pBKMH5 indeed turned out to be expressed efficiently in a stable and fully soluble form, representing up to 22% of the total *E. coli* protein after induction (Fig. 2, lane 6). It was reported that cleavage between Phe-440 and Tyr-441 in the p66 subunit of the HIV-1 RT, most probably by HIV-1 protease, is responsible for the formation of the C terminus of p51 as found in the heterodimer p66/p51 (32–34) and the release of the p15 protein, corresponding to RNase H (35). Our original intent was to use HIV-1 protease for *in vitro* release of an authentic p15 from the purified DHFR–p15 fusion protein. However, we observed no cleavage with HIV-1 protease at pH 6.0, the pH optimum for this enzyme (36). A prominent cleavage was nevertheless observed when pH values were around 4.5 (data not shown). Surprisingly, N-terminal sequencing of the products of this reaction revealed that the cleavage occurred upstream from the predicted cleavage site, in a region of the amino acid sequence created arbitrarily during construction of the plasmid pBKMH5 (Fig. 4). Data from limited proteolysis by α-chymotrypsin showed that the DHFR–p15 fusion protein is indeed composed of two domains, with the interdomain region accessible to proteolytic attack (Fig. 2, lane 8). To avoid the effects of acidic pH as well as the presence of amino acids at the N terminus unrelated to RT, the limited proteolysis by α-chymotrypsin was performed on a preparative scale. The released p15 protein with Gln-429 as its N terminus (determined by N-terminal sequencing) was purified to homogeneity (lane 9). This protein behaves as a monomer in our analytical gel-filtration system.

Analysis of RNase H Activity. The RNase H activity was assayed with an RNA·DNA hybrid substrate shown in Fig. 5.

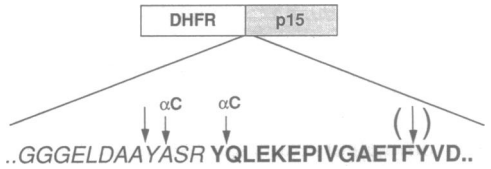

FIG. 4. Amino acid sequence of the junction between the two portions of the DHFR–p15 fusion protein BKMH5. The sequence immediately following the Arg-159 of DHFR is shown. The site cleaved by HIV-1 protease is indicated by a plain arrow. Sites cleaved by α-chymotrypsin are indicated by αC above the arrow. Cleavage reported to generate the C terminus of p51 in the p66/p51 heterodimer (32–34) is shown by an arrow in parentheses. Amino acid sequence newly created by genetic manipulation is in italics. Sequence from the HIV-1 RT region is in boldface.

Incubation of this substrate with different RNase activities led to the appearance of characteristic patterns of degradation products (Fig. 6). Action of RNase H associated with RTs from AMV, MoMuLV, and HIV-1 resulted in all cases in formation of a predominant band ≈28 nucleotides long. Formation of this band may be explained by a combination of endonucleolytic cleavage in the heteroduplex portion of this region as shown by several groups (29, 37, 38), followed by 3′–5′ exonuclease action as proposed recently (39) or, alternatively, by action of a directional endonuclease, which in our assay stops uniformly at position VI (cf. Fig. 5). Extension of the RNA transcript by 8 nucleotides at the 3′ end (by using the *Bam*HI-digested template) did not result in change of the size of the predominant band, further supporting our interpretation that this band originates from the 5′ end of the RNA transcript.

The *E. coli* RNase H and pancreatic RNase A, included as controls, produced different patterns of degradation products (Fig. 6, lanes 11 and 12). Appearance of the RNA band of ≈40 nucleotides (lane 12) is explained by protection from RNase A of the RNA portion hybridized to the complementary oligodeoxyribonucleotide.

Trans-Complementation of RNase H Activity. In contrast to the wild-type RTs described above, the isolated C-terminal domain of HIV-1 RT displayed no detectable RNase H activity in this assay. However, the addition of the purified N-terminal domain of HIV-1 RT (p51) to this system led to the appearance of the same pattern of degradation products as observed with other RTs. Isolated p15 protein, released by α-chymotrypsin cleavage, as well as the original DHFR–p15 fusion protein (BKMH5) were both effective in reconstitution of the specific RNase H activity when combined with the p51 form of HIV-1 RT. Only the native form of p51 was able to complement the RNase H activity. Neither heat denatured p51, nor other proteins such as bovine serum albumin or DHFR, was able to reconstitute the RNase H activity when combined with p15 in this assay (data not shown).

Some preparations of p51 exhibited various levels of residual RNase H activity. Judging from the pattern of degradation products, this activity seems to be caused by contamination with *E. coli* RNase H, which is abundantly present in bacterial lysates. Interestingly, a batch of p51 with a rela-

FIG. 5. Substrate for detection of RNase H activity. A portion of the *gag* region of HIV-1 [nucleotides 629–694 in the nucleotide sequence of BH-10 (31)], shown to contain a cluster of HIV-1 RNase H cleavage sites numbered I–VI (29), was cloned in the plasmid pTZ18R (Pharmacia). Uniformly labeled runoff transcripts (boldface) of this region were prepared from the resulting plasmid with an RNA synthesis kit (Stratagene) and UTP[α-^{35}S]. The 3′ ends of these transcripts were generated by *Pvu* II or *Bam*HI digestions of the template DNA in the positions indicated. The transcripts were gel purified and hybridized with a complementary synthetic oligodeoxyribonucleotide, the sequence of which is shown in italics.

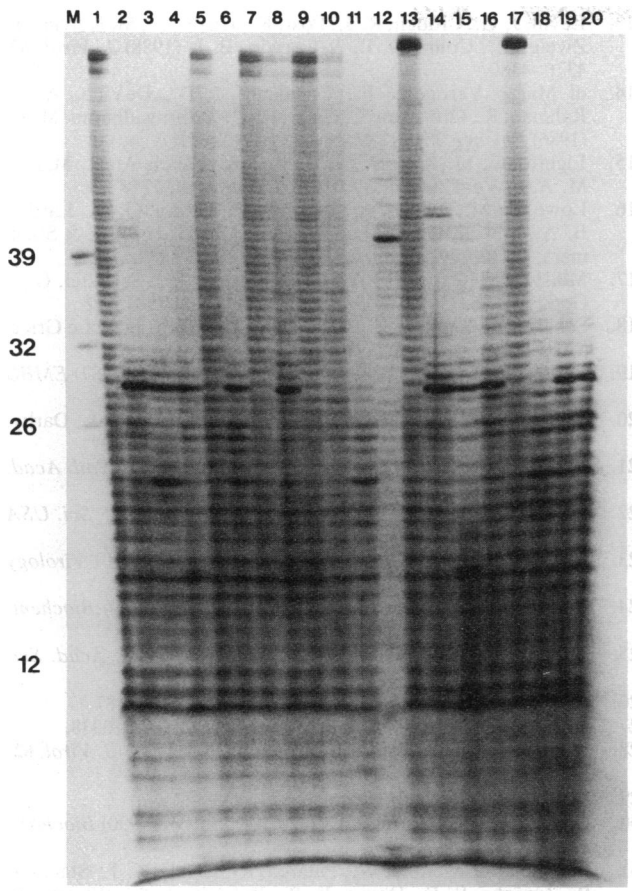

FIG. 6. Analysis of RNase H activity. Three nanograms of hybrid substrate (150,000 cpm) prepared as shown in Fig. 5, with the 3' end from either *Pvu* II (lanes 1–12) or *Bam*HI (lanes 13–20) digested template, was incubated in 10 μl of buffer containing 50 mM Tris·HCl, pH 8.0/50 mM KCl/7 mM $MgCl_2$/5 mM dithiothreitol with the following proteins. Lanes: 1, control with no protein; 2, 9 units of AMV RT; 3, 20 units of MoMuLV RT; 4, 1.5 μg of HIV-1 RT (form p66/p51); 5, 1.3 μg of p51; 6, 1.3 μg of p51 + 1.0 μg of p15; 7, 1.0 μg of p15; 8, 1.3 μg of p51 + 1.5 μg of DHFR–p15 fusion protein (BKMH5); 9, 1.5 μg of DHFR–p15 fusion protein (BKMH5); 10, 2.6 μg of p51 + 2.0 μg of DHFR; 11, 0.008 unit of *E. coli* RNase H; 12, 10 ng of RNase A; 13, control with no protein; 14, 9 units of AMV RT; 15, 1.5 μg of HIV-1 RT (p66/p51 form); 16, 1.3 μg of p51 + 1.5 μg of DHFR–p15 fusion protein (BKMH5); 17, 1.5 μg of DHFR–p15 fusion protein (BKMH5); 18, 1.3 μg of p51; 19, 1.3 μg of p51 + 1.0 μg of p15; 20, 1.3 μg of p51 + 30 μg of DHFR–p15 fusion protein (BKMH5). Batch 13 of the p51 preparation was used in lanes 5, 6, 8, 10, and 16; batch 10 of the p51 preparation was used in lanes 18–20. No RNase inhibitor was used in this analysis. After incubation for 5 min at 37°C, reactions were terminated by adding 2 μl of formamide with bromophenol blue and then boiling for 3 min. Five microliters of the sample was analyzed by electrophoresis in 10% polyacrylamide gel containing 8 M urea. After electrophoresis, the gel was soaked in Amplify (Amersham), dried, and autoradiographed at −70°C. Selected labeled oligodeoxyribonucleotides are in lane M, with size in nucleotides indicated on the left.

tively high level of the presumed contaminating activity, when combined with isolated p15 or with DHFR–p15 fusion, exhibited the same HIV-1 RNase H-specific pattern as the wild-type enzyme (cf. Fig. 6, lanes 18–20). This result may be due to protection of the hybrid substrate from the contaminating activity by the reconstituted RT RNase H.

DISCUSSION

Studies with MoMuLV RT have shown that this enzyme can be divided into two domains: the N-terminal, having DNA polymerase, and the C-terminal, having RNase H, activities. The domains can be expressed separately while retaining their respective activities (10, 11). In the case of HIV-1 RT, which has a clear structural homology with MoMuLV RT as well as with other retroviral RTs, it has been observed that the expression of the p51 DNA polymerase domain without the C-terminal p15 protein leads to a reduction of DNA polymerase activity (19–21). One of the attempts to explain this observation was the proposal that sequences in the C-terminal domain of HIV-1 RT might be important in the proper folding of the DNA polymerase domain (40).

In this communication, we describe expression of p15 that is fused with bacterial DHFR. The data from limited proteolysis by α-chymotrypsin, together with high solubility of the fusion protein when overexpressed in *E. coli*, suggest that the C-terminal p15 portion of this protein forms a distinctly folded domain. We also show that this domain can be released and purified to homogeneity, but it has no detectable RNase H activity. However, when the isolated p15 domain is combined with the purified p51 domain, RNase H activity can be detected with the same RNA processing specificity as that of the wild-type RT. This result shows a close functional relationship among the domains of HIV-1 RT, as suggested previously by mutagenesis studies (22, 23).

One group has reported nonprocessive RNase H activity of a p15 protein derived from the C terminus of the HIV-1 RT (35). In contrast, we describe that the purified p15 protein is enzymatically inactive; however, the RNase H activity in our assay can be reconstituted by the addition of purified p51. The results presented in this paper were obtained with proteins expressed in *E. coli*, whereas the results reported by Hansen *et al.* (35) were based on protein precipitated from the lysate of virus particles by a monoclonal antibody raised against a bacterially expressed C-terminal portion of p66.

As has been shown in several laboratories (16, 17), the most active form of HIV-1 RT is a p66/p51 heterodimer. We can envision two possibilities regarding which of the two p51 domains interacts with the p15 domain. In the heterodimer, the productive interaction can take place either between covalently connected p15 and p51 domains of the p66 subunit (interaction in cis) or between the p15 domain of the p66 subunit and the p51 subunit (interaction in trans).

The productive functional interaction, as demonstrated in our complementation assay, implies a direct structural affinity between the p15 and p51 domains, which may be one of the factors contributing to dimerization of HIV-1 RT. Our observation that the p51 form of RT (the form without the p15 domain) is monomeric leads us to favor this possibility. The productive interaction in the heterodimer would then be of the trans type.

An alternative explanation would involve a cis interaction in which a covalent link between the p51 and p15 domains would be required to mediate a conformational change in the p66 subunit leading to dimerization with the p51 subunit.

In contrast to the reconstitution of the RNase H activity, the combination of the isolated p15 and p51 domains did not result in any increase of the residual DNA polymerase activity that was originally present in the p51 preparation (data not shown). Thus, while the covalent junction between the p15 and p51 domains of the p66 subunit does not seem to be relevant for the productive interaction leading to RNase H activity, this covalent junction seems to be required for high levels of DNA polymerase activity, perhaps because it brings the other p51 domain within close contact with the p51 subunit. This concept might be further supported by our observation that the p51 domain, normally attached to the N terminus of the p15 domain, can be replaced with DHFR without disturbing the productive functional interaction between p15 and the p51 of the other subunit. As shown in our assay, the DHFR–p15 fusion seems to have activity similar to that of the p15 domain alone.

If the interaction of two p51 domains is required for efficient DNA polymerase activity, the residual activity of our preparation of the p51 form of RT might be explained by a low level formation of p51/p51 dimers undetected in our analytical gel filtration experiments. The weak subunit interaction in these dimers may be further diminished by increasing salt concentration, as the observed sensitivity to KCl of the DNA polymerase activity of p51 may suggest.

In connection with the formation of RT heterodimer, presumably by proteolytic processing of the p66/p66 homodimer, the question of the fate of the released p15 arises. In some of our protease digestion experiments during prolonged incubation, we observed the p15 being preferentially degraded (data not shown). Other investigators have also noticed the disappearance of this protein in their assays (16, 41). This, together with our data about lack of activity in isolated p15, may indicate that HIV-1 RNase H functions only as part of a complex with DNA polymerase. Released p15 as a by-product of RT processing would have no function and as such may be targeted for rapid degradation.

Further experiments addressing the relative contributions of individual domains for productive interaction remain to be done. This information, together with data from mutagenesis studies, will be undoubtedly helpful in interpretation of the awaited crystal structure of HIV-1 RT. The understanding of details of the interactions among the domains of RT may eventually help in development of efficient inhibitors of this important drug target.

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