Purification and Characterization of Recombinant Equine Infectious Anemia Virus Reverse Transcriptase

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A 1.67-kb segment of the equine infectious anemia virus *pol* gene, encoding a 66-kDa reverse transcriptase (RT), was cloned and expressed in *Escherichia coli*. Recombinant RT, purified by a combination of metal chelate affinity chromatography and ion-exchange chromatography, displays both RNA-dependent DNA polymerase and RNase H activity. The affinity of purified RT for its replication primer, tRNA₃^{Lys} was equivalent to that observed for human immunodeficiency virus RT. Our data suggest that an additional domain between RT-RNase H and integrase on the equine infectious anemia virus *pol* open reading frame is not an integral component of the RT polypeptide.

Equine infectious anemia virus (EIAV) is a member of the lentivirus subfamily of retroviruses. This group includes human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2), simian, feline, and bovine immunodeficiency viruses, caprine arthritis-encephalitis virus of goats, and visna-maedi virus of sheep (9-11, 29, 30, 36), which share common structural and biological features, including replication in cells of the immune system, establishment of persistent infections, and high mutation rates. EIAV causes a unique episodic disease in horses; i.e., chronic equine infectious anemia is characterized by recurring cycles of fever and viremia, which correlate with emergence of envelope gene variants capable of escaping host immune responses (26, 33). EIAV is thus a useful model system for studying the consequences of envelope variation during a persistent infection.

Initiation of minus-strand DNA synthesis in EIAV is similar to that in other retroviruses, involving extension of a cellular tRNA primer hybridized to a specific region of the viral RNA genome, the primer-binding site (37). DNA sequence analysis (14, 36) implicates tRNA₃^{Lys} as the replication primer for EIAV reverse transcriptase (RT) (19). The same primer is used by RT of HIV-1 and HIV-2 (12, 38), the causative agents of AIDS (4, 31). In recent studies, a specific complex between the HIV-1 enzyme and its replication primer, involving a dodecanucleotide encompassing the anticodon domain, could be demonstrated (1, 2, 5, 34). The ability of cordycepin analogs of 2',5'-oligoadenylic acid to displace tRNA₃^{Lys} from HIV-1 RT (28), presumably by base pairing with uridine residues in the anticodon domain, suggests a novel therapeutic approach to inhibition of HIV replication by preventing association of RT with its replication primer (17). Inhibition of HIV RT prior to initiation of replication may have advantages over impairment of polymerase function with agents such as zidovudine (AZT) and ddC, which also interrupt DNA synthesis by host polymerases. A more detailed understanding of the domain(s) of RT involved in this interaction would be beneficial to these studies. Since the HIV and EIAV enzymes share a common

replication primer, a comparative study of ribonucleoprotein complexes involving these enzymes would also be useful.

In the work described in this communication, we have genetically engineered a 66-kDa N-terminally polyhistidine-extended form of EIAV RT and purified it to more than 80% homogeneity by a combination of metal chelate and ion-exchange chromatography. Cloning of EIAV RT involved polymerase chain reaction amplification (32) of a 1.67-kb region of the *pol* gene, the protocol for which is outlined schematically in Fig. 1. From the sequence reported by Stephens et al. (36), amino acid sequence homology between the *pol*-encoded protease (PR) and RT domains of EIAV and HIV (13, 23) was used to approximate the 5' end of the RT coding sequence at position 1911. Location of the C-terminal RNase H boundary was complicated by the fact that the *pol* genes of EIAV, FIV, and visna virus contain an additional domain directly preceding integrase (IN), believed to encode either a pseudoprotease (22, 25) or deoxyuridine triphosphatase (24) (Fig. 1a). Consequently, the 3' terminus of EIAV RT (position 3587) was based on homology between the RNase H domains of EIAV and other retroviral enzymes (13, 23). The 1,677-bp RT-containing gene was introduced into a bacterial expression system, generating plasmid p6HEIAV RT. This plasmid was maintained in *Escherichia coli* under inducible control of *lac* regulatory elements (6).

Enzyme purification. Previously, we demonstrated the use of nitrilotriacetic acid (NTA)-Sephacrose metal chelate affinity chromatography for purification of recombinant HIV-1 RT from the high-speed supernatant of a bacterial homogenate (15). The same approach has been used to purify the EIAV enzyme (Fig. 1b), except that elution from NTA-Sephacrose was accomplished with a gradient of 0 to 0.5 M imidazole rather than a decreasing pH gradient (adsorption to the matrix occurs via coordination of the imidazole nitrogen of histidine and matrix-bound nickel). NTA-Sephacrose-purified RT was dialyzed and subjected to S-Sepharose ion-exchange chromatography. A summary of the purification protocol is given in Fig. 2; this indicates that EIAV RT was at least 80% pure following these two chromatographic steps. The same figure shows that immunological

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[a]

[b]

FIG. 1. (a) Organization of the EIAV *pol* open reading frame. In addition to the PR, RT-RNase H, and IN genes, another gene, encoding either a pseudoprotease (22, 25) or deoxyuridine triphosphatase (24), is located between RT-RNase H and IN (PR/dU). The present work has assumed that the additional open reading frame is not an essential component of EIAV RT. The 5' and 3' endpoints of EIAV RT, against which primers were prepared for its cloning, have been indicated. Base pair numbering is from Stephens et al. (36). Underlined portions of the oligonucleotides for amplification represent homology with the EIAV RT domain. (b) Strategy for purification of recombinant EIAV RT. The chromatographic procedures used are essentially similar to those we have previously reported for HIV RT purification (15). Buffer RT A/78 is 50 mM sodium phosphate (pH 7.8) and 0.3 M NaCl. Buffer RT A/60 is 50 mM sodium phosphate (pH 6.0), 0.3 M NaCl, and 10% (vol/vol) glycerol. Buffer D is 50 mM Tris · HCl (pH 7.0), 25 mM NaCl, 1 mM EDTA, and 10% (vol/vol) glycerol.

analysis following each purification step indicated the presence of 66- and 51-kDa polypeptides.

Preliminary characterization of recombinant EIAV RT. RNA-dependent DNA polymerase activity of S-Sepharose-purified EIAV RT was fully dependent on the presence of a divalent cation, with a preference for Mg^{2+} (64 pmol of TTP incorporated every 15 min into polynucleotide in 15 mM $MgCl_2$ versus 18.3 pmol in 1 mM $MnCl_2$). In contrast, activity could be supported in the absence of a monovalent cation (15.4 pmol of TTP incorporated per 15 min). A slight preference for K^+ was observed (43 pmol of TTP incorporated per 15 min in 25 mM KCl versus 33.7 pmol in 25 mM NaCl). The metal ion requirements reported here are in accordance with data recently obtained with EIAV RT purified from virions (37a). By using a poly(rA)-oligo(dT) template primer, a specific activity of 25,000 U/mg has been calculated for recent enzyme preparations, which agrees

FIG. 2. Summary of EIAV RT purification. Peak fractions from NTA-Sepharose metal chelate affinity chromatography (left) and S-Sepharose ion-exchange chromatography (right) are presented in the analysis. Immunological analyses were performed with a pool of serum samples from EIAV-infected horses as primary antibody. The migration positions of the two immunoreactive RT polypeptides are indicated. Notations for protein molecular mass markers are given in kilodaltons.

well with values obtained for RT of avian myeloblastosis virus and of Moloney murine leukemia virus (1 U is defined as the amount catalyzing incorporation of 1 nmol of TTP into polynucleotide in 10 min at 37°C).

RNase H activity. The presence in EIAV of either a pseudoprotease or deoxyuridine triphosphatase between the RT-RNase H and IN domains (22, 24, 25) made it difficult to accurately define the C-terminal boundary of the EIAV RT-RNase H polypeptide. Furthermore, we could not exclude the possibility that this novel domain was an integral component of RT. In the present study, we assumed this not to be the case, and chose bp 3587 as the RT-RNase H C terminus, based on amino acid homology between the EIAV RNase H domain and those of *E. coli* and related retroviruses. Conclusive evidence that the C-terminal portion of p66 EIAV RT contains a functional RNase H domain was provided by assaying this activity of the purified enzyme. Figure 3 illustrates the ability of purified EIAV RT to degrade the RNA component of a radiolabeled RNA-DNA hybrid. For comparison, the homodimeric (p66-p66) and heterodimeric (p66-p51) forms of equivalently purified HIV-1 RT were analyzed. Our results indicate that the three enzymes degrade the RNA-DNA hybrid with almost equal efficiency. From these observations, we believe that the C terminus of our recombinant EIAV RT contains most, if not all, of the amino acids constituting its RNase H domain. The possibility that contaminating *E. coli* RNase H accounts for the activity observed can be excluded by the facts that (i) the selectivity of NTA-Sepharose requires recombinant proteins bearing a polyhistidine extension (the equivalent array of histidine residues cannot be achieved with *E. coli* RNase H) and (ii) HIV-1 p66 and p66-p51 RTs carrying a point mutation in their RNase H domain (Glu-478 → Gln), identically purified from the same *E. coli* strain, are completely devoid of RNase H activity, assayed either in situ or as reported here (35).

Primer-binding characteristics. In addition to enzymatic properties, we compared the binding of $tRNA_{Lys}^3$ to recombinant EIAV and HIV-1 RT by gel mobility shift analysis (Fig. 4). Incubation of heterodimeric p66-p51 HIV-1 RT with

FIG. 3. RNase H activity of S-Sepharose-purified EIAV RT. Activity is presented as loss of radioactivity from an RNA-DNA hybrid within which the RNA component was internally labeled with [³²P]CTP. As controls, RNase H-proficient forms of recombinant p66-p66 and p66-p51 HIV-1 RTs have been included. Results are the average of duplicate analyses.

a twofold excess of natural tRNA^{Lys} (0.4 pmol of enzyme, 0.8 pmol of tRNA) gave incomplete complex formation (Fig. 4a, lane 2). When the RT/tRNA ratio is increased to 1.5 (1.2 pmol of enzyme, 0.8 pmol of tRNA), tRNA^{Lys} is quantitatively bound to the enzyme (Fig. 4a, lane 4). A similar stoichiometry of tRNA^{Lys} binding is observed with recombinant EIAV RT (Fig. 4a, lanes 5 to 7), indicating that the HIV and EIAV enzymes display equivalent affinity for their cognate replication primer. Figure 4b illustrates a similar experiment with in vitro-synthesized tRNA. Both the HIV and EIAV enzymes are capable of forming a ribonucleoprotein complex with the synthetic replication primer, although binding was not fully quantitative. Although synthetic tRNA^{Lys} was heated and slowly cooled to allow proper

FIG. 4. Analysis of the interaction of HIV and EIAV with natural and synthetic forms of their replication primer, tRNA^{Lys}, by gel mobility shift experiments. (a) Natural bovine tRNA^{Lys}. Each reaction mixture contained ca. 0.8 pmol of ³²P-end-labeled tRNA. Lane 1, no RT; lanes 2 to 4, 0.4, 0.8, and 1.2 pmol of p66-p51 HIV-1 RT, respectively; lanes 5 to 7, 0.4, 0.8, and 1.2 pmol of EIAV RT, respectively. Migration positions of free and RT-bound tRNA^{Lys} are indicated. (b) Interaction with in vitro-synthesized tRNA. Each reaction mixture contained ca. 1.2 pmol of internally labeled tRNA. Lanes 1 to 3, 0.4, 0.8, and 1.2 pmol of HIV RT, respectively; lanes 4 to 6, 0.4, 0.8, and 1.2 pmol of EIAV RT, respectively; lane 7, no RT.

refolding, it is possible that a portion remains incorrectly folded and inaccessible to RT. In parallel experiments, a 5- to 10-fold excess of tRNA^{Pro} failed to displace tRNA^{Lys} from either the HIV or EIAV enzyme (data not shown).

Finally, an interesting aspect of the present study is the presence of 66- and 51-kDa polypeptides in the purified enzyme, similar to the heterodimeric form of HIV-1 and HIV-2 RT (7, 18, 20, 21, 27), in which p51 RT results from partial HIV PR-mediated maturation of p66. In the present work, EIAV RT was expressed in the absence of its *pol*-encoded PR, suggesting processing of the 66-kDa polypeptide by an *E. coli* protease during preparation. This observation is not without precedent, since both Barr et al. (3) and Furman et al. (8) have observed accumulation of heterodimer HIV-1 RT in recombinant *Saccharomyces cerevisiae* and *E. coli*, respectively, following expression of solely the 66-kDa subunit. In support of our observations, EIAV RT isolated from virions likewise appears to be composed of 66- and 51-kDa subunits (37a). To address this more directly, we have now cloned an EIAV PR-RT polypeptide, similar to that previously derived from the HIV-1 *pol* gene (16). Initial results indicate accumulation of 66- and 51-kDa polypeptides (data not shown), suggesting that the N terminus of our recombinant enzyme is close to that produced by PR maturation.

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