Distinct Subsets of Retroviruses Encode dUTPase

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The nonprimate lentiviruses feline immunodeficiency virus, equine infectious anemia virus, visna virus, and caprine encephalitis virus contain a gene segment in the polymerase gene that is lacking in the primate lentiviruses. A related sequence has been noted in other retroviruses, most notably the type D retroviruses. Computer searches have indicated a relatedness between this unique gene segment, termed proteaselike element and elements of both the aspartate proteinase and the dUTPase enzyme families. In this report, we show that members of both nonprimate lentiviruses and type D retroviruses possess dUTPase activity and present a formal demonstration that in feline immunodeficiency virus, the activity is encoded by the proteaselike element.

In a computer survey of viral sequences, McClure et al. noted that a diverse group of viruses share a gene product distinct from, but related to, the aspartate proteinases (13, 14). The list of viruses includes the nonprimate lentiviruses equine infectious anemia virus (27), visna virus (25), and caprine encephalitis virus (23); the type D retroviruses SRV-1 (21) and Mason-Pfizer monkey virus (26); hamster intracisternal A particles (19); vaccinia virus (24); mouse mammary tumor virus (16); and human endogenous retrovirus (18). The *pol* gene of feline immunodeficiency virus (FIV) contains a similar predicted sequence between the coding regions for reverse transcriptase and integrase (17, 20, 28). Whether the predicted protein, termed proteaselike (PrL) protein because of its apparent derivation via tandem duplication and divergence of an aspartate proteinase (13, 14, 21), actually exists as a distinct species has not been demonstrated for any of the above-mentioned viruses. We therefore prepared site-directed antibodies to all the proteins predicted to be encoded by the *pol* gene of FIV and performed Western blot (immunoblot) analyses against purified virions (Fig. 1). This study showed that the expected *pol* gene products, including proteinase (data not shown), both the full-length and the truncated (lacking the C-terminal RNase H domain, as in other lentiviruses; 11) forms of reverse transcriptase (Fig. 1, lanes 1 and 2), and integrase (Fig. 1, lanes 4), were present in gradient-purified FIV. However, an additional gene product was revealed by an antiserum directed to the predicted N terminus of the PrL protein (Fig. 1, lanes 3). These results demonstrate that the FIV PrL protein is indeed a distinct protein species rather than an extension of either reverse transcriptase or integrase. Furthermore, the PrL protein is packaged into virions, implying that it performs a role in the early stages of the virus life cycle.

Recently, McGeoch, by using computer analyses, made the observation that the PrL sequences are also related to the sequences of the dUTPase (DU) family of enzymes (15). On the strength of this finding, we tested FIV for DU activity (Fig. 2). Gradient-purified preparations of FIV demonstrated strong DU activity, with only negligible cleavage of dATP,

dCTP, or dTTP. In addition, competition experiments done with unlabeled deoxynucleoside triphosphates to compete for radioactive dUTP cleavage indicated that only unlabeled dUTP would block cleavage (data not shown).

We next tested a battery of other retroviruses predicted from previous studies to encode DU (13, 14, 21; Fig. 3). Enzyme activity was noted in the nonprimate lentiviruses FIV and equine infectious anemia virus as well as the type D retroviruses SRV-1 and Mason-Pfizer monkey virus. No DU activity was detected in viruses lacking PrL sequences, including the type C retroviruses Rauscher murine leukemia virus and feline leukemia virus, as well as the arenavirus lymphocytic choriomeningitis virus. In addition, material that was from noninfected cells used to produce FIV and that banded in sucrose gradients at the same density as the virus was inactive (data not shown).

We have cloned the entire *pol* gene of FIV in *Escherichia coli*; the gene is expressed under the control of the T7 polymerase promoter. The polyprotein is processed properly into proteinase, reverse transcriptase, DU, and integrase by the viral protease (data not shown). Partial purification of the virus-encoded DU protein expressed in *E. coli* revealed that the reactivity of the enzyme coincided precisely with the eluted protein, as shown by Western blot analysis (Fig. 4). Activity was negligible in similar fractions from this strain of *E. coli* expressing the same plasmid bearing feline leukemia virus *env* (Fig. 4). This result confirms that the protein species responsible for the DU activity coincides with the appropriate protein encoded by the *pol* gene and is not a cellular contaminant in the virus preparation.

Deletion mutants are being prepared to assess the role of DU in DU-positive (DU⁺) retroviral strains. In the herpesviruses, the DU gene is a nonessential accessory gene, as defined in vitro (5). Likewise, the F2L gene segment of vaccinia virus which, on the basis of the findings presented here, is likely to encode DU, is in a nonessential region of the genome (4, 22). However, the relevance of observations made in vitro are limited. Recent findings with the Nef protein of simian immunodeficiency virus (9) indicate that Nef is required for pathogenesis in vivo, even though it had been shown previously not to be required for in vitro growth of the virus (7, 10). Therefore, a full assessment of the role of DU should await completion of in vivo studies with mutants.

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FIG. 1. Characterization of the protein products encoded by the *pol* gene of FIV. Synthetic peptides corresponding to sequences of the predicted protein products of FIV *pol* were synthesized (6), coupled to keyhole limpet hemocyanin (12), and injected into rabbits for antibody preparation. Western blotting (8) was then performed against proteins of FIV (15 μ g per analysis) purified by gradient centrifugation. The peptide sequences are shown in the single-letter code, with relative locations. Lanes marked - represent Western blotting done with antisera directed to the synthetic peptides, as indicated by arrows. Lanes marked + represent Western blotting done with the corresponding antibodies after treatment with cognate peptide. Note that the reactivity in each case is blocked specifically by the addition of cognate peptide. Pr, aspartate protease; RT, reverse transcriptase; PLP, proteaslike element; In, integrase.

The degree to which DU is conserved (28) and the fact that it is maintained in the relatively small retrovirus genome imply that it is used to advantage by DU⁺ viruses. In particular, the enzyme might serve as a localized scavenger to decrease the inappropriate incorporation of dUTP into viral DNA. This function should have the net effect of reducing the error rate, with a concomitant increase in genetic stability in DU⁺ versus DU-negative (DU⁻) viral strains. Sufficient sequences are not available at present for any of the DU⁺ viruses to allow an accurate assessment of divergence relative to hypermutable retroviruses, such as human and simian immunodeficiency viruses, which are DU⁻. However, it is notable that DU⁺ Mason-Pfizer monkey virus from persistently infected macaques shows very little sequence variation (1). Other studies have shown that the frequency of spontaneous, nonconditional, replication-defective mutants of Mason-Pfizer monkey virus is very low (<1%), compared with that of the DU⁻ type C Moloney leukemia virus (>30%; 2). Whether this difference relates to the presence of DU in the former and not in the latter remains to be determined. In bacteria, DU⁻ strains exhibit hyperrecombination as a function of small Okazaki DNA fragments created via excision and repair of misincorporated dUTP (29). Thus, the lack of DU might explain a high

FIG. 2. Demonstration of DU activity in preparations of FIV. Assays were performed at 37°C as described previously (30) with [³H]dUTP as the substrate. Gradient-purified FIV was used at 100 ng of viral protein per assay. Negligible cleavage was observed with dATP, dCTP, or dTTP. Data represent the percent decrease in counts bound to DE81 paper, as a function of the conversion of dUTP to dUMP over time (30).

incidence of recombination observed with certain DU⁻ retroviruses, such as the type C retroviruses. Many of these speculations can be tested and suggest interesting experiments to define the role of DU in the life cycles of distinct subsets of retroviruses.

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FIG. 3. Relative DU activities in virus preparations. Assay mixtures were incubated for 10 min at 37°C as described previously (30) and included 100 ng of gradient-purified viral protein per assay. LCMV, lymphochoriomeningitis virus; MPMV, type D Mason-Pfizer monkey virus (26); EIAV, equine infectious anemia virus (27); SRV, simian type D retrovirus SRV-1 (21); R-MuLV, Rauscher leukemia virus; FeLV, feline leukemia virus.

FIG. 4. Partial purification of recombinant FIV DU from *E. coli* extracts and analysis by activity and Western blotting. The entire *pol* gene of FIV was cloned into the T7-7 vector (kindly provided by S. Taybor), from the *EcoRI* site at base 1871 to the *BglII* site at base 6455 (28). *E. coli* BL 21.DE3, which contains stably integrated T7 polymerase, was transformed with the *pol*-containing plasmid (termed 34POL). Positive transformants were selected and grown in CircleGrow medium (Bio 101) containing 25 μ g each of spectinomycin and streptomycin per ml, 30 μ g of chloramphenicol per ml, and 100 μ g of ampicillin per ml to an optical density at 550 nm of 0.5. The cultures were induced in the presence of 5 mM isopropyl- β -D-thiogalactopyranoside at 30°C overnight. The cells were lysed as described previously (3) and centrifuged at $10,000 \times g$ for 20 min. The supernatant fraction was passed over an S-Sepharose column (Pharmacia) and eluted with a 0 to 1 M KCl linear gradient. Fractions were assayed for DU activity as described previously (3) and by Western blotting (inset) with a monoclonal antibody prepared against a peptide corresponding to the region of FIV DU shown in Fig. 1. Symbols: $\square$, DU activity in fractions derived from 34POL-bearing cells; $\circ$, DU activity of a negative control plasmid bearing feline leukemia virus *env* (C11D8). Western blotting was also performed on fractions from the C11D8 plasmid-bearing cells and was negative (data not shown).

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