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Expression of Reverse Transcriptase from Feline Immunodeficiency Virus in *Escherichia coli*

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Reverse transcriptase from feline immunodeficiency virus (FIV) has been cloned and expressed in *Escherichia coli*. We have purified this recombinant enzyme and shown that it is a 66-kDa protein that is indistinguishable from virion-derived FIV reverse transcriptase in sensitivity to the 5′-triphosphates of 3′-azido-3′-deoxythymidine and the four 2′,3′-dideoxynucleosides. The availability of large quantities of the FIV reverse transcriptase will allow more detailed physical and pharmacological studies.

Feline immunodeficiency virus (FIV) is a lentivirus that causes an immune deficiency in domestic cats that is very similar to AIDS in humans (13, 17). FIV-infected cats develop an AIDS-related complex-like disease which progresses to the final AIDS-like stage (6). Like AIDS in humans, the immune dysfunction in cats involves depletion of the $CD4^+$ T-lymphocyte subset, diminished in vitro lymphocyte proliferative response to phytomitogens and interleukin-2, decreased humoral immune responsiveness to T-dependent immunogens, hypergammaglobulinemia, and diminished interleukin-2 production (1, 2, 12, 15, 16). These features make FIV a valuable animal model for studies of AIDS.

We have previously purified the FIV reverse transcriptase (RT) from detergent-lysed virions and shown that it is similar to the human immunodeficiency virus type 1 (HIV-1) RT in physical properties, catalytic activities (10), and sensitivity to the 5′-triphosphates of several nucleoside analogs that display anti-HIV activity, including zidovudine (3′-azido-3′-deoxythymidine) 5′-triphosphate, 2′,3′-dideoxynucleotides (ddATP, ddCTP, ddGTP, and ddTTP), and 2′,3′-dideoxy-2′,3′-didehydrothymidine 5′-triphosphate (4, 9, 11). The FIV and HIV RTs are also similar in sensitivity to phosphonoformate but differ in that the FIV enzyme is not sensitive to other nonnucleoside inhibitors such as nevirapine (BIRG-587) and tetrahydroimadazo[4,5,1-j,k][1,4]benzodiazepin-2(1H)-one and -thione (TIBO) compounds, which are potent inhibitors of the HIV-1 RT. Although very similar to one another, the FIV and HIV-1 RTs are quite different from the RT of avian myeloblastosis virus in susceptibility to antiviral nucleotide analogs (11).

Work with the FIV RT has been limited by availability of the purified enzyme, which has previously been purified from cell-free virus (10). Larger quantities are needed for physical studies, production of antibody reagents, and studies with heteropolymer templates. We report here the cloning and expression of the FIV RT in *Escherichia coli* and a comparison of the recombinant enzyme with the FIV RT purified from virus.

Construction of a clone expressing FIV RT. Plasmid DNA containing the entire FIV genome, pFIV-14 Petaluma, was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program and used as a template for amplification of the RT-encoding region of the *pol* gene. On the basis of sequence homologies with HIV, the region chosen for amplification spanned nucleotides 2332 to 4013 of pFIV-14 Petaluma. Primers used for PCR amplification contained add-on sequences for restriction endonuclease sites and signals for transcription and translation (Fig. 1). The 5′ primer (5′-AGTCTGAATTCTAGGAGATCTATAATG GCTCAAATTTCTGATAAG-3′) directed synthesis of an *Eco*RI site (GAATTC), a Shine-Dalgarno sequence (AG GAGA), and a translation initiation codon (ATG) and had 17 bases (underlined) complementary to the antisense strand of the putative 5′ end of the RT-encoding region (nucleotides 2332 to 2348 of pFIV-14 Petaluma). The 3′ primer (5′-AGTC TCTGCAGGTACTCATTACCCTTCTATTATCATCA TTGT-3′) contained a *Pst*I site (CTGCAG), two tandemly placed translation stop codons (TCATTA), and 21 bases (underlined) complementary to the sense strand of the putative 3′ end of the RT-encoding region (nucleotides 3993 to 4013 of pFIV-14 Petaluma). Samples were heated to 94°C for 5 min; then PCRs were carried out for 25 cycles. Each cycle consisted of 94°C for 2 min, 55°C for 1.5 min, and 72°C for 3 min (with an extension of 5 s at 72°C per cycle), using *Thermus aquaticus* (*Taq*) DNA polymerase under conditions recommended by the supplier (Perkin-Elmer Cetus). Amplification products were separated from primers by electrophoresis on 1% agarose gels. The desired fragment (1,731 bp) was purified by using Geneclean (Bio 101, La Jolla, Calif.) according to the manufacturer's protocol and then resuspended in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA. This fragment was digested with *Eco*RI and *Pst*I and ligated to the *Eco*RI-*Pst*I-digested plasmid expression vector pKK223-3 (Pharmacia). Plasmid DNA was introduced into *E. coli* JM109, and several ampicillin-resistant clones were selected. Crude bacterial lysates of these clones were prepared after isopropylthiogalactoside (IPTG) induction, and RT activities were assayed by using poly(rC) · oligo(dG) as a template-primer as described previously (14). The clone expressing the highest RT activity, designated pRTF14, was selected for further study. DNA sequence analysis of the insert was carried out on both strands with an ABI 373A automated DNA sequencer by using multiple primers which generated overlapping sequences. The sequence obtained was identical to the published sequence for nucleotides 2332 to 4013 of pFIV-14 Petaluma. No PCR-induced mutations were observed.

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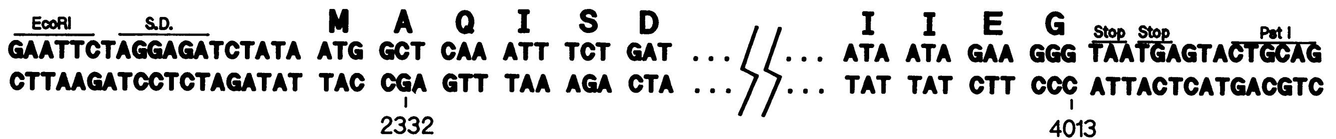

FIG. 1. Expression of recombinant FIV-14 RT from a prokaryotic vector. A PCR-amplified FIV *pol* gene fragment containing the RT-encoding region (*Eco*RI-*Pst*I; 1.7 kb) was cloned into the expression vector pKK223-2 as described in the text. The predicted amino acid sequence of the recombinant RT has the same amino acid sequence from nucleotides 2334 to 4013 as FIV-14 plus two additional amino acids, M and A, at the N terminus. S.D., Shine-Dalgarno sequence.

Purification of recombinant FIV RT. Recombinant FIV RT was purified from *E. coli* containing the clone pRTF14. Bacteria were grown in 1 liter of L broth (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter [pH 7.5]) at 37°C to an optical density at 550 nm of 0.55. IPTG was added to a final concentration of 1 mM, and incubation continued at 37°C for 3 h. Cells were harvested by centrifugation at 3,000 × *g* for 5 min, and all subsequent steps were carried out at 4°C. Cells were washed with 50 mM Tris-HCl, pH 7.5, containing 0.5 mM EDTA and 0.15 mM NaCl and then resuspended in 10 ml of 10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 10% sucrose, 0.3 M NaCl, and 1 mM phenylmethylsulfonyl fluoride. NaCl was then added to a final concentration of 1 M, and cells were disrupted by sonic oscillation. Debris was removed by centrifugation at 30,000 × *g* for 30 min, and Triton X-100 was added to a final concentration of 0.2%. This mixture was dialyzed three times for a total of 12 to 16 h against 1.5 liter of 50 mM Tris-HCl, pH 7.9, containing 1 mM EDTA, 1 mM dithiothreitol, and 5% glycerol and then used for purification of RT by chromatography on DEAE-cellulose and then phosphocellulose, as previously described (10).

During purification, fractions were assayed for RT activity with poly(rA) · oligo(dT) as a template-primer. Other homopolymer and heteropolymer templates were used in characterization of RT preparation. Poly(rA) · oligo$(dT)_{10}$, poly (rC), oligo$(dG)_{10}$, poly(dA-dT), and 2′,3′-dideoxynucleoside 5′-triphosphates (ddATP, ddCTP, ddGTP, and ddTTP) were purchased from Pharmacia LKB, Piscataway, N.J. The zidovudine 5′-triphosphate (N_3dTTP) was provided by Wayne Miller of the Burroughs Wellcome Co., Research Triangle Park, N.C. The 2′-deoxyribonucleoside 5′-triphosphates (dATP, dCTP, dGTP, and dTTP) were purchased from Sigma Chemical Co., St. Louis, Mo. M13 DNA was provided by Brad Preston, Rutgers University, Piscataway, N.J. The isotopes used in this study, [*methyl*-^{3}H]dTTP, [5-^{3}H]dCTP, [8-^{3}H]dATP, and [8-^{3}H]dGTP, were purchased from Dupont, NEN Research Products, Boston, Mass.

DEAE-cellulose chromatography yielded three peaks of activity during purification of RT. The first peak, which was eluted at approximately 180 mM NaCl, had a high level of activity with the DNA alternating copolymer template poly(dA-dT) and was presumed to be contaminated with *E. coli* DNA polymerase I. The second peak, which was eluted at approximately 300 mM NaCl, was active with poly(rA) · oligo(dT) but not with poly(dA-dT), as expected for the FIV RT. Fractions containing this peak of activity were pooled for phosphocellulose chromatography. We have not characterized the activity present in the third peak because it was eluted from the DEAE-cellulose column at a much higher salt concentration than the virion FIV RT. Further purification of the activity in peak 2 by chromatography on phosphocellulose yielded a single peak of activity at the position expected for the FIV RT (10).

We have analyzed the purified RT by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (immunoblot) analysis using a monoclonal antibody (MAb) known to recognize the virion-derived FIV RT (Fig. 2). This antibody, MAb 42, which was obtained from mice immunized with the HIV-1 RT (5), cross-reacts with the FIV RT. MAb 42 recognizes two polypeptides of approximately 66 and 51 kDa in preparations of virion-derived FIV RT (lane 2). The recombinant FIV RT eluted from the phosphocellulose column contains only the 66-kDa protein (lane 1). This preparation of FIV RT was more than 98% pure as determined by SDS-PAGE and silver staining. The specific activity of the purified recombinant FIV RT was approximately 30,000 U/mg of protein. This is slightly lower than the value of 48,000 U/mg

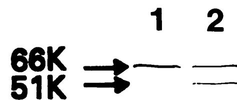

FIG. 2. Western blot analysis of purified recombinant and virion FIV RTs. Polyacrylamide gels (12.5%) were prepared as described by Laemmli (8). Sample preparation and gel electrophoresis were as previously described (10). Proteins were visualized by staining with silver nitrate (Sigma Chemical Co.). Molecular weight standards (Bio-Rad) were run with each gel. Following electrophoresis, samples were transferred to nitrocellulose paper by electroblotting, as previously described (7). Blocking and washing were performed with 0.05% Tween 20 in Dulbecco's phosphate-buffered saline (3). Immunostaining was performed as described by Ferris et al. (5). Samples of recombinant FIV RT (lane 1) and virion FIV RT (lane 2) were analyzed with MAb 42. The positions of p66 and p51, as determined from molecular weight standards, are indicated.

TABLE 1. Template specificities of DNA polymerase activities[a]

Template	% Activity[b]		
	FIV RT		*E. coli* DNA polymerase I
	Virion[c]	Recombinant	
Poly(rA) · oligo(dT)	100	100	2.5 ± 1.4
Poly(rC) · oligo(dG)	60 ± 12	50 ± 11	0.7 ± 0.1
Poly(dA-dT)	1.1 ± 0.3	1.1 ± 0.1	100

[a] The conditions for assay of RT using RNA homopolymers primed with complementary oligonucleotides were as previously described (9, 10). For assays with poly(dA-dT), the conditions were identical to those with poly(rA) · oligo (dT) except that poly(dA-dT) was used as a template-primer rather than poly (rA) · oligo(dT) and reaction mixtures contained both dATP and [^{3}H]dTTP, each at 20 μM.

[b] Values are means ± standard errors for three or more determinations and are normalized to activity with the template that gave the highest activity—poly(rA) · oligo(dT) for RT preparations and poly(dA-dT) for DNA polymerase I. These values correspond to 3.9 ± 1.2, 2.6 ± 0.8, and 2.1 ± 0.2 nmol of deoxynucleoside monophosphate incorporated per h for the recombinant FIV RT, virion FIV RT, and *E. coli* DNA polymerase I, respectively.

[c] FIV RT was purified as previously described (10).

TABLE 2. Kinetic constants for virion and recombinant RTs in reactions with poly(rA) · oligo(dT) as a template-primer[a]

FIV RT	K_m for dTTP (μM)	K_i (nM) for:	
		ddTTP	N_3dTTP
Virion	4.6 ± 0.6	5.5 ± 0.2	6.9 ± 0.7
Recombinant	5.2 ± 0.8	4.2 ± 0.8	5.8 ± 0.4

[a] Kinetic parameters (K_m and K_i) were determined as previously reported (4, 9) by using intercept values calculated from double-reciprocal plots. The values are means ± standard errors for three or more determinations.

we estimated for the purified virion FIV RT (10). The recombinant and virion RTs are different in that the former is a homodimer of p66 and the latter is a heterodimer of p66 and p51, and the apparent difference in specific activity may be attributable to this.

Comparison of recombinant and virion-derived FIV RTs. Comparisons of template specificities of recombinant and virion-derived FIV RTs are shown in Table 1. Both preparations effectively use poly(rA) · oligo(dT) and poly(rC) · oligo (dG) as template-primers but are inactive with poly(dA-dT) · poly(dA-dT). In contrast, *E. coli* DNA polymerase I uses poly(dA-dT) · poly(dA-dT) preferentially and has poor activity with the RNA homopolymer templates. These data demonstrate similar template specificities of recombinant and virion-derived FIV RTs and confirm that the recombinant FIV RT preparation is not significantly contaminated with *E. coli* DNA polymerase I.

Of particular importance to studies of antiviral agents is the degree of similarity of these two RT preparations in their sensitivities to antiviral inhibitors. Accordingly, we have compared the sensitivities of the two enzymes to a dideoxynucleotide (ddTTP) and to the active form of zidovudine (N_3dTTP) in experiments with poly(rA) · oligo(dT) as a template-primer. As shown in Table 2, there is no significant difference between these two preparations of FIV RT with respect to K_m for dTTP or K_i for either inhibitor.

We previously showed that virion-derived FIV RT will effectively utilize φX174 DNA as a template and characterized inhibition of this reaction with analogs of dATP and dTTP (4). In order to extend this work, we have characterized reactions of recombinant and virion-derived FIV RT with M13 DNA, which is more readily available than φX174 DNA. The velocity of FIV RT with M13 DNA is 15 to 20% of that obtained with poly(rA) · oligo(dT), which is slightly higher than what we reported with φX174 DNA (4). We have determined K_m values for all four deoxynucleoside triphosphate (dNTP) substrates and K_i values for competitive inhibition by the four ddNTPs and N_3dTTP. These data are summarized in Table 3. The recombinant and virion RTs were indistinguishable from one another in the ability to use this heteropolymeric DNA template.

We have not had sufficient quantities of virion-derived FIV RT to precisely determine the N and C termini. Also, it is not possible to infer these termini from the nucleotide sequence, because the virion RT is derived by proteolysis of a larger protein. Therefore, our selection of the region of the FIV *pol* gene to clone and express was based upon sequence homology with HIV-1 and likely sites for proteolysis. The enzyme we have cloned and expressed is a 66-kDa protein that is indistinguishable from the virion-derived FIV RT in catalytic activity and sensitivity to antiviral nucleotides. Our ability to purify large quantities of the recombinant FIV RT will make the enzyme more readily available and enable more detailed physical studies.

TABLE 3. Kinetic constants for virion and recombinant RTs in reactions with primed M13 DNA[a]

Substrate or inhibitor	Virion RT		Recombinant RT	
	K_m (μM)	K_i (nM)	K_m (μM)	K_i (nM)
Substrates				
dATP	0.5 ± 0.1		0.5 ± 0.1	
dCTP	1.6 ± 0.4		1.5 ± 0.2	
dGTP	0.11 ± 0.01		0.10 ± 0.01	
dTTP	1.2 ± 0.1		0.9 ± 0.1	
Inhibitors				
ddATP		10.5 ± 1.2		6.3 ± 1.2
ddCTP		19.5 ± 5.7		29 ± 8.4
ddGTP		10.9 ± 0.8		6.8 ± 0.5
ddTTP		12.5 ± 1.5		12.0 ± 2.8
N_3dTTP		22.4 ± 2.2		21.0 ± 2.8

[a] RT assays with M13 DNA were carried out as previously described for reactions with φX174 DNA (4). For preparation of primed M13 DNA, 5.22 μg of M13 DNA was mixed with 0.2 μg of primer (5'-TTGAGGGAGGGAAG GTA-3'). This mixture was heated to 95°C for 1 min and then cooled at 65°C for 15 min, 37°C for 15 min, room temperature for 15 min, and 4°C for 15 min. Primed M13 DNA was stored at −20°C until use. Reaction mixtures (50 μl each) contained 0.013 A_{260} units of primed M13 DNA and 10 μM each dNTP except in kinetic experiments in which the concentration of one dNTP was varied. All other conditions for RT assay were as previously described for reactions with φX174 DNA (4). The values are means ± standard errors for three or more determinations.

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