A Single 66-Kilodalton Polypeptide Processed from the Human Immunodeficiency Virus Type 2 *pol* Polyprotein in *Escherichia coli* Displays Reverse Transcriptase Activity

STUART F. J. LE GRICE, ROSWITHA ZEHNLE, AND JAN MOUS*

Central Research Units, F. Hoffmann-LaRoche & Co. Ltd., CH-4002 Basel, Switzerland

Received 16 November 1987/Accepted 18 March 1988

We have cloned the entire *pol* gene of human immunodeficiency virus type 2 into a high-level *Escherichia coli* expression system. Induction of cultures containing the recombinant plasmid, p2RTL1, leads to rapid accumulation of polypeptides of 66, 54, and 34 kilodaltons. We have designated the larger polypeptides reverse transcriptase, and we have designated the smaller polypeptide endonuclease. Purification of reverse transcriptase via ion-exchange and affinity chromatography yields the 66-kilodalton polypeptide, with which reverse transcriptase activity is associated. Purified enzyme furthermore displays a higher apparent molecular weight than its counterpart from human immunodeficiency virus type 1.

Recently, a new type of human immunodeficiency virus, designated human immunodeficiency virus type 2 (HIV-2), has been isolated from West African patients with the acquired immunodeficiency syndrome (AIDS) (2, 3). This virus is related to human immunodeficiency virus type 1 (HIV-1), the causative agent of the AIDS epidemic, by its morphology, lymphotropism, and in vitro cytopathic effect on CD4-positive cells. Despite this conservation in biological properties, a genetic comparison of the two human AIDS viruses reveals only limited sequence homology (6). For example, comparison of the polymerase (*pol*) genes of HIV-1 and HIV-2 shows a 60% homology at the amino acid level. Since the reverse transcriptase component of the *pol* open reading frame is receiving increasing attention as a target for antiviral therapy (8, 12, 13), it is important to determine the relationship and possible differences between these enzymes from HIV-1 and HIV-2.

Progress towards the characterization of HIV-1 reverse transcriptase has been greatly facilitated by recent observations that an enzymatically active form is released from the *pol* precursor polyprotein expressed in bacterial systems (5, 7, 10, 16), thus making available large amounts for study. Using this as precedent, we decided to follow the same strategy for HIV-2 reverse transcriptase.

Figure 1 illustrates cloning of the entire HIV-2 *pol* gene in our high-level *Escherichia coli* expression system (15). As starting material, we used a 4.8-kilobase *Hind*III fragment, containing *pol* flanked at each end with extra HIV-2 sequences (6) (Fig. 1b). Since the *pol* open reading frame contains a unique *Apa*I site near the amino terminus of the putative protease moiety, the 5' end of the *Hind*III fragment was eliminated by *Apa*I digestion (Fig. 1c) and the *Apa*I-*Hind*III fragment was ligated with the following synthetic adaptor sequence:

-G-A-T-C-T-G-G-C-A-G-G-T-T-T-T-T-A-G-G-A-C-T-G-G-G-C-C-
*Bgl*III -A-C-C-G-T-C-C-A-A-A-A-A-T-C-C-T-G-A-C- *Apa*I

This adaptor sequence replaces those missing protease amino acids; the *pol* open reading frame was thus tailored for insertion into our high-level expression system as a *Bgl*III-*Hind*III fragment (Fig. 1d).

In an initial experiment, *E. coli* cultures containing the recombinant plasmid p2RTL1 were induced by isopropyl-β-D-thiogalactopyranoside (IPTG) and analyzed for immunoreactive polypeptides by using serum containing antibodies to HIV-2 (Fig. 2). As can be seen, there was rapid accumulation of a 66-kilodalton (kDa) polypeptide, and lesser amounts of a 54-kDa polypeptide (Fig. 2a). For comparison, Fig. 2b illustrates processing of reverse transcriptase from the HIV-1 *pol* open reading frame expressed in the same *E. coli* system; the similarity in the two processing profiles allowed us to tentatively designate these HIV-2 polypeptides as reverse transcriptase. In addition, serum containing antibodies to HIV-2 reveals a 34-kDa polypeptide. This corresponds to the predicted molecular weight of the endonuclease portion of *pol*; in Fig. 3, we have compared immunoreactivity of the products of the HIV-2 *pol* open reading frame from *E. coli* and total HIV-2 viral antigen with serum containing antibodies to HIV-2. This analysis illustrates, in both cases, immunoreactive polypeptides of 66, 54, and 34 kDa, strengthening our postulation that the larger polypeptides correspond to reverse transcriptase and that the 34-kDa polypeptide is endonuclease. From molecular mass predictions, the other product of the *pol* open reading frame, protease, should be present as an 18-kDa polypeptide. Such a polypeptide was also observed, but confirmation of this as protease awaits the production of specific antibodies against this polypeptide.

Interestingly, the cleavage kinetics of the HIV-2 *pol* polyprotein differ from those of the HIV-1 counterpart, as indicated in Fig. 2. The polyprotein of HIV-1 is detected shortly after induction and thereafter rapidly processed to two polypeptides of 64 and 52 kDa; reverse transcriptase activity has been demonstrated to be associated with the presence of these polypeptides (4, 5, 7, 10, 11). In contrast, we detect no precursor polyprotein in cells containing p2RTL1, but rather rapid accumulation of polypeptides of 66 and 54 kDa. These results suggest that, at least in *E. coli*, the

* Corresponding author.

FIG. 1. Strategy for cloning of the HIV-2 *pol* open reading frame in *E. coli*. (a) Genomic organization of HIV-2 (6). The *Hind*III, *pol*-containing fragment (b) was further digested with *Apal* (c) and inserted into the expression vector pDS56/RBSII (d), within which the missing protease amino acids (6) had been added via a synthetic *Bgl*III-*Apal* adaptor (BA). *Hind*III and *Apal* sites are abbreviated H and A, respectively. P/O, Synthetic T5 promoter/lac operator fragment (15); R, ribosome-binding site; to and T1, transcriptional terminators (14); *cat* and *bla*, structural genes for chloramphenicol acetyltransferase and β-lactamase, respectively; *ori*, origin of replication. In plasmid p2RTL1, the shaded portion at the 3' end of the *pol* open reading frame represents the Q, R, and X open reading frames which are harbored by the original *Hind*III fragment. Kb, Kilobase.

FIG. 2. Processing of reverse transcriptase from the *pol* polyproteins of HIV-2 and HIV-1 in *E. coli*. The HIV-1 *pol*-containing plasmid pRTL10 is a derivative of the plasmid pRTL11 previously reported (10). Cultures containing recombinant clones were grown in antibiotic-supplemented L broth until an optical density at 600 nm of 0.7 was reached, after which gene expression was induced by addition of IPTG to a final concentration of 400 μg/ml. Post-IPTG induction times (in minutes) at which samples were removed for immunological analysis were 0 (o), 15 (a), 30 (b), 45 (c), 60 (d), 120 (e), and 180 (f). At these times, cells from 1 ml of culture were suspended in 200 μl of sodium dodecyl sulfate-polyacrylamide gel buffer and heated 10 min at 90°C. Samples (10 μl) were fractionated through 12.5% polyacrylamide gels containing a 3.3% stacking gel (9). For Western immunoblot analysis, fractionated proteins were transferred to nitrocellulose as previously described (17). Colorimetric detection of immunoreactive polypeptides was as described by Certa et al. (1). Notations pp and RT refer to precursor polyprotein and reverse transcriptase, respectively. M, Prestained protein markers (in kilodaltons).

FIG. 3. Comparison of HIV-2 reverse transcriptase produced in *E. coli* with total HIV-2 viral antigen. The immunoblot presented here has been analyzed with serum containing antibodies to HIV-2. Lane M, Prestained molecular mass protein markers (indicated to the left in kilodaltons); lane 1, total viral antigen from HIV-1; lane 2, HIV-1 reverse transcriptase produced in *E. coli*; lane 3, products of the HIV-2 *pol* polypeptide in *E. coli*; lane 4, total HIV-2 viral antigen. Note that lane 2 contains a purified preparation of reverse transcriptase, while lane 3 contains a bacterial lysate following induction. RT, Reverse transcriptase; Endo, endonuclease.

pol polypeptides of HIV-1 and HIV-2 follow different kinetics of processing. Whether this difference lies in a more efficient HIV-2 protease remains to be elucidated. In this respect, we are presently constructing hybrid *pol* open reading frames in which the protease moieties can be interchanged.

To determine whether the 66- and 54-kDa HIV-2 polypep-

tides produced from *pol* in *E. coli* were indeed forms of reverse transcriptase, these were purified by chromatography over DEAE-Sephacel and DNA-cellulose. In Fig. 4, we illustrate the elution profile from DNA-cellulose, in which a large peak of reverse transcriptase activity is eluted with 0.17 M NaCl. Immunological analysis, using a mixture of HIV-1- and HIV-2-specific antibodies, of the DNA-cellulose-purified enzyme (Fig. 4c) now detects a single polypeptide of 66 kDa; the same analysis illustrates that this form is indeed larger than the comparably purified HIV-1 reverse transcriptase.

In Fig. 5a and b we present a titration curve and time course of incorporation of precursor dGTP into polynucleotide by purified HIV-2 reverse transcriptase. Under these conditions, we estimate that our isolation yields 150 U of enzyme per mg of cells, where 1 U catalyzes incorporation of 1 pmol of precursor dGTP into polynucleotide in 10 min at 37°C, using poly(rC) primed with oligo(dG) primer-template system (10). Incorporation of precursor is linear over a period of 20 min. In addition, we have compared the monovalent and divalent cation requirements of similarly purified HIV-1 and HIV-2 enzymes (Fig. 5c through f). As can be seen, neither enzyme has an absolute dependence for monovalent cations; for each, the calculated K^+ and Na^+ optima were 120 and 75 mM, respectively (Fig. 5c and d). However, both enzymes display an absolute divalent cation requirement, with Mg^{2+} being preferred to Mn^{2+} (Fig. 5e and f). In this respect, we can conclude that the 66-kDa enzyme from HIV-2 resembles the 64-kDa/52-kDa enzyme of HIV-1.

In summary, we show here that appreciable amounts of enzymatically active HIV-2 reverse transcriptase can be produced in *E. coli*. Since there is a reasonable amount of divergence at the amino acid level between this enzyme and the HIV-1 counterpart, it would therefore be important to compare the effect of inhibitors on both enzymes.

FIG. 4. Purification and electrophoretic characterization of HIV-2 reverse transcriptase from *E. coli* harboring p2RTL1. (A) Activity profile from DNA-cellulose affinity chromatography. The y axis to the left shows the amount of [32 P]dGTP incorporated, while the y axis to the right shows the NaCl concentration. (B) Analysis of purified enzyme by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. M, protein markers; DEAE, sample of DEAE-Sephacel-purified enzyme; DNA, sample of pooled and concentrated DNA-cellulose-purified enzyme. Purification of reverse transcriptase by DEAE-Sephacel ion-exchange chromatography has been previously described (10). This enzyme preparation was further purified by application to a 4.5-ml DNA-cellulose column equilibrated in a buffer of 50 mM Tris hydrochloride (pH 8.0), 1 mM EDTA, 0.5 mM dithiothreitol, and 10% (vol/vol) glycerol. Once unbound proteins were removed, reverse transcriptase was eluted with a linear gradient of 0 to 0.7 M NaCl in the same buffer. Assay for reverse transcriptase was as previously described (10). The arrowhead indicates the position of the 66-kDa HIV-2 reverse transcriptase polypeptide. (C) Immunological analysis of DNA-cellulose-purified HIV-1 (lane 1) and HIV-2 (lane 2) reverse transcriptases. Lane M contains prestained molecular mass protein markers (in kilodaltons). Conditions for electrophoresis, transfer, and immunological detection were as described in the legend to Fig. 2.

FIG. 5. Characterization of DNA-cellulose-purified reverse transcriptases from HIV-1 and HIV-2. (a) Titration curve for HIV-2 enzyme; (b) incorporation time course for HIV-2 enzyme; (c) through (f), dependence of HIV-1 and HIV-2 reverse transcriptase on K^+ , Na^+ , Mg^{2+} , and Mn^{2+} , respectively. In all assays, the vertical axis depicts dGTP incorporation into polynucleotide. Assay conditions were as described by Le Grice et al. (10). Symbols: ▲, HIV-1 enzyme; ■, HIV-2 enzyme.

We thank M. Alizon of the Pasteur Institute (Paris, France) for the gift of plasmid pROD4.8 containing the HIV-2 *pol* open reading frame, B. Garcia and S. Reutener for technical assistance, D. Stüber for *E. coli* expression vectors, W. Lücke (Deutsches Primatenzentrum, Göttingen, Federal Republic of Germany) for HIV-2 antigen, and W. Bannwarth for synthetic oligonucleotides.

LITERATURE CITED

1. Certa, U., W. Bannwarth, D. Stüber, R. Gentz, M. Lanzer, S. F. J. Le Grice, F. Guillot, I. Wendler, G. Hunsmann, H. Bujard, and J. Mous. 1986. Subregions of a conserved part of the HIV gp41 transmembrane protein are differentially recognized by antibodies of infected individuals. *EMBO J.* 5:3051-3056.
2. Clavel, F., D. Guétard, F. Brun-Vezinet, S. Chamaret, M.-A. Rey, M. O. Santos-Ferreria, A. G. Laurent, C. Dauget, C. Katlama, C. Rousieux, D. Klatzmann, J. L. Champlimaud, and L. Montagnier. 1986. Isolation of a new human retrovirus from West African patients. *Science* 233:343-346.
3. Clavel, F., M. Guyader, D. Guetard, R. Salle, L. Montagnier,

- and M. Alizon. 1986. Molecular cloning and polymorphism of the human immune deficiency virus type 2. *Nature (London)* **324**:691-695.
4. Di Marzo Veronese, F., T. D. Copeland, A. L. DeVico, R. Rahman, R. C. Gallo, and M. G. Sarngadharan. 1986. Characterisation of highly immunogenic p66/p51 as the reverse transcriptase of HTLV-III/LAV. *Science* **231**:1289-1291.
 5. Farmerie, W. G., D. D. Loeb, N. C. Casavant, C. A. Hutchison, M. E. Edgell, and R. Swanstrom. 1987. Expression and processing of the AIDS reverse transcriptase in *Escherichia coli*. *Science* **236**:305-308.
 6. Guyader, M., M. Emerman, P. Sonigo, F. Clavel, L. Montagnier, and M. Alizon. 1987. Genome organisation and transactivation of the human immunodeficiency virus type 2. *Nature (London)* **326**:662-669.
 7. Hansen, J., T. Schultz, and K. Moelling. 1987. RNase H activity associated with bacterially expressed reverse transcriptase of human T-cell lymphotropic virus III/lymphadenopathy-associated virus. *J. Biol. Chem.* **262**:12393-12396.
 8. Jentsch, K. D., G. Hunsmann, H. Hartmann, and P. Nickel. 1987. Inhibition of human immunodeficiency virus type I reverse transcriptase by suramin-related compounds. *J. Gen. Virol.* **68**:2183-2192.
 9. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
 10. Le Grice, S. F. J., V. Beuck, and J. Mous. 1987. Expression of biologically active human T-cell lymphotropic virus type III reverse transcriptase in *Bacillus subtilis*. *Gene* **55**:95-103.
 11. Lightfoote, M. M., J. E. Coligan, T. M. Folks, A. S. Fauci, M. A. Martin, and S. Venkatesan. 1986. Structural characterization of reverse transcriptase and endonuclease polypeptides of the acquired immunodeficiency syndrome retrovirus. *J. Virol.* **60**:771-775.
 12. Mitsuya, H., and S. Broder. 1986. Strategies for antiviral therapy in AIDS. *Nature (London)* **325**:773-778.
 13. Mitsuya, H., K. J. Weinhold, P. A. Furman, M. H. St. Clair, S. Nusinoff Lehrman, R. C. Gallo, D. Bolognesi, D. W. Barry, and S. Broder. 1985. 3'-Azido-3' deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus *in vitro*. *Proc. Natl. Acad. Sci. USA* **82**:7096-7100.
 14. Peschke, U., V. Beuck, H. Bujard, R. Gentz, and S. F. J. Le Grice. 1985. Efficient expression of *Escherichia coli* transcriptional signals in *Bacillus subtilis*. *J. Mol. Biol.* **186**:547-555.
 15. Stüber, D., I. Ibrahimi, D. Cutler, B. Dobberstein, and H. Bujard. 1984. A novel *in vitro* transcription-translation system: accurate and efficient synthesis of single proteins from cloned DNA sequences. *EMBO J.* **3**:3143-3148.
 16. Tanese, N., J. Sodroski, W. A. Haseltine, and S. P. Goff. 1986. Expression of reverse transcriptase activity of human T-lymphotropic virus type III (HTLV-III/LAV) in *Escherichia coli*. *J. Virol.* **59**:743-745.
 17. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350-4354.