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HIV-1 PROTEINASE IS REQUIRED FOR SYNTHESIS OF PRO-VIRAL DNA

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HIV-1 proteinase activity is thought to occur primarily post-integration by cleaving the viral Gag and Gag-Pol polyproteins. Its role in the pre-integration stages of viral replication, however, has not been studied in detail. Here we report that a synthetic peptide analogue, UK-88,947, which is a specific inhibitor of purified HIV-1 proteinase, inhibits the processing of the viral polyproteins in cultures of HIV-1 infected cells and prevents the formation of mature, infectious virions. Analysis of DNA from HIV-1 infected cells treated with UK-88,947 showed that viral DNA synthesis was inhibited when the compound was added to cultures one hour before infection. Similar results were obtained when AZT was used. Neither HIV-1 reverse transcriptase or the replication of FIV are inhibited by UK-88,947.

The proteolytic cleavage of Gag (p55) and Gag-Pol (p165) polyproteins of human immunodeficiency virus type 1 (HIV-1) by the virus encoded proteinase (PR) is required for viral maturation and infectivity (1-4). In the absence of PR, non-infectious viral particles devoid of electron dense cores are produced (2,3). The PR is enzymatically active as a dimer and is thought to function primarily post-integration (5-10). Studies with equine infectious anemia virus (EIAV) (11), however, showed that the nucleocapsid (NC) protein purified in capsids, undergoes further proteolytic processing, which suggests the viral PR may play a role in the early stages of the replication cycle. This report describes how a synthetic peptide analogue UK-88,947, which inhibits HIV-1 PR in both cell-free and cell-based assays, is used to demonstrate that this enzyme is involved in the steps after the entry of the virus prior to viral DNA synthesis.

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The sequence of amino acids in S28 was searched for internal duplications and one possible repeat was found: MDTSR (at positions 1-5) and MDDTSR (at positions 35-40).

The determination of the sequence of amino acids in rat S28 is a contribution to a data set which it is hoped will eventually include the structure of all the proteins in the ribosomes of this mammalian species. The primary purpose for the accumulation is its anticipated use in arriving at a solution of the structure of the organelle. However, the information may also help in understanding the evolution of ribosomes, in unraveling the function of the proteins, in defining the rules that govern the interaction of the proteins and the rRNAs, and in uncovering the amino acid sequences that direct the proteins to the nucleolus for assembly on nascent rRNA.

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REFERENCES

1. Wool, I.G., Endo, Y., Chan, Y.L. and Glück, A. (1990) in The Ribosome: Structure, Function and Evolution (Hill, W.E., *et al.* ed.), pp. 203-214, Amer. Soc. Microbiol., Washington, D.C.
2. Chan, Y.L., Lin, A., McNally, J. and Wool, I.G. (1987) J. Biol. Chem. 262, 12879-12886.
3. Chan, Y.L. and Wool, I.G. (1988) J. Biol. Chem. 263, 2891-2896.
4. Beaucage, S.L. and Caruthers, M.H. (1981) Tetrahedron Lett. 22, 1859-1862.
5. Kozak, M. (1986) Cell 44, 283-292.
6. Proudfoot, N.J. and Brownlee, G.G. (1976) Nature 263, 211-214.
7. Levy, S., Avni, D., Hariharan, N., Perry, R.P. and Meyuhas, O. (1991) Proc. Natl. Acad. Sci. USA 88, 3319-3323.
8. Goerl, M., Welfle, H. and Bielka, H. (1978) Biochim. Biophys. Acta 519, 418-427.
9. Flinta, C., Persson, B., Jörnvall, H. and von Heijne, G. (1986) Eur. J. Biochem. 154, 193-196.
10. Dayhoff, M.O. (1978) in Atlas of Protein Sequence and Structure (Dayhoff, M.O., ed.) Vol. 5, Suppl. 3, pp. 1-8, National Biomedical Research Foundation, Washington, D.C.
11. Leer, R.J., van Raamsdonk-Duin, M.M.C., Schoppink, P.J., Cornelissen, M.T.E., Cohen, L.H., Mager, W.H. and Planta, R.J. (1983) Nucleic Acids Res. 11, 7759-7768.

798-829. The blots were washed three times with 6xSSC containing 1% SDS at room temperature, three times at 70°C and exposed to X-ray film for 24 hours.

Feline Immunodeficiency Virus (FIV) replication assay: anti-FIV activity was determined by assaying viral core antigen (p26) production in Crandle feline kidney cells infected with FIV (Glasgow 8 strain, donated by Professor O. Jarnett, Veterinary School, Glasgow). UK-88,947 was added to cells at the time of infection. One week later FIV antigen present in the supernatant of infected cells was quantitated using an ELISA kit (Idexx, Inc).

RESULTS AND DISCUSSION

The activity of UK-88,947 (Figure 1) against purified HIV-1 PR was determined using an *in vitro* assay system. This compound was 90-fold more active against the HIV-1 PR than the aspartyl proteinase inhibitor, pepstatin A (Table 1). Specificity of UK-88,947 for the viral enzyme was apparent as its IC_{50}s against HIV-1 PR, renin and pepsin were 12nM, 580nM and 450nM respectively. The antiviral activity of UK-88,947 was determined by quantitating infectious virus produced from cells initially infected and subsequently treated with inhibitor. UK-88,947 showed similar activity to AZT as it reduced p24 levels by 90% at 3μM and blocked production of infectious virus at 6μM. Pepstatin A showed no antiviral activity at 15μM (Table 1). UK-88,947 at 60μM was not toxic to proliferating H9 cells since it had no effect on cellular DNA, RNA or protein synthesis. To confirm that HIV-1 PR was the target of UK-88,947, the cleavage of HIV-1 p55 in treated H9IIIB cells was studied. Western blot analysis (Figure 2a) showed that processing of viral p55 to p24 was inhibited by UK-88,947 (12μM). When viewed by electron microscopy, the virus from treated cells (Figure 2c), consisted of predominantly (97%) immature particles lacking the condensed cone-shaped core (capsid) which is characteristic of mature infectious virus (Figure 2b), indicating that this compound does not prevent post-integration synthesis of viral proteins, virus assembly and budding.

To determine whether PR has a role in viral replication prior to integration, DNA synthesis in H9 cells infected with HIV-1 in the presence of UK-88,947 was analysed with the PCR, using

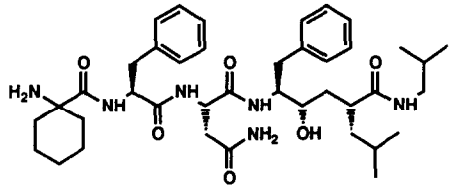

Figure 1. Chemical structure of UK-88,947: N-isopentyl-(S)-5-[1-aminocyclohexylcarbonyl-(S)-phenylalanyl-(S)-asparaginylamino]-(S)-4-hydroxy-(R)-2-isobutyl-6-phenylhexanamide, hydrochloride.

Table 1. Inhibition of HIV-1 replication and viral replication by UK-88,947. [a] - p24, IC_{90}: concentration that inhibits p24 levels by 90%. [b] - IT, IC_{100}: concentration that inhibits infectious titre by 100%.

COMPOUND	HIV-1 PROTEINASE IC_{50} (nM)	ANTI-HIV-1_{IIIB} ACTIVITY	
		p24[a], IC_{90} (μM)	IT[b], IC_{100} (μM)
UK-88,947	12	3	6
PEPSTATIN	1100	>15	>15
AZT	-	3.78	7.5

primers that hybridize to the *gag* region of HIV-1 DNA. As the length of one cycle of HIV-1 replication in H9 cells has been reported to be approximately 24 hours (16), a time course experiment was performed whereby a single dose of the inhibitor (12 μM) was added 1 h before infection, at infection, or 2.5, 5 and 18 hours post-infection. The cells were harvested 18 hours post-infection to ensure that only first-round DNA synthesis was analysed, separated into nuclear and cytoplasmic fractions from which DNA was isolated and subjected to PCR. When UK-88,947 was added to cells one hour prior to infection, no viral DNA was detected in the cytoplasmic or nuclear fractions (Figure 3). Increasing the time interval before addition of compound resulted in a steady increase in the amount of HIV-1 DNA synthesised. This suggests that viral uncoating and DNA synthesis occur more rapidly than diffusion of the compound to its site of action, as cytoplasmic proviral DNA was detected even when compound was added simultaneously with virus. When compound was added 2.5 h after infection, proviral DNA synthesis was depressed but not completely inhibited. This implies that UK-88,947 achieves this affect without interfering with virus binding to the cell. This was investigated further with a cell-based assay using Jurkat-tat-III cells (17). Transformation with the plasmid pIIIenv3-1, encoding the gp120 protein (17), and production of normal levels of CD4-gp120-induced syncytia could not be inhibited with UK-88,947 (120μM) (data not shown). The quantity of virus specific DNA detected in the nucleus was consistently lower than that observed in the cytoplasm (Figure 3). Proviral DNA was first detected in the nucleus when UK-88,947 was added 2.5 h after infection, but not when compound was added simultaneously, indicating that a minimum of 2.5 hours is required for HIV-1 DNA to be translocated to the nucleus. Identical results were obtained in a parallel experiment using AZT (7.5μM), which when added 1 h before infection completely inhibited viral DNA synthesis (Figure 3). Addition of AZT at increasing time intervals after infection was progressively less effective at inhibiting viral DNA synthesis in the cytoplasm and the nucleus (Figure 3).

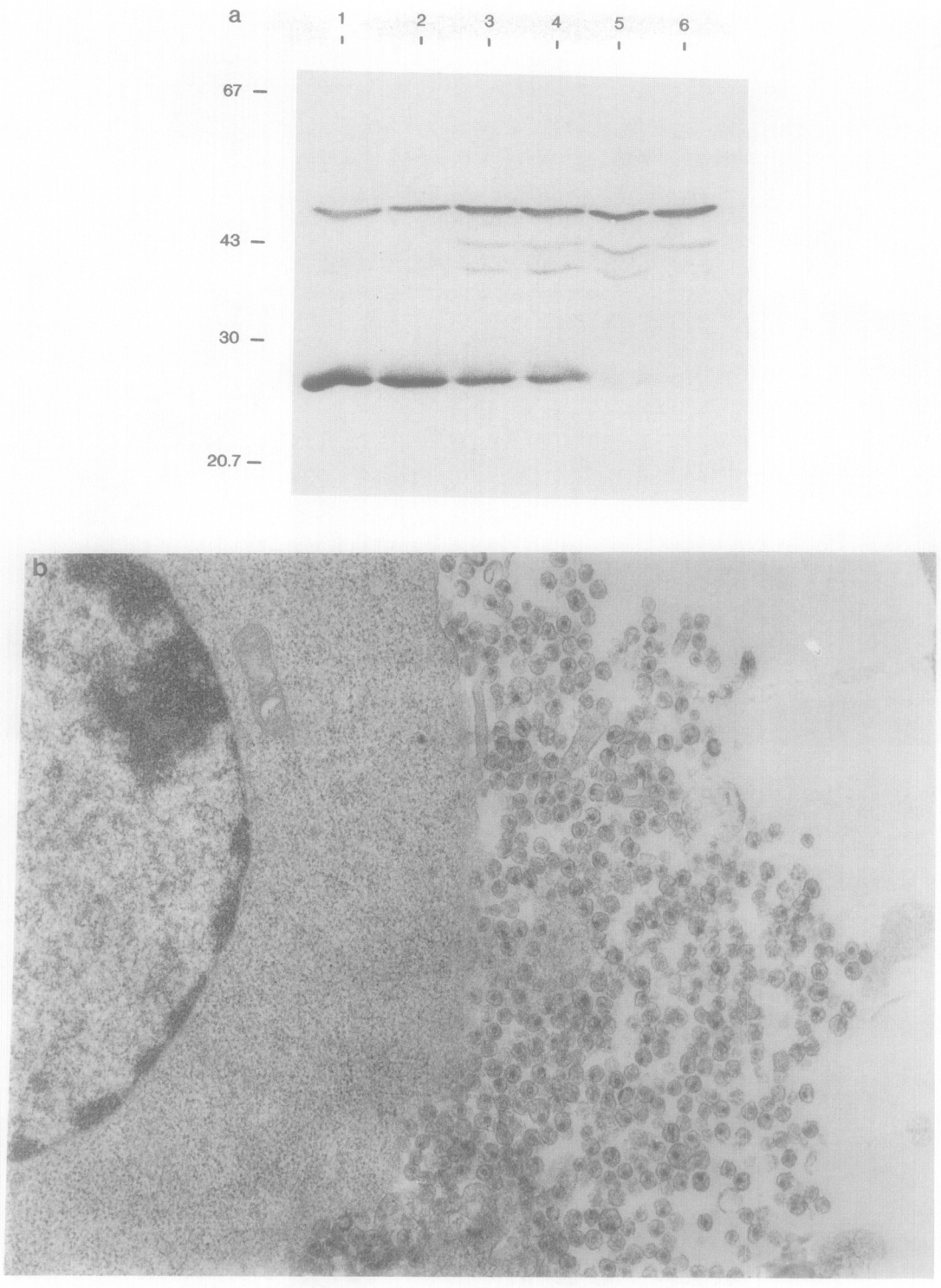

Figure 2. Inhibition of HIV-1 p55 processing in H9IIIB cells by UK-88,947. (a) Western blot analysis: proteins profiles from lysates of H9IIIB cells cultured with 0, 1.5, 3, 6, or 12μM UK-88,947 (lanes 1 and 3-6) respectively, and with 7.5μM AZT (lane 2) are shown. (b and c) Electron Microscopy: samples of the H9IIIB cells either untreated (b) or treated with 12μM UK-88,947 (c).

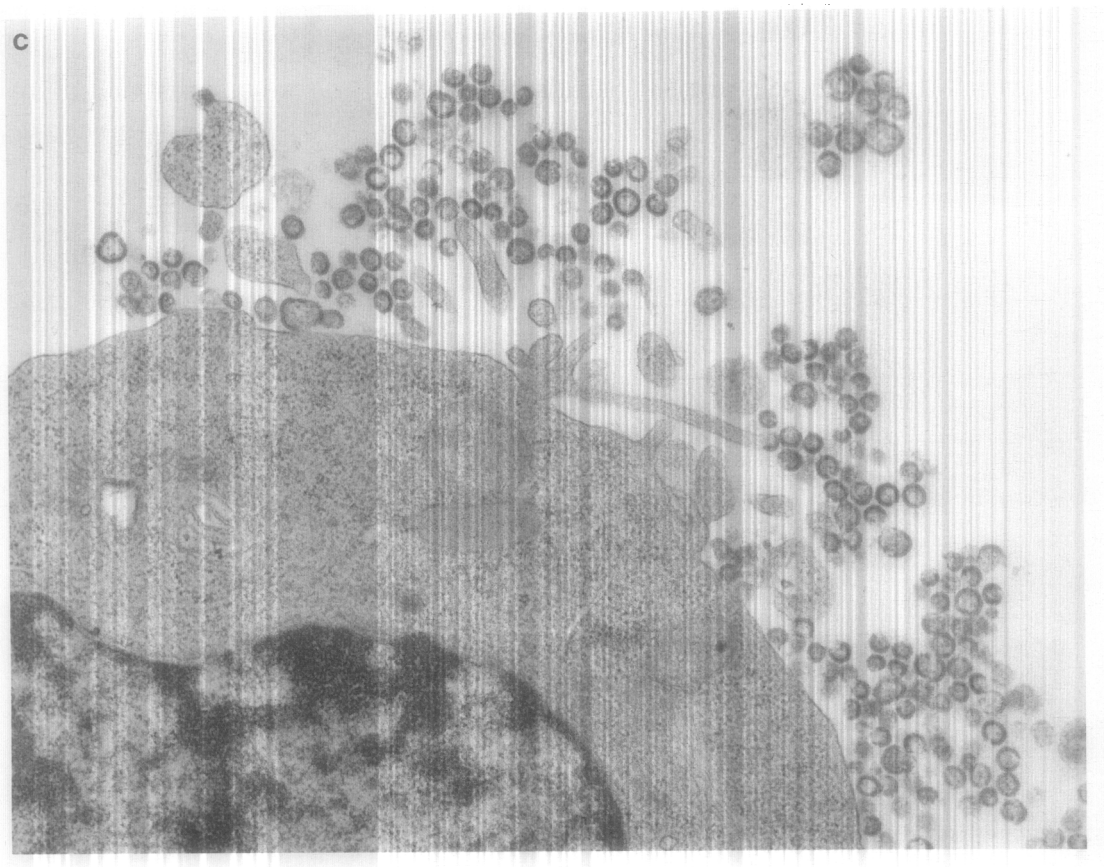

Figure 2 - Continued

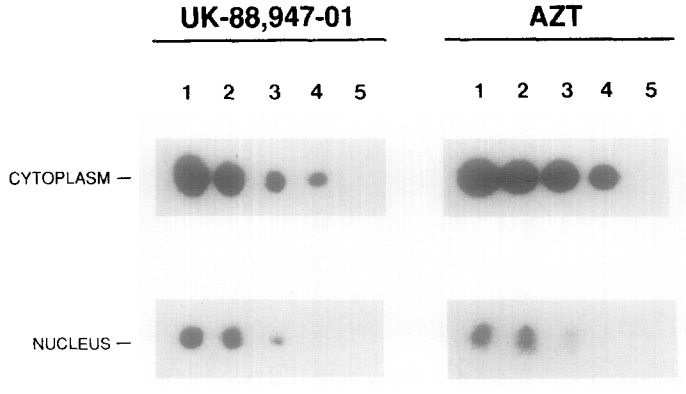

Figure 3. Southern blot analysis of HIV-1 DNA synthesis in acutely infected H9 cells cultured in the presence of UK-88,947. UK88,947 (12μM) or AZT (7.5μM) was added to the cultures one hour prior to infection (5), 0 hours (4), 2.5 hours (3), 5 hours (2) and 18 hours (1) post infection. Cell cultures were harvested at the 18 hour time point.

Table 2. The effect of UK-88,947 on replication of feline immunodeficiency virus

COMPOUND	CONCENTRATION (μM)	FIV p26 (%)
UK-88,947	0	100
	3	95
	6	100
	12	109
AZT	7.5	4.4

Additional evidence that UK-88,947 specifically inhibits HIV-1 PR is that the compound at 12μM (i) did not affect replication of another lentivirus, FIV, in CRFK cells as determined by ELISA (Table 2), and (ii) did not inhibit HIV-1 reverse transcriptase activity at 12μM in an *in vitro* assay (18) (data not shown). These results suggest that, as well as preventing late phase maturation of virions, UK-88,947 restricts productive infection at an earlier phase in the HIV-1 replication cycle, i.e. after binding of the virus to cellular receptors, up to and including reverse transcription of viral DNA. The role of PR at this stage of the replicative cycle is unknown.

When EIAV particles are stripped of their envelope, the packaged PR in these capsids is able to cleave the p11 NC protein to p6 and p4 proteins (19) *in situ*. The p11 cleavage is inhibited when UK-88,947 (12μM) is incubated with isolated capsids (M. Roberts, P.J. Whittle, and S. Oroszlan, unpublished). This cleavage was not observed in the whole virus which implies that the early PR cleavage activity is invoked only when internalisation has occurred. It is not known whether cleavage of the corresponding HIV-1 NC protein, p7, occurs upon entry into susceptible cells.

The role of p11 cleavage in EIAV is uncertain but it may be required to allow access for reverse transcriptase to transcribe genomic viral RNA. A similar mechanism may be occurring in HIV-1.

As the inhibitor is effective at preventing proviral DNA synthesis only when present in the culture prior to infection, it is likely that protein cleavage is required before viral DNA can be made, and that this occurs rapidly upon entry and uncoating of HIV-1 in H9 cells.

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REFERENCES

1. Gottlinger, G., Sodroski, J., and Haseltine, W. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5781-5785.
2. Kohl, N.E. Emini, E.A., Schleif, W.A., Davis, L.J., Heimbach, J.C., Dixon, R.A.F., Scolnick, E.M., and Sigal, I.S. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**,4686-4690.
3. Peng, C., Ho, B., Chang, T., and Chang, N. (1989) *J. Virol.* **63**, 2550-2556.
4. Seelmeier, S., Schmidt, H., Turk, V., and von der Helm, K. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6612-6616.
5. Henderson, L.E., Copeland, T. D., Sowder, R.C., Schultz, A.M., and Oroszlan, S. (1987) in *Human Retroviruses, Cancer, and AIDS: Approaches to Prevention and Therapy* 135-147 (Alan Liss, New York).
6. Lapatto, R., Blundell, T.L., Hemmings, A., Overington, J., Wilderspin, A., Wood, S., Merson, J.R., Whittle, P.J., Danley, D.F., Geoghegan, K.F., Hawrylik, S.J., Lee, S.E., Scheld, K.G., and Hobart, P.M. (1989) *Nature* **342**, 299-302.
7. Meek, T.D., Dayton, B.D., Metcalf, B.W., Dreyer, G.B., Strickler, J.E., Gorniak, J.G., Rosenberg, M., Moore, M.L., Magaard, V.W., and Debouck, C. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1841-1845.
8. Navia, M.A., Fitzgerald, P.M.D., McKeever, B.M., Leu, C.T., Heimbach, J.C., Herber, W.K., Sigal, I.S., Drake, P.L., and Springer, J.P. (1989) *Nature* **337**, 615-620.
9. Nutt, R.F., Brady, S.F., Drake, P.L., Ciccarone, T.M., Colton, C.D., Nutt, E.M., Rodkey, J.A., Bennett, C.D., Waxman, L.H., Sigal, I.S., Anderson, P.S., and Verber, D.F. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7129-7133.
10. Veronese, F.D., Copeland, T.D., Orozlan, S., Gallo, R.C., and Sarngadharan, M.G.(1988) *J. Virol.* **62**, 795-801.
11. Roberts, M. and Oroszlan, S. (1989) *Biochem. Biophys. Res. Commun.* **160**, 486-494.
12. Danley, D. E. Geoghegan, K.F., Scheld, K.G., Lee, S.E., Merson, J.R., Hawrylik, S.J. Rickett, G.A., Ammirati, M.J., and Hobart, P.M. (1989) *Biochem. Biophys. Res. Commun.* **165**, 1043-1050.
13. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) *Molecular Cloning.* (Cold Spring Harbour, N.Y.).
14. Innes, M., Gelfand, D., Sninsky, J., and White, T. (1990) *PCR Protocols - A Guide to Methods and Application.* (Academic Press, London).
15. Myers, G., Rabson, A.B., Bersofsky, J.A., Smith, T.F. and Wong-Staal, F. (1990) *Human Retroviruses and AIDS* (Los Alamos, New Mexico).
16. Kim, S., Byrn, R., Groopman, J., and Baltimore, D. (1989) *J. Virol.* **63**, 3708-3713.
17. Sodroski, J., Goh, W.C., Rosen, C., Campbell, K., and Haseltine, W.A. (1986) *Nature* **322**, 470-474.
18. Matthes, E., Lehmann, C., Scholz, D., Von Janta-Lipinski, M., Gaertner, K., Rosenthal, H.A., and Langen P. (1987) *Biochem. Biophys. Res. Commun.* **148**, 78-85.
19. Roberts, M., Copeland, T., and Oroszlan, S. (1991) *Protein Engineering.* In Press.