Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 11241–11245, December 1991
Microbiology

Human immunodeficiency virus type 1 mutants resistant to nonnucleoside inhibitors of reverse transcriptase arise in tissue culture

DOUGLAS RICHMAN*†, CHENG-KON SHIH‡, ISRAEL LOWY§, JANICE ROSE‡, PATRICIA PRODANOVICH¶, STEPHEN GOFF§, AND JOHANNA GRIFFIN‡

*Departments of Pathology and Medicine, University of California, and the Veterans Affairs Medical Center, San Diego, CA 92161; ‡Boehringer Ingelheim Pharmaceuticals, Inc., 900 Ridgebury Road, Box 368, Ridgefield, CT 06877; §Columbia University, College of Physicians and Surgeons, 630 168th Street, New York, NY 10032; and ¶Life Sciences Research Laboratory, Baxter Diagnostics, P.O. Box 85200, San Diego, CA 92186

Communicated by Edwin D. Kilbourne, September 9, 1991 (received for review July 20, 1991)

ABSTRACT **We have recently described a nonnucleoside compound that specifically inhibits the reverse transcriptase of human immunodeficiency virus type 1 (HIV-1), the causative agent of AIDS. This compound, nevirapine (BI-RG-587), interacts with highly conserved tyrosine residues at positions 181 and 188 in the reverse transcriptase to inhibit the recombinant enzyme and virus replication in cell culture with 50% inhibitory concentrations in the 40 nM range. HIV-1 variants resistant to nevirapine emerged with passage in cell culture in the presence of drug. This resistant phenotype was stable with continued passage in the absence of drug. These mutants had a substitution of cysteine for the tyrosine at position 181. Introduction of this mutation into the recombinant enzyme increased the inhibitory concentration of nevirapine 100-fold. Substitution of cysteine for tyrosine at residue 181 into the wild-type viral genome conferred a similar reduction in susceptibility to nevirapine. Mutants were also resistant to a tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-one and -thione derivative and two 6-phenylthiouracil derivatives but retained their sensitivity to the other reverse transcriptase inhibitors, 3′-azido-3′-deoxythymidine and foscarnet.**

Infection by human immunodeficiency virus type 1 (HIV-1) requires reverse transcriptase (RT)-catalyzed synthesis of double-stranded DNA (1, 2). Inhibition of this enzymatic reaction by the nucleoside analogue drug zidovudine (3′-azido-3′-deoxythymidine; AZT) has provided the first clinical benefit for HIV-infected patients (3–5). Prolonged therapy with AZT has resulted in the emergence of drug-resistant variants with changes in the RT gene (6, 7). A class of nonnucleoside compounds including nevirapine (BI-RG-587) and the tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-one and -thione (TIBO) series inhibit RT by a different mechanism (8–11) and, therefore, are effective against the AZT-resistant mutant viruses (12). Nevirapine is a potent, highly specific inhibitor that binds to HIV-1 RT by a mechanism that is noncompetitive with respect to primer, template, nucleotide, and tRNA (9, 10). The residues to which nevirapine binds, Tyr-181 and -188 (13, 14), flank an amino acid sequence that has been shown by mutational analysis to be critical for enzyme activity and that is conserved among all RNA-dependent polymerases but whose functional role is not understood (15). Although the two tyrosine residues have been absolutely conserved among HIV-1 isolates, there are different amino acids at that site in other related lentiviral RTs (16). Therefore, we anticipated that some mutations might be tolerated in the nevirapine binding sites of the HIV-1 enzyme that would permit replication but render the virus resistant to the drug. Such mutations would be expected to emerge under the selective pressure of prolonged therapy with nevirapine in AIDS patients. In these studies, we describe several independent isolates of nevirapine escape mutants selected in cell culture in both a laboratory strain and a low passage patient isolate of HIV-1. It seems likely that the single nucleotide transition in the codon for amino acid 181 from TAT for tyrosine to TGT for cysteine that we observed in these variants may arise in patients undergoing nevirapine treatment.

MATERIALS AND METHODS

Virus Passage and Selection for Resistance in Cell Culture. HIV-1_{BRU} and the AZT-sensitive San Diego isolate A018A (6) were passaged in 0, 1, or 10 μM nevirapine in wells of 48-well plates (Costar) containing 10^5 CEM cells in 1.0 ml of RPMI 1640 medium containing 100 units of penicillin per ml, 100 μg of streptomycin per ml, 2 mM glutamine, 1 μg of Polybrene per ml, and 10% fetal bovine serum. The multiplicity of the initial infection was 1 tissue culture ID_{50} per cell. Passages were performed weekly by adding 150 μl of the infected culture supernatant to a final culture vol of 1 ml containing 10^5 uninfected CEM cells. The remaining supernatant was frozen in aliquots at −70°C.

Drug Susceptibility Assays by Plaque Reduction. HeLa cells expressing CD4 (HT4-6C cells) (17) were used in a plaque (syncytial focus) reduction assay as described (6, 18). Briefly, 24-well culture plates containing HT4-6C cell monolayers were inoculated with virus in various concentrations of antiviral drug in medium. Duplicate wells were prepared for each dilution, and the percentage plaque reduction was based on the control value without drugs. The IC_{50} was calculated with the median effect plot (19). Nevirapine and R82913, a TIBO derivative (8), were synthesized in-house. Foscarnet was purchased from Fairfield Chemicals (Blythwood, SC). 1-Ethoxymethyl-5-ethyl-6-(phenylthio)uracil and 1-benzyloxymethyl-5-ethyl-6-phenylthio-2-thiouracil were the generous gift of M. Baba (Fukushima, Japan) (20).

Nucleic Acid Sequencing. Viral isolates found to be resistant to nevirapine and their parental strains were sequenced by asymmetric PCR (21). Both strands were sequenced to confirm results. The primers obtained from Genosys (The Woodlands, TX) used for sequencing are indicated with their nucleotide numbers (22): sense (2583–2597), 5′-CCAAAGTAGCATGAC-3′; antisense (2795–2768), 5′-ACCCATCCAAAGGAATGGAGGTTCTTTC-3′.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HIV, human immunodeficiency virus; RT, reverse transcriptase; AZT, 3′-azido-3′-deoxythymidine; TIBO, tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-one and -thione.

†To whom correspondence should be addressed.

Preparation of Recombinant RT Enzymes. The HIV-1 RT expression clone, which is also available from the National Institutes of Health AIDS Research and Reference Reagent Program was obtained from Yale University (23). Mutant construct HIV-1 RT Cys-181 was made by oligonucleotide-directed mutagenesis (24) using protocols available commercially (Bio-Rad, Richmond, CA). The mutant RT construct was first cloned into the *Nco* I/*Hin*dIII sites of phagemid vector pGEM3Zf(+) (Promega), which was modified to contain an *Nco* I site in the polylinker region, and the clones were selected by nucleotide sequence analysis. The construct was then subcloned into pKK233-2 (Pharmacia), the same expression vector as wild-type RTs. The mutation was again confirmed by nucleotide sequencing. Highly purified wild-type and mutant RTs were prepared as described (10).

Western Blot Analysis. Highly purified mutant and wild-type RT enzymes were run on an SDS/polyacrylamide gel and blot-transferred onto a nitrocellulose filter. The blot reacted with monoclonal antibody directed against HIV-1 RT and was developed with reagents and protocols from Promega.

Binding of BI-RJ-70, Photoaffinity Analog of Nevirapine. Wild-type and mutant RTs were labeled with [^{3}H]BI-RJ-70, a photoaffinity analog probe that binds to the same site on HIV-1 RT as nevirapine under conditions in which the photoaffinity probe labeled only the p66, but not the p51, subunit of the wild-type HIV-1 RT heterodimer (10). Samples were then fractionated by SDS/PAGE, and the gel was fluorographed with Entensify (NEN) and exposed to x-ray film at −70°C for 3 days.

Polymerase Assays. AZT 5′-triphosphate was obtained from Raymond F. Schinazi through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. The other drugs were obtained as indicated. Highly purified enzymes were used for measurements of 50% inhibitory concentrations (IC_{50}) with compounds. IC_{50} values for nevirapine, R82913, and foscarnet were derived from RNA-directed DNA polymerase assays, using poly(rC)·oligo(dG) as template primer in the following conditions: 50 mM Tris·HCl, pH 7.8/60 mM NaCl/2 mM $MgCl_2$/24 nM poly(rC)·oligo$(dG)_{10}$ (Pharmacia)/600 nM [^{3}H]dGTP (NEN) (specific activity, 11 Ci/mmol; 1 Ci = 37 GBq)/20 mM dithiothreitol/0.02% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate in a total vol of 60 μl. IC_{50} values for AZT 5′-triphosphate were measured on poly(rA)·oligo(dT) as template primer with the following reaction components: 50 mM Tris·HCl, pH 7.8/60 mM NaCl/2 mM $MgCl_2$/5 nM poly$(rA)_{300}$·oligo$(dT)_{12-18}$ (Pharmacia)/700 nM [^{3}H]TTP (NEN; specific activity, 20 Ci/mmol). The reaction mixture was incubated for 60 min at 25°C. Acid-insoluble products were collected on glass fiber filters, and radioactivity was determined by LKB Beta Plate scintillation counting.

Construction and Analysis of Mutant Viral Genomes. A recombinant provirus containing the tyrosine to cysteine mutation at amino acid 181 of the polymerase was constructed by transfer of an *Eco*RV/*Pfl*MI restriction enzyme fragment spanning the mutation in the recombinant RT enzyme into a plasmid containing the remainder of a replication-competent provirus, R7/3/BH10; this provirus was used for the wild-type control. One microgram of wild-type or mutant proviral DNA was introduced into 10^6 Jurkat cells by the DEAE-dextran method (25). After distributing the transfected cells into wells with different concentrations of nevirapine, the course of viral replication was monitored by the appearance of cytopathic effect and quantitation of RT activity and p24 antigen in the supernatant.

Detection of Wild-Type and Mutant Sequence at Amino Acid 181. Wild-type or mutant sequence present in the supernatants of passaged virus was detected by amplification with the isothermal, self-sustained sequence replication method (26) and differential hybridization using a bead-based sandwich hybridization technique that has been applied to AZT resistance mutations (27). Trisacryl Oligobeads 90–46, and ^{32}P-labeled detection oligonucleotides 91–115, 91–87, and 91–86 were used to detect the following amplification products: 90–46, GT AGC ATG ACA AAA ATC TTA GAG CC; 91–115, CTTA GAA ATA GGG CAG CA; 91–87, ATA GTT ATC **TAT** CAA TAC AT; 91–86, ATA GTT ATC **TGT** CAA TAC AT. Oligonucleotide 91–115 detects all RT amplification products independent of genotype. Oligonucleotides 91–87 and 91–86 span the codon for amino acid 181 and are specific for the wild-type sequence (Tyr-181) or the mutant sequence (Cys-181), respectively. Complementary and noncomplementary targets were differentiated by a series of increasingly more stringent washes with continuous monitoring for labeled detection oligonucleotide. The total radioactivity of the pooled washes and the detection oligonucleotide remaining bound to the Oligobeads were determined by Cerenkov counting, and the femtomoles of product captured were calculated based on the percentage of the total cpm that remained hybridized to the beads (27).

RESULTS

Selection of Nevirapine-Resistant Virus. Two strains of HIV-1 were each passaged in the presence of either 1 or 10 μM nevirapine. Passaged supernatants were tested for infectivity and drug-susceptibility phenotype. With the laboratory strain HIV-1_{BRU}, plaques were detected in supernatants from the first passage in the presence of 1 μM nevirapine and between passages 6 and 12 at 10 μM. The virus population in supernatants after 20 passages in 1 μM nevirapine was ≈100-fold less susceptible than the parental virus, although passage 12 supernatants contained virus with an apparently intermediate susceptibility (Fig. 1). Strain A018A, a 1986 virus isolate, generated sufficiently high titers of virus to detect plaques at passage 3 with 1 μM nevirapine and at passage 7 with 10 μM nevirapine. The drug susceptibilities of these isolates were 100-fold reduced compared to the control virus passaged in the absence of drug.

Cross-Resistance of the Resistant Phenotype. The nevirapine-resistant virus displayed no cross-resistance to foscarnet or AZT; however, the susceptibility to other nonnucleoside inhibitors, a TIBO derivative, and two 6-phenylthiouracil derivatives, was significantly reduced (Table 1). Cross-resistance to neither dideoxycytosine nor dideoxyinosine was observed (data not shown).

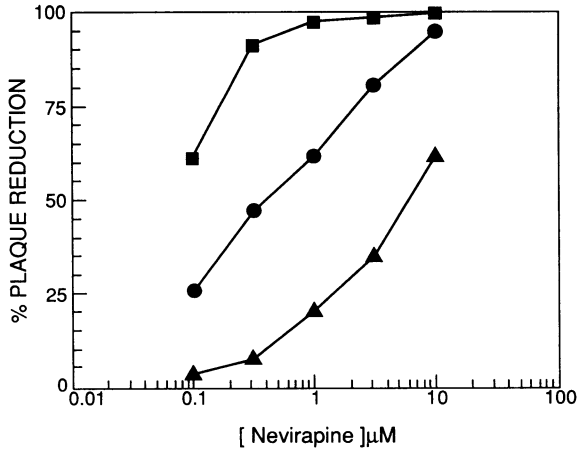

FIG. 1. Susceptibility of HIV-1_{BRU} passaged in the presence of 1 μM nevirapine. ■, Passage 0; ●, passage 12; ▲, passage 20.

Table 1. Susceptibility of nevirapine-resistant variant to other RT inhibitors

	IC_{50}, μM					
	Nevirapine	AZT-TP	Foscarnet	TIBO	E-EPU	E-BPU-S
Virus						
Passage 0	0.04	0.01	30	0.05	0.02	0.003
Passage 12	10.0	0.01	30	0.2	1.0	1.0
Recombinant enzyme						
Wild type	0.06 ± 0.01	0.04 ± 0.02	18.6 ± 11.1	0.13 ± 0.03	ND	ND
Y181C mutant*	4.83 ± 0.71	0.02 ± 0.01	17.2 ± 9.3	1.62 ± 0.08	ND	ND

The 1 μM nevirapine passage 12 supernatant of strain A018A and the parental virus were used. Polymerase assays were performed in triplicate at each drug concentration, and averages of triplicate data were used for analysis. Results from two independent experiments were analyzed to derive IC_{50} ± SE. ND, not done; E-EPU, 1-ethoxymethyl-5-ethyl-6-(phenylthio)uracil; E-BPU-S, 1-benzyloxymethyl-5-ethyl-6-phenylthio-2-thiouracil.
*Tyr-181 to Cys-181 mutation.

Stability of the Resistant Phenotype. The passage-12-resistant variant of A018A passaged in 10 μM nevirapine was then passaged 19 additional times in drug-free medium. The resistant phenotype remained unchanged in the absence of selective pressure, and its growth rate was not distinguishable from the parental virus (data not shown).

DNA Sequence Analysis of the Nevirapine Binding Site. To determine the mechanism by which these mutants had become resistant to the nonnucleoside inhibitors, we sequenced the region of the viral genome that encodes the nevirapine binding site in RT. The sequence of 171 bases of DNA from two independent isolates of A018A and one of HIV-1_{BRU} revealed a single nucleotide change from the wild-type sequence in the codon for the tyrosine residue at codon 181 (TAT to TGT), resulting in a mutation to cysteine.

Emergence of the Cys-181 Mutation by *in Vitro* Selection. The supernatants from each passage of virus in the presence of nevirapine were monitored for the presence of the wild-type and mutant sequence at codon 181, part of the nevirapine binding site (13, 14), utilizing a recently devised methodology of self-sustained sequence replication amplification and differential bead-based sandwich hybridization. In experiments utilizing two strains of HIV-1 and two drug concentrations, mutant sequence was detectable in a mixture as early as the first passage and a pure population of mutant sequence developed in the second passage in one experiment (Table 2). The virus examined in Fig. 1 proved to be a mixture of wild-type and mutant virus at passage 12 and mutant alone at passage 20.

Effect of the Resistance Mutation of Recombinant RT. To determine whether or not the single change of Tyr-181 to Cys-181 was sufficient to confer nevirapine resistance to the RT enzyme, the substitution was made in the recombinant clone of the gene encoding HIV-1 RT. Purified recombinant wild-type or mutant RT proteins were tested for enzyme activity and for sensitivity to nevirapine. The recombinant enzyme containing Cys-181 was as active as the wild-type enzyme containing Tyr-181 (data not shown). However, the IC_{50} of the Cys-181 mutant for nevirapine and TIBO was 100- and 10-fold higher, respectively, than that of the wild-type enzyme (Table 1). To determine the mechanism by which the Cys-181 mutation conferred resistance to nevirapine, we tested the ability of the mutant enzyme to bind to [^{3}H]BI-RJ-70, the radiolabeled photoaffinity analog of nevirapine (13). BI-RG-70 has IC_{50} values for wild-type and mutant enzymes of 0.06 and 0.51, respectively. The labeling of the mutant was dramatically reduced compared with the labeling of wild-type RT, even though Western blot analysis indicated that comparable amounts of wild-type and mutant enzyme were analyzed (Fig. 2). These data indicated that a single A to G mutation in the RT gene of HIV-1 was sufficient to inhibit binding of the photoaffinity analog and to render the enzyme resistant to nevirapine.

Table 2. Passages first displaying indicated genotype

	HIV-1_{BRU}		A018A	
Genotype	10 μM	1 μM	10 μM	1 μM
Wild type	1	1		1
Mixture	3	17*	1	
Cys-181 only	5		5	2

*Only 20 passages were tested.

Effect of Cys-181 Substitution in the HIV-1 Genome. It was possible that the single base change in the RT gene was sufficient to render the enzyme insensitive to nevirapine but that some compensatory mutation elsewhere in the viral genome was required for efficient viral replication. This possibility was tested by introducing that mutation into the cloned wild-type viral genome. After transfection of the mutant provirus into Jurkat cells, the virus grew with kinetics and titers similar to wild type (data not shown). The mutant virus, however, like the originally selected isolates, was 100-fold more resistant to nevirapine when assayed by syncytial plaque reduction (Fig. 3), as well as by inhibition of accumulation of supernatant RT activity and p24 antigen (data not shown). Although it is possible that mutations elsewhere in the RT gene could contribute to nevirapine resistance, these results confirm that the introduction of a single nucleotide change into the wild-type HIV-1 genome, of A to G in the codon for Tyr-181 to Cys-181, respectively, is sufficient to confer the same level of resistance to nevirapine

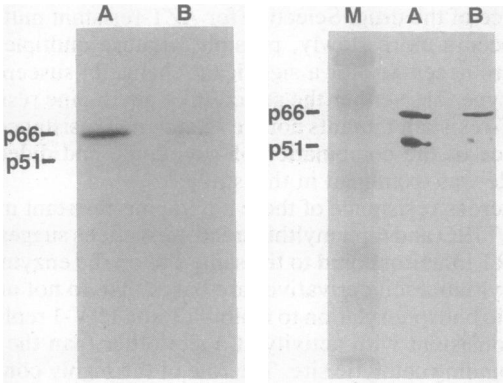

FIG. 2. Photoaffinity probe labeling (*Left*) and Western blot analysis (*Right*) of wild-type and mutant Cys-181 HIV-1 RTs. Experimental conditions described in *Materials and Methods* were used to react BI-RJ-70 with purified recombinant enzymes. Lanes: A, wild-type HIV-1 RT; B, mutant enzyme; lane M, prestained SDS/PAGE molecular size standard (Bio-Rad). Positions of the heterodimer, p66 and p51, of HIV-1 RT are indicated.

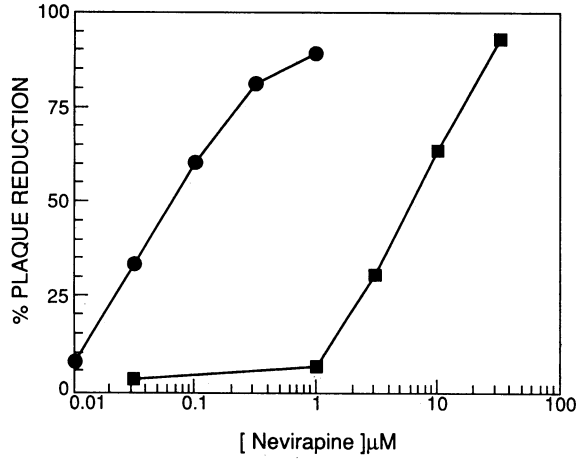

FIG. 3. Susceptibility to nevirapine of HIV reconstructed to contain the Cys-181 mutation in RT. Virus was prepared by transfecting Jurkat cells with mutant or wild-type proviral DNA. Nevirapine susceptibility of the progeny virus was then assayed by plaque reduction in CD4 HeLa (HT4-6C) cells. ●, Wild type; ■, Cys-181 mutant.

as that of tissue culture-selected virus grown in the presence of the drug.

DISCUSSION

Nevirapine, a dipyridodiazepinone, is one of a class of nonnucleoside inhibitors of HIV-1 RT that does not inhibit HIV-2 RT or any of several other viral and mammalian DNA polymerases (9). It inhibits p24 antigen production and syncytium formation of wild-type virus in cell culture at concentrations >8000-fold lower than the toxic dose for those cells. Nevirapine inhibits mutant isolates of HIV resistant to AZT and displays synergy with AZT in inhibiting AZT-sensitive virus (12). Promising toxicologic and pharmacokinetic data have prompted phase I/II trials to assess the compound as a candidate drug for HIV infection.

The unusually high rate of genetic mutation of HIV-1 portends that variants resistant to this chemotherapeutic agent will emerge as they have to AZT. To confirm the site at which the drug acts and to identify the mutations that are most likely to arise in patients treated with the nonnucleoside inhibitor nevirapine, we have selected for resistant strains in cell culture. Resistant mutants with two strains of virus were obtained after only a few passages of infected cells in the presence of the drug. Selection for AZT-resistant mutants *in vitro* occurs more slowly, possibly because multiple mutations are necessary for a significant change in susceptibility phenotype (7). Neither the selection of nevirapine resistance in AZT-resistant mutants nor the selection of resistance in the presence of the combination of nevirapine and dideoxynucleoside was examined in this study.

The cross-resistance of these nevirapine-resistant mutants for the TIBO and 6-phenylthiouracil derivatives suggests that these RT inhibitors bind to the same site on the enzyme. The 6-phenylthiouracil derivatives are bases that do not undergo anabolic phosphorylation to inhibit RT and HIV-1 replication (28), consistent with activity at a site other than the nucleotide-binding catalytic site. The role of the highly conserved amino acid sequences flanked by Tyr-181 and -188, the nevirapine binding sites in HIV-1 RT, in the function of the RT enzyme is not understood. These drugs that specifically bind to sites in this domain and escape mutants that do not bind the drugs will be useful reagents in understanding the functional significance of these sequences in viral enzyme activity. Although the full-length RT gene was not sequenced, the single mutation at amino acid 181 fully accounted for the phenotypic change in susceptibility observed in the mutants selected in culture. Two features of these mutants suggest that they may be amenable to attack should they arise in patients being treated with nevirapine. First, both the mutant virus and the recombinant enzyme with the A to G substitution retained full sensitivity to AZT and foscarnet. The prospects of combination therapy are thus encouraging because resistance to either drug confers no cross-resistance to the other. Second, all resistant virus isolates contained the same base change. This suggested that mutation may be the only one that is well tolerated for replication by the virus at that site and that a second generation compound that inhibits such a mutant could effectively control the virus infection.

Note Added in Proof. After submission of this manuscript Nunberg *et al.* (29) published a report of the selection for HIV-1 mutants resistant to pyridinone containing RT inhibitors with cross resistance to nevirapine and a TIBO compound. These mutants showed 1000-fold reductions in susceptibility and contained a lysine to asparagine mutation at codon 103 in addition to the Cys-181 mutation. Sequencing of the nevirapine mutants has demonstrated the codon 103 mutation not to be present. It is unclear whether the lack of a codon 103 mutation and the 10-fold lower change in susceptibility with nevirapine selection is attributable to a difference in the drugs or to a difference in selection conditions.

We gratefully acknowledge the excellent technical assistance of Patricia Ley and Lydia Chiang and the helpful advice of Thomas Gingeras. We also thank Gary Caviness and Dr. Jan Glinski for enzyme purification. This work was supported by Grants HB-67019, AI-27670, AI-30457, and AI-29164 from the National Institutes of Health; by Boehringer Ingelheim Pharmaceuticals, Inc.; and by the Research Center for AIDS and HIV Infection of the San Diego Veterans Affairs Medical Center. I.L. was supported by a Clinician-Scientist Award from the American Heart Association/Squibb Corporation and by the Aaron Diamond Foundation.

1. Mitsuya, H., Yarchoan, R. & Broder, S. (1990) *Science* **249**, 1533–1544.
2. Goff, S. P. (1990) *J. AIDS* **3**, 817–831.
3. Fischl, M. A., Richman, D. D., Grieco, M. H., Gottlieb, M. S., Volberding, P. A., Laskin, O. L., Leedom, J. M., Groopman, J. E., Mildvan, D., Schooley, R. T., Jackson, G. G., Durack, D. T., King, D. & AZT Collaborative Working Group (1987) *N. Engl. J. Med.* **317**, 185–191.
4. Fischl, M. A., Richman, D. D., Hansen, N., Collier, A. C., Carey, J. T., Para, M. F., Hardy, W. D., Dolin, R., Powderly, W. G., Allan, J. D., Wong, B., Merigan, T. C., McAuliffe, V. J., Hyslop, N. E., Rhame, F. S., Balfour, H. H., Jr., Spector, S. A., Volberding, P. A., Pettinelli, C., Anderson, J. & AIDS Clinical Trials Group (1990) *Ann. Intern. Med.* **112**, 727–737.
5. Volberding, P. A., Lagakos, S. W., Koch, M. A., Pettinelli, C., Myers, M. W., Booth, D. K., Balfour, D. H., Reichman, R. C., Bartlett, J. A., Hirsch, M. S., Murphy, R. L., Hardy, W. D., Solero, R., Fischl, M. A., Bartlett, J. G., Merigan, T. C., Hyslop, N. E., Richman, D. D., Valentine, F. T., Corey, L. & AIDS Clinical Trials Group (1990) *N. Engl. J. Med.* **322**, 941–949.
6. Larder, B. A., Darby, G. & Richman, D. D. (1989) *Science* **243**, 1731–1734.
7. Larder, B. A. & Kemp, S. D. (1989) *Science* **246**, 1155–1158.
8. Pauwels, R., Andires, K., Desmyter, J., Schols, D., Kukla, M. J., Bresline, H. J., Raeymaeckers, A., Van Gelder, J., Woestenborghs, R., Heykants, J., Schellekens, K., Janssen, M. A. C., De Clercq, E. & Janssen, P. A. J. (1990) *Nature (London)* **343**, 470–474.
9. Merluzzi, V. J., Hargrave, K. D., Labadia, M., Grozinger, K., Skoog, M., Wu, J., Shih, C.-K., Eckner, K., Hattox, S., Adams, J., Rosenthal, A. S., Faanes, R., Eckner, R. J., Koup, R. A. & Sullivan, J. L. (1990) *Science* **250**, 1411–1413.
10. Wu, J. C., Warren, T. C., Adams, J., Proudfoot, J., Skiles, J.,

Raghaven, P., Perry, C., Potocki, I., Farina, P. R. & Grob, P. M. (1991) *Biochemistry* **30**, 2022–2026.
11. Debyser, Z., Pauwel, R., Andries, K., Desmyter, J., Kukla, M., Janssen, P. A. J. & De Clercq, E. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 1451–1455.
12. Richman, D. D., Rosenthal, A. S., Skoog, M., Eckner, R. J., Chou, T.-C., Sabo, J. P. & Merluzzi, V. J. (1991) *Antimicrob. Agents Chemother.* **35**, 305–308.
13. Cohen, K. A., Hopkins, J., Ingraham, R. H., Pargellis, C., Wu, J. C., Palladino, D. E. H., Kinkade, P., Warren, T. C., Rogers, S., Adams, J., Farina, P. R. & Grob, P. M. (1991) *J. Biol. Chem.* **266**, 14670–14674.
14. Shih, C.-K., Rose, J. M., Hansen, G. L., Wu, J. C., Bacolla, A. & Griffin, J. A. (1991) *Proc. Natl. Acad. Sci. USA*, in press.
15. Xiong, Y. & Eickbush, T. H. (1988) *Mol. Biol. Evol.* **5**, 675–690.
16. Myers, G., ed. (1990) Theoretical Biology and Biophysics, (Los Alamos, NM).
17. Chesebro, B. & Wehrly, K. (1988) *J. Virol.* **62**, 3779–3788.
18. Larder, B. A., Chesebro, B. & Richman, D. D. (1990) *Antimicrob. Agents Chemother.* **34**, 436–441.
19. Chou, J. & Chou, T.-C. (1987) *Dose–Effect Analysis with Microcomputers: Quantitation of ED50, LD50, Synergism, Antagonism, Low-Dose Risk, Receptor-Ligand Binding and Enzyme Kinetics* (Elsevier-Biosoft, Cambridge, UK), pp. 1–93.
20. Baba, M., De Clercq, E., Tanaka, H., Ubasawa, M., Takashima, H., Sekiya, K., Nitta, I., Umezu, K., Nakashima, H., Mori, S., Shigeta, S., Walker, R. T. & Miyasaka, T. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 2356–2360.
21. Gyllenstein, U. B. & Erlich, H. A. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7652–7656.
22. Sanchez-Pescador, R., Power, M. D., Barr, P. J., Steimer, K. S., Stempien, M. M., Brown-Shimer, S. L., Gee, W. W., Renard, A., Randolph, A., Levy, J. A., Dina, D. & Luciw, P. A. (1985) *Science* **227**, 484–492.
23. D'Aquila, R. T. & Summers, W. C. (1989) *J. AIDS* **2**, 579–587.
24. Kunkel, T. A., Roberts, J. D. & Zakour, R. A. (1987) *Methods Enzymol.* **154**, 367–382.
25. Ausubel, F. A., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K., eds. (1990) *Current Protocols in Molecular Biology* (Wiley, New York), pp. 2.1–2.6.
26. Guatelli, J. C., Whitfield, K. M., Kwoh, D. Y., Barringer, K. J., Richman, D. D. & Gingeras, T. R. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1874–1878.
27. Gingeras, T. R., Prodanovich, P., Latimer, T., Guatelli, J. C., Richman, D. D. & Barringer, K. J. (1991) *J. Infect. Dis.*, in press.
28. Baba, M., Shigeta, S., Tanaka, H., Miyasaka, T., Ubasawa, M., Umezu, K., Walker, R. T., Pauwels, R. & De Clercq, E. (1991) *Antiviral Res.*, in press.
29. Nunberg, J. H., Schlief, W. A., Boots, E. J., O'Brien, J. A., Quintero, J. C., Hoffman, J. M., Emini, E. A. & Goldman, M. E. (1991) *J. Virol.* **65**, 4887–4892.