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# Mutational Analysis of the Human Immunodeficiency Virus *vpr* Open Reading Frame

KOJI OGAWA,† RIRI SHIBATA, TAKAHIRO KIYOMASU, IKUKO HIGUCHI, YOSHIKO KISHIDA, AKINORI ISHIMOTO, AND AKIO ADACHI*

*Laboratory of Gene Analysis, Department of Viral Oncology, Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606, Japan*

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**Mutations were introduced by recombinant DNA techniques into the *vpr* open reading frame of an infectious molecular clone of human immunodeficiency virus type 1. The effect of these changes on the replicative and cytopathologic properties of the virus recovered from transfected cells was studied in several human CD4$^+$ lymphocyte cell lines. In all cases, mutant viruses were infectious and cytopathic. However, when a low-input dose was used, mutants grew significantly more slowly than the wild-type virus. The growth kinetics of *vpr* mutants were distinct from those of *vif* and *vpu* mutants.**

Human immunodeficiency virus type 1 (HIV-1) is the primary etiological retrovirus of acquired immunodeficiency syndrome and related diseases (4, 15, 18). The complete nucleotide sequences of several distinct strains of HIV-1 have revealed a very complex genetic structure (17, 20, 23, 34). In addition to the three structural genes, *gag*, *pol*, and *env*, in common with other retroviruses, six extra genes have already been identified. Of these, two *trans*-activator genes, *tat* and *rev*, are essential for virus replication (5, 7, 27, 32). *nef*, *vif*, and *vpu* are not required for virus infectivity, although mutations in these genes greatly alter the phenotypes of the virus (2, 6, 17, 26, 28, 31, 32, 34). Recently, Wong-Staal et al. reported the presence of another gene, *vpr*, which could encode a protein recognizable by sera of some HIV-1-seropositive people (37). Functional analysis of *vpr*, however, has not yet been carried out. Therefore, we investigated the effects of mutations in *vpr* on the replication and cytopathogenicity of the virus.

Figure 1 shows the structure of the genomes used for this analysis, including plasmids pNL-Nd and pNL-Ss, which are *vif* and *vpu* mutants, respectively. An infectious proviral DNA, pNL432, which expressed all known HIV-1 proteins (1, 2, 30, 31, 36), was used to generate all mutants. The mutants were constructed by insertional frameshift mutations at the restriction endonuclease sites shown in Fig. 1.

The nucleotide sequence of the *vpr* region of pNL432 was determined to compare it with the published data (Fig. 2A). pNL432 contains *vpr*, which can encode 102 codons (96 from the first methionine codon). It overlaps with the *vif* gene and terminates after the first *tat* coding exon. The deduced amino acid sequence of *vpr* is highly conserved among seven proviruses used for comparison (Fig. 2B). Of note is the presence of an infectious clone which can encode only 78 amino acids. This short version of *vpr* is due to a frameshift in the 3′ portion of *vpr* of clone HXB-2. This frameshift is observed in other clones derived from the HIV-IIIB cell line (37). *vpr* mutants in this communication all have a short amino acid sequence relative to the wild-type clone pNL432. pNL-Af2 contains 27 amino acids, and pNL-Ec and pNL-Sl contain 79.

The effects of alterations in the virus genome on the production of virus particles were determined in transfection assays. The mutants and pNL432 were introduced into SW480 cells, which efficiently express transfected DNA and produce high reverse transcriptase (RT) in the culture fluids within 24 h (1). The amount of particles produced by the mutants was comparable to that produced by wild-type DNA following transfection into SW480 cells, as determined by RT activity (Fig. 1). However, the results obtained with mutants of other regulator genes were quite different. *tat* and *rev* mutants showed no RT activity, while *nef* mutants produced higher RT activity than the wild type (not shown).

The effects of virus infection were first monitored in the Molt-4 clone 8 cell line (M4-8) by measurements of viable cell number and cell-free RT activity. This cell line was shown to be highly sensitive to cytopathic effects (CPE) caused by HIV (14). Cell-free samples of *vpr* mutants and wild-type virus particles were prepared from the supernatants of SW480 cells transfected with pNL-Af2, pNL-Ec, pNL-Sl, or pNL432 by low-speed centrifugation and filtration through a 0.22-μm filter. M4-8 cells were infected with equivalent amounts (RT units) of each virus preparation, and HIV replication was monitored (Fig. 3). M4-8 cells infected with wild-type virus displayed strong CPE, including ballooning, multinuclear giant cells, and ghost cells as early as day 7. This profound CPE was followed by a reduction in the number of viable cells (Fig. 3A). The viable cell number reached a peak at day 7 and fell gradually during the observation period. RT activity in the culture fluids began to increase on day 7 and was maximum on day 16 (Fig. 3B). The kinetics of *vpr* mutant viruses in M4-8 cell were quite different. The appearance of CPE, reduction of cell number, and RT production were observed with delayed kinetics, occurring about 7 to 10 days after confirmation of wild-type virus infection. No significant difference of kinetics was found among cells infected with three mutants. Interestingly, when 10-fold-higher input multiplicities were used, infection

* Corresponding author.
† Present address: Research Institute, Ono Pharmaceutical Co., Ltd., Osaka 618, Japan.

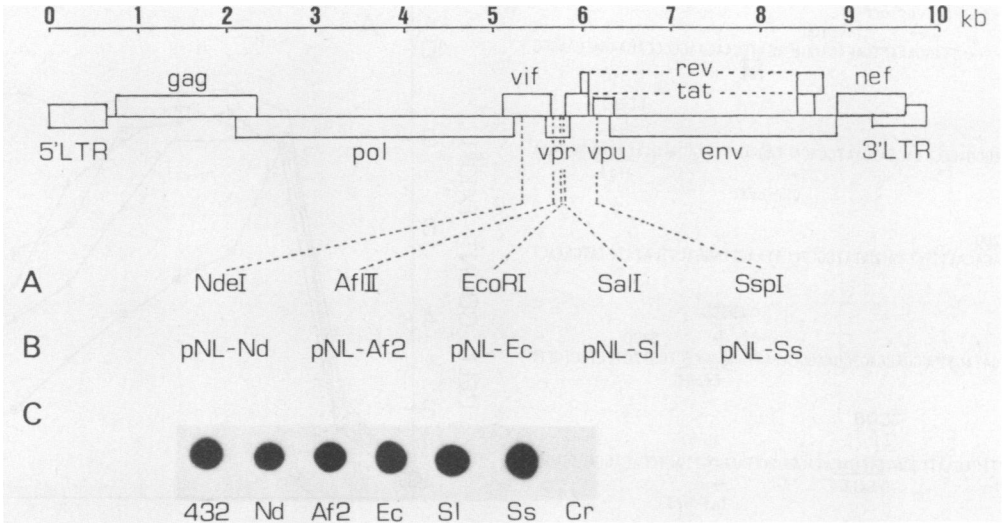


FIG. 1. Mutants used in this study. Schematic representation of HIV-1 proviral genome at the top. (A) Restriction enzyme sites used to generate mutants; (B) mutant designations; (C) transient expression of RT activity in transfected SW480 cells (ATCC CCL228). All mutants except pNL-Ss were constructed by cleaving plasmid DNA with the enzyme indicated, blunt ended by T4 DNA polymerase, and resealed by T4 DNA ligase. Thus, pNL-Nd has a 2-base-pair insertion; pNL-Af2, pNL-Ec, and pNL-Sl have 4-base-pair insertions. pNL-Ss was generated by digesting DNA with *Ssp*I, inserting an 8-base-pair *Cla*I linker by T4 DNA ligase, and reclosing by T4 DNA ligase. When necessary, the appropriate DNA fragment was first subcloned into pUC19, the mutation was introduced, and the mutated DNA was put back into the infectious clone, pNL432. DNA structure was confirmed by restriction mappings and partial sequencings. For transient transfection assays, proviral DNA was introduced into SW480 cells by the calcium phosphate coprecipitation method (11, 35). Cr in part C shows nondetectable RT activity in pUC19-transfected cells. RT activity was measured as reported previously (36). kb, Kilobases.

kinetics were indistinguishable between wild-type virus and *vpr* mutants (not shown).

We also examined cell line variation in *vpr* mutant responsiveness, using other human $CD4^+$ leukemia cell lines infected with wild-type and mutant viruses. Infection experiments were carried out as above, and CPE and RT activity were monitored at intervals (Fig. 4). In two cell lines, Molt-3 (JCRB9048) and TALL-1 (JCRB0086), similar delayed kinetics of the mutant virus infection were noted. In A3.01 cells (8), however, the time course of CPE and RT production was similar between wild-type- and mutant virus-infected cells, although RT activity persisted longer in cells infected with the mutant virus. This "tailing" of RT production in *vpr* mutant-infected A3.01 cells was always seen in several independent experiments. A3.01 cells differed from the other two cell lines in that they could produce large amounts of virus shortly after infection (Fig. 4). This property of A3.01 cells was considered to result in high multiplicities of infection at a very early stage in the time course experiment. Therefore, A3.01 cells were infected with two different doses of virus and RT activity was monitored (Fig. 5). Virus growth kinetics differed little among the three viruses when high multiplicities were used (Fig. 5, bottom). A 10-fold reduction of input dose clearly delayed the infection kinetics, particularly in *vpr* mutant-infected cells. The effect of multiplicities was not as drastic on the time course of RT production in wild type virus-infected cells. By contrast, in mutant-infected cells, the peak day of RT activity was delayed several days and the level of activity was low. Progeny viruses, produced in these four cell lines after inoculation with the mutant virus (pNL-Af2), behaved exactly like the *vpr* mutant, suggesting that "reversion" had not occurred (not shown).

The phenotype of the *vpr* mutant was compared with those of *vif* and *vpu* mutants. Published reports have demonstrated that *vif* is required for efficient virus transmission (6, 30) and *vpu* is required for virus maturation or assembly (31). A3.01 cells were infected with various viruses with quite high multiplicities. Figure 6 shows the virus growth curves of *vif*, *vpr*, *vpu*, and wild-type virus as determined by RT assays. Again, little difference was seen between wild-type and *vpr* mutant viruses. The *vif* mutant, pNL-Nd, grew so poorly that faint RT activity was detected as late as day 23 (Fig. 6A). RT activity was detected even 60 days after infection in *vif* mutant-infected A3.01 cells with very weak CPE, whereas cells infected with *vpr* mutant or wild-type virus did not produce RT activity 40 days after infection (A. Adachi and K. Ogawa, unpublished observation). Figure 6B gives a phenotypical comparison of *vpr* and *vpu* mutants. The growth curve of the *vpu* mutant was striking, although the kinetics was similar to that of the wild-type virus. A several-fold reduction in progeny virions (as determined by RT assays) relative to wild-type virus was observed, as reported (31) after the infection of A3.01 cells with a *vpu* mutant, in contrast to the phenotype of the *vpr* mutant. This growth characteristic of the *vpu* mutant was seen in another infection experiment that used 10-fold-lower input multiplicities (not shown).

Our results suggest that *vpr* is necessary for efficient replication of the virus and concurrent CPE in $CD4^+$ cell cultures. In particular, pNL-Af2, which was constructed to eliminate expression of most of the *vpr* open reading frame, is clearly inefficient for virus growth. The three *vpr* mutants, pNL-Af2, pNL-Ec, and pNL-Sl, showed similar delayed kinetics of infection relative to wild-type virus. This suggests that the C-terminal portion of *vpr* is important for *vpr* function (Fig. 2B). However, *vpr* is dispensable for growth of the virus in $CD4^+$ cells. In our assay system, the function of *vpr* is only evident after multiple rounds of productive infection have occurred, since the defective phenotype of

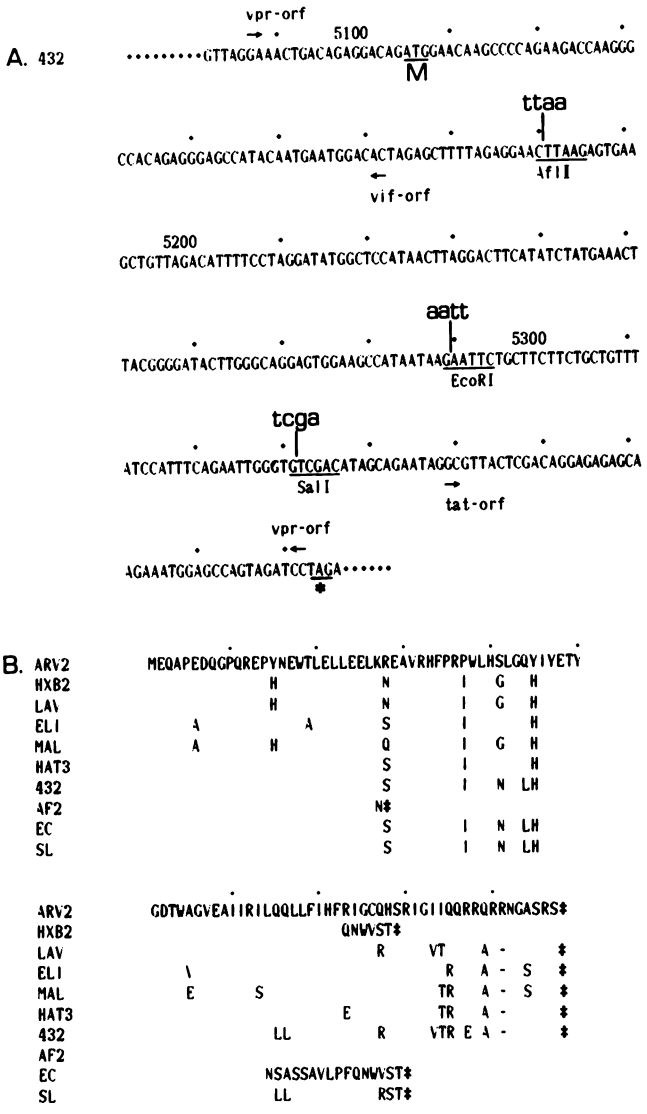


FIG. 2. Sequence of pNL432 *vpr* region. (A) Nucleotide sequence of the *vpr* region. The nucleotide numbering system of Wain-Hobson et al. (34) was used. Sequence was determined by the dideoxy sequencing method (24). Both strands were sequenced. The positions of the first methionine (M) and the stop codon (asterisk) in *vpr* are indicated. A 4-base-pair insertion is shown above the enzyme cleavage site (position also indicated). (B) Deduced amino acid sequences of *vpr* of several HIV-1 strains, ARV2 (23), HXB2 (19), LAV (34), ELI (3), MAL (3), and HAT3 (29), are shown for comparison. A one-letter amino acid code was used. Sequence is from the first methionine codon (top left) to the stop codon (asterisk, bottom right). Blank represents an amino acid identical to that of the ARV2 strain. −, Deletion of the amino acid.

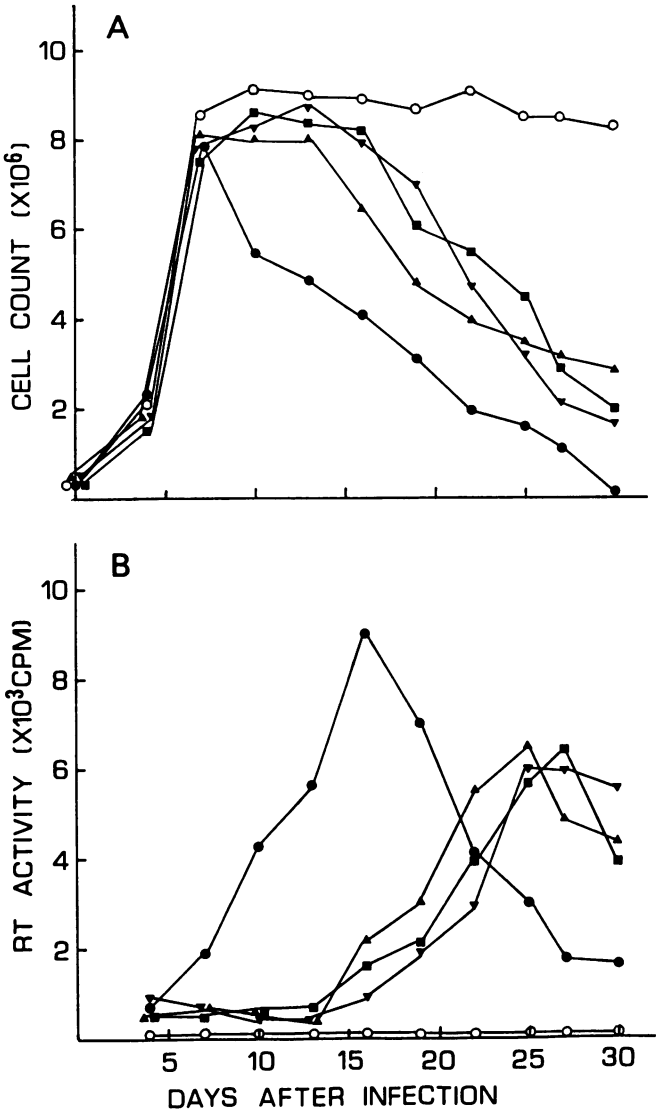


FIG. 3. Growth kinetics of *vpr* mutants (Af2, Ec, and Sl) and wild-type virus (432) in M4-8 cells. (A) Viable cell counts per culture as determined by trypan blue exclusion. (B) RT activity in culture fluids (1.5 μl of supernatants). Cells were infected with the virus in the presence of Polybrene, as reported previously (8). Equivalent amounts of virus ($2 \times 10^5$ cpm) were used to initiate infection. Symbols: ○, mock; ●, pNL432; ■, pNL-Af2; ▲, pNL-Ec; ▼, pNL-Sl.

*vpr* mutants is very dependent on input multiplicities. This mild effect of *vpr* mutation on virus replication is puzzling, since this reading frame is well conserved not only among many strains of HIV but also in the distantly related ungulate lentivirus, visna virus (28). Moreover, some sera of infected individuals recognize the bacterially expressed *vpr* products (37). Conservation of the *vpr* open reading frame suggests that it plays an important role in the life cycle of the virus. Two possible functions for *vpr* can be considered. *vpr* may be essential for the growth of HIV in cell types other than the $CD4^+$ lymphocytes used in this report. Productive infection of monocytes and macrophages has been described recently (10, 22). HIV has also been reported to propagate in the central nervous system (13, 21, 25). *vpr* may perform a function needed in these tissues. Alternatively, *vpr* may exert a function not readily measured in the tissue culture system. Nonpathogenic simian immunodeficiency virus isolated from African green monkey lacks the *vpr* open reading frame (9).

At present, the exact nature of *vpr* is not clear. *vpr* can affect any step of the virus growth cycle (adsorption, penetration, uncoating, transcription, mRNA processing, translation, protein processing, assembly, or maturation) and

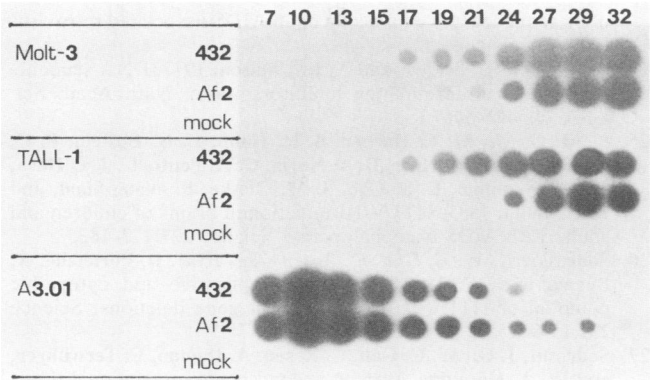


FIG. 4. Growth kinetics of a *vpr* mutant (Af2) and wild-type virus (432) in three human lymphocyte cell lines, Molt-3, TALL-1, and A3.01, by RT assays. Values at the top indicate day after infection. Equivalent dose ($5 \times 10^4$ cpm) was used for infection. Molt-3 and TALL-1 lines were obtained from the Japanese Cancer Research Resources Bank.

mutants exhibiting the phenotype described here. Several characteristics of *vpr* mutants should be pointed out. No significant difference in the transient expression of either wild-type or *vpr* mutant plasmids was observed by immunoblotting or electron microscopy (N. Ono, K. Ogawa, A. Adachi, and S. Ueda, manuscript in preparation), and RT activities in the culture fluids were indistinguishable. Growth kinetics of the *vpr* mutant is clearly distinct from those of *vif* and *vpu* mutants. These results suggest that HIV *vpr* is not important in the transmissibility, in the regulation of viral gene activity, or in the assembly and release of progeny virions. However, detailed genetic analysis coupled with biochemical study needs to be carried out to determine the function of *vpr*. In this respect, we have generated mutants affecting other genes, and complementation experiments are in progress in our laboratory. A comparative functional study on *vpr* of HIV-2 (12) is also important to evaluate this question further.

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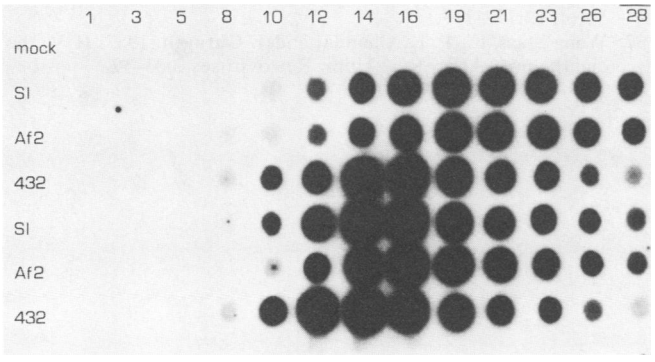


FIG. 5. Effect of input multiplicities on growth kinetics of *vpr* mutants (Af2 and Sl) and wild-type virus (432) in A3.01 cells by RT assays. Values at the top indicate day after infection. Input amounts of virus used for infection were $3 \times 10^3$ cpm (from 2nd line 2 to line 4) and $3 \times 10^4$ cpm (from line 5 to line 7).

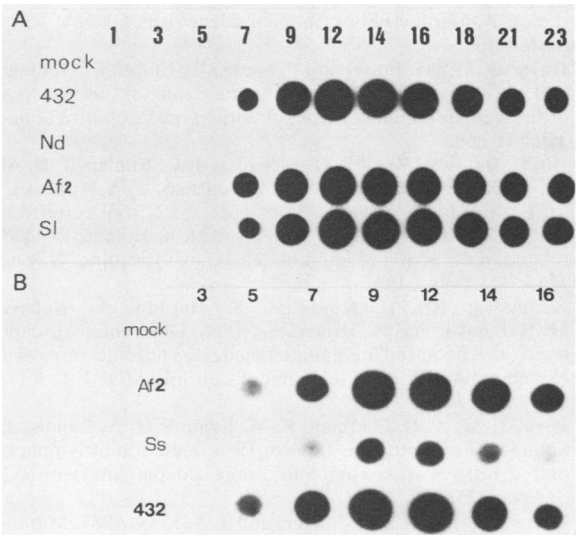


FIG. 6. Growth kinetics of *vif* (Nd), *vpr* (Af2), and *vpu* (Ss) mutants and wild-type virus (432) in A3.01 cells by RT assays. Values at the top indicate day after infection. Input dose for infection was $3 \times 10^4$ cpm. Experiments in parts A and B were carried out independently.

## LITERATURE CITED

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59**:284–291.
2. **Ahmad, N., and S. Venkatesan.** 1988. *Nef* protein is a transcriptional repressor of HIV-1 LTR. Science **241**:1481–1485.
3. **Alizon, M., S. Wain-Hobson, L. Montagnier, and P. Sonigo.** 1986. Genetic variability of the AIDS virus: nucleotide analysis of two isolates from African patients. Cell **46**:63–74.
4. **Barré-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, D. Dauguet, and C. Axler-Blin.** 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science **220**:868–871.
5. **Dayton, A. I., J. G. Sodroski, C. A. Rosen, W. C. Goh, and W. A. Haseline.** 1986. The trans-activator gene of the human T-cell lymphotropic virus type III is required for replication. Cell **44**:941–947.
6. **Fisher, A. G., B. Ensoli, L. Ivanoff, M. Chamberlain, S. Petteway, L. Ratner, R. C. Gallo, and F. Wong-Staal.** 1987. The sor gene of HIV-1 is required for efficient virus transmission in vitro. Science **237**:888–893.
7. **Fisher, A. G., M. B. Feinberg, S. F. Josephs, M. E. Harper, L. M. Marselle, G. Reyes, M. A. Gonda, A. Aldovini, C. Debouk, R. C. Gallo, and F. Wong-Staal.** 1986. The trans-activator gene of HTLV-III is essential for virus replication. Nature (London) **320**:367–371.
8. **Folks, T., S. Benn, A. Rabson, T. Theodore, M. D. Hoggan, M. Martin, M. Lightfoote, and K. Sell.** 1985. Characterization of a continuous T-cell line susceptible to the cytopathic effects of the acquired immune deficiency syndrome (AIDS)-associated retrovirus. Proc. Natl. Acad. Sci. USA **82**:4539–4543.
9. **Fukasawa, M., T. Miura, A. Hasegawa, S. Morikawa, H. Tsujimoto, K. Miki, T. Kitamura, and M. Hayami.** 1988. Sequence of simian immunodeficiency virus from African green monkey, a new member of the HIV/SIV group. Nature (London) **333**: 457–461.
10. **Gartner, S., P. Markovits, D. M. Markovitz, M. H. Kaplan, R. C. Gallo, and M. Popovic.** 1986. The role of mononuclear phagocytes in HTLV-III/LAV infection. Science **233**:215–219.
11. **Graham, F. L., and A. J. Van der Eb.** 1973. A new technique for

the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.

12. **Guyader, M., M. Emerman, P. Sonigo, F. Clavel, L. Montagnier, and M. Alizon.** 1987. Genome organization and transactivation of the human immunodeficiency virus type 2. Nature (London) **326**:662–669.
13. **Ho, D. D., T. R. Rota, R. T. Schooley, J. C. Kaplan, J. D. Allan, J. E. Groopman, L. Resnick, D. Felsenstein, C. A. Andrews, and M. S. Hirsch.** 1985. Isolation of HTLV-III from cerebrospinal fluid and neural tissues of patients with neurologic syndromes related to the acquired immunodeficiency syndrome. N. Engl. J. Med. **313**:1493–1497.
14. **Kikukawa, R., Y. Koyanagi, S. Harada, N. Kobayashi, M. Hatanaka, and N. Yamamoto.** 1986. Differential susceptibility to the acquired immunodeficiency syndrome retrovirus in cloned cells of human leukemic T-cell line MOLT-4. J. Virol. **57**:1159–1162.
15. **Levy, J. A., A. D. Hoffman, S. M. Kramer, J. A. Landis, J. M. Shimabukuro, and L. S. Oshiro.** 1984. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. Science **225**:840–842.
16. **Luciw, P. A., C. Cheng-Mayer, and J. A. Levy.** 1987. Mutational analysis of the human immunodeficiency virus. The orf-B region down-regulates virus replication. Proc. Natl. Acad. Sci. USA **84**:1434–1438.
17. **Muesing, M. A., D. H. Smith, C. D. Cabradilla, C. V. Benton, L. A. Lasky, and D. J. Capon.** 1985. Nucleic acid structure and expression of the human AIDS lymphoadenopathy retrovirus. Nature (London) **313**:450–463.
18. **Popovic, M., M. G. Sarngadharan, E. Read, and R. C. Gallo.** 1984. Detection, isolation and continuous production of cytopathic retroviruses from patients with AIDS and pre-AIDS. Science **224**:497–500.
19. **Ratner, L., A. Fisher, L. L. Jagodzinski, H. Mitsuya, R.-S. Liou, R. C. Gallo, and F. Wong-Staal.** 1987. Complete nucleotide sequences of functional clones of the AIDS virus. AIDS Res. Hum. Retroviruses **3**:57–69.
20. **Ratner, L., W. A. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. J. Josephs, E. K. Doran, J. A. Rafalski, E. A. Whitehorn, K. Baumeister, L. Ivanoff, S. R. Petteway, Jr., M. L. Pearson, J. A. Lautenberger, T. S. Papas, J. Ghrayeb, N. J. Chang, R. C. Gallo, and F. Wong-Staal.** 1985. Complete nucleotide sequence of AIDS virus. HTLV-III. Nature (London) **313**:277–284.
21. **Resnick, L. F., DiMarzo-Veronese, J. Schopbach, W. W. Tourtellotte, D. D. Ho, F. Muller, P. Shapshak, M. Vogt, J. E. Groopman, P. D. Markham, and R. C. Gallo.** 1985. Intra-blood brain-barrier synthesis of HTLV-III-specific IgG in patients with neurologic symptoms associated with AIDS or AIDS-related complex. N. Engl. J. Med. **313**:1498–1504.
22. **Salahuddin, S. A., R. M. Rose, J. E. Groopman, P. D. Markham, and R. C. Gallo.** 1986. Human T lymphotropic virus type III infection of human alveolar macrophages. Blood **68**:281–284.
23. **Sanchez-Pescador, R., M. D. Power, P. J. Barr, K. S. Steimer, M. M. Stempien, S. L. Brown-Shimer, W. W. Gee, A. Renard, A. Randolph, J. A. Levy, D. Dina, and P. A. Luciw.** 1985. Nucleotide sequence and expression of an AIDS-associated retrovirus (ARV-2). Science **227**:484–492.
24. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
25. **Shaw, G. M., M. E. Harper, B. H. Hahn, L. G. Epstein, P. C. Gajdusek, R. W. Prince, B. A. Novia, C. K. Petito, C. J. O'Hara, J. E. Groopman, E. S. Cho, J. M. Oleske, F. Wong-Staal, and R. C. Gallo.** 1985. HTLV-III infection in brains of children and adults with AIDS encephalopathy. Science **227**:177–182.
26. **Sodroski, J., W. C. Goh, C. Rosen, A. Tartar, D. Portetelle, A. Burny, and W. Haseltine.** 1986. Replicative and cytopathic potential of HTLV-III/LAV with sor gene deletions. Science **231**:1549–1553.
27. **Sodroski, J. G., W. C. Goh, C. Rosen, A. Dayton, E. Terwilliger, and W. A. Haseltine.** 1986. A second posttranscriptional transactivator gene required for HTLV-III replication. Nature (London) **321**:412–417.
28. **Sonigo, P., M. Alizon, K. Staskus, D. Klatzman, S. Cole, O. Danos, E. Retzel, P. Tiollais, A. Haase, and S. Wain-Hobson.** 1985. Nucleotide sequence of the visna lentivirus: relationship to the AIDS virus. Cell **42**:369–382.
29. **Starcich, B. R., B. H. Hahn, G. M. Shaw, P. D. McNeely, S. Modrow, H. Wolf, E. S. Parkes, W. P. Parks, S. F. Josephs, R. C. Gallo, and F. Wong-Staal.** 1986. Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS. Cell **45**: 637–648.
30. **Strebel, K., D. Daugherty, K. Clouse, D. Cohen, T. Folks, and M. A. Martin.** 1987. The HIV "A" (sor) gene product is essential for virus infectivity. Nature (London) **328**:728–730.
31. **Strebel, K., T. Klimkait, and M. A. Martin.** 1988. A novel gene of HIV-1, *vpu*, and its 16-kilodalton product. Science **241**: 1221–1223.
32. **Terwilliger, E., R. Burghoff, R. Sia, J. G. Sodroski, W. A. Haseltine, and C. Rosen.** 1988. The *art* gene product of human immunodeficiency virus is required for replication. J. Virol. **62**:655–658.
33. **Terwilliger, E., J. C. Sodroski, C. A. Rosen, and W. A. Haseltine.** 1986. Effects of mutations within the 3′ *orf* open reading frame region of human T-cell virus type III (HTLV-III/LAV) on replication and cytopathogenicity. J. Virol. **60**:754–760.
34. **Wain-Hobson, S., P. Sonigo, O. Danos, S. Cole, and M. Alizon.** 1985. Nucleotide sequence of the AIDS virus, LAV. Cell **40**:9–17.
35. **Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin.** 1979. DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. Proc. Natl. Acad. Sci. USA **76**:1373–1376.
36. **Willey, R. L., D. H. Smith, L. A. Lasky, T. S. Theodore, P. L. Earl, B. Moss, D. J. Capon, and M. A. Martin.** 1988. In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. J. Virol. **62**:139–147.
37. **Wong-Staal, F., P. K. Chanda, and J. Ghrayeb.** 1987. HIV: the eighth gene. AIDS Res. Hum. Retroviruses **3**:33–39.