JOURNAL OF VIROLOGY, May 1990, p. 2448–2451
0022-538X/90/052448-04$02.00/0

Vol. 64, No. 5

Removal of Cryptic Poxvirus Transcription Termination Signals from the Human Immunodeficiency Virus Type 1 Envelope Gene Enhances Expression and Immunogenicity of a Recombinant Vaccinia Virus

PATRICIA L. EARL,* AMBROS W. HÜGIN, AND BERNARD MOSS

Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892

Received 30 November 1989/Accepted 5 February 1990

The in vivo role of the proposed poxvirus early transcription termination signal TTTTTNT was confirmed by analysis of the RNA species made by recombinant vaccinia viruses. Premature transcription termination occurred following each of two TTTTTNT sequences present naturally within the coding region of the human immunodeficiency virus type 1 envelope gene. Alteration of the TTTTTNT sequences, without changing the encoded amino acids, resulted in production of full-length early mRNAs, improved protein expression, and a more consistent immune response.

Poxviruses encode and package their own transcription system, contributing to their ability to replicate within the cytoplasm of cells (B. Moss, Annu. Rev. Biochem., in press). Gene expression is regulated by different *cis*- and *trans*-acting factors at early and late stages of infection. In vitro studies, carried out with a soluble template-dependent transcription system, revealed that the sequence TTTTTNT (T_5NT) signals termination approximately 50 nucleotides downstream (22, 31). Further studies indicated that the vaccinia virus capping enzyme is required for termination (24) and that the signal is actually recognized in RNA as UUUUUNU (25). The presence of T_5NT near the ends of many early genes, and its infrequent occurrence near the start or middle of those genes, suggested that this signal is also used in vivo (30). Despite the common occurrence of T_5NT in the middle of late genes, late mRNAs are long and varied in size (8, 18). One explanation for this difference is that the early transcription system uniquely recognizes or utilizes the termination signal. Another possibility is that an unrecognized T_5NT sequence context, present exclusively in early genes, is needed for termination in vivo. The presence of T_5NT sequences in foreign genes that have been expressed by vaccinia virus vectors presented us with a double opportunity. Firstly, we could test predictions regarding transcription termination in vivo made from previous in vitro studies. Secondly, we could evaluate whether the elimination of cryptic termination signals from foreign genes enhances the expression of those genes.

A survey of genes expressed in vaccinia virus revealed the presence of T_5NT within the coding regions of many genes, including the hepatitis B surface antigen (27), respiratory syncytial virus fusion (7) and nucleocapsid protein (6), vesicular stomatitis virus glycoprotein (28) and L protein (23), and *Plasmodium falciparum* circumsporozoite protein (16) genes. In addition, most of the human immunodeficiency virus type 1 (HIV-1) envelope (*env*) genes in the acquired immune deficiency syndrome database (19) contain two copies of the T_5NT motif at approximately the same locations within the coding region. Despite the presence of the T_5NT sequence, foreign gene expression was detected in each of the above cases. The use of the compound early-late P7.5 promoter may have obscured the deleterious effects of the T_5NT sequence on early RNA synthesis. Alternatively, T_5NT may not function as a transcription signal within the context of foreign DNA. To assess the importance of T_5NT in vivo, we constructed a recombinant vaccinia virus containing an HIV-1 *env* gene in which both T_5NT sequences were altered without changing the amino acids encoded. The effects of this manipulation on expression and immunogenicity are documented here.

The recombinant vaccinia virus vPE7 (29) contains the HIV-1 *env* gene from the BH8 clone (21), the sequence of which contains T_5NT sequences starting at nucleotides 6324 and 6945 (Fig. 1). The first T_5NT actually consists of overlapping signals with N representing T in one signal and A in the other. In the second T_5NT, N is a C. Expression is directed by the compound early-late P7.5 promoter (5). By site-directed mutagenesis, a new recombinant virus, vPE16, was constructed. In this virus, the TTT phenylalanine codons in both T_5NT motifs were changed to TTC phenylalanine codons (Fig. 1). Thus, the sequence of the expressed protein would be unchanged.

The existence of two sets of T_5NT sequences within the coding region of gp160 was predicted to cause premature termination of transcripts made early in infection. The protein synthesis inhibitor cycloheximide arrests vaccinia virus in the early stage of the life cycle and thereby increases the amount of early mRNAs synthesized. The *env*-specific RNAs produced by cells infected with vPE7 or vPE16 under these conditions were compared by Northern (RNA) blotting (Fig. 1). Cells infected with vPE7 produced three *env*-specific RNA species. The 600- and 1,200-nucleotide bands represent transcripts prematurely terminated at the two T_5NT sequences within the gene, while the barely detectable 2,900-nucleotide band represents readthrough of these signals and termination of the T_5NT near the end of the adjacent vaccinia virus thymidine kinase gene. Densitometry of an autoradiogram indicated that the efficiency of termination was 75 to 80% at each T_5NT, consistent with previous data that suggested that termination of natural vaccinia virus

* Corresponding author.

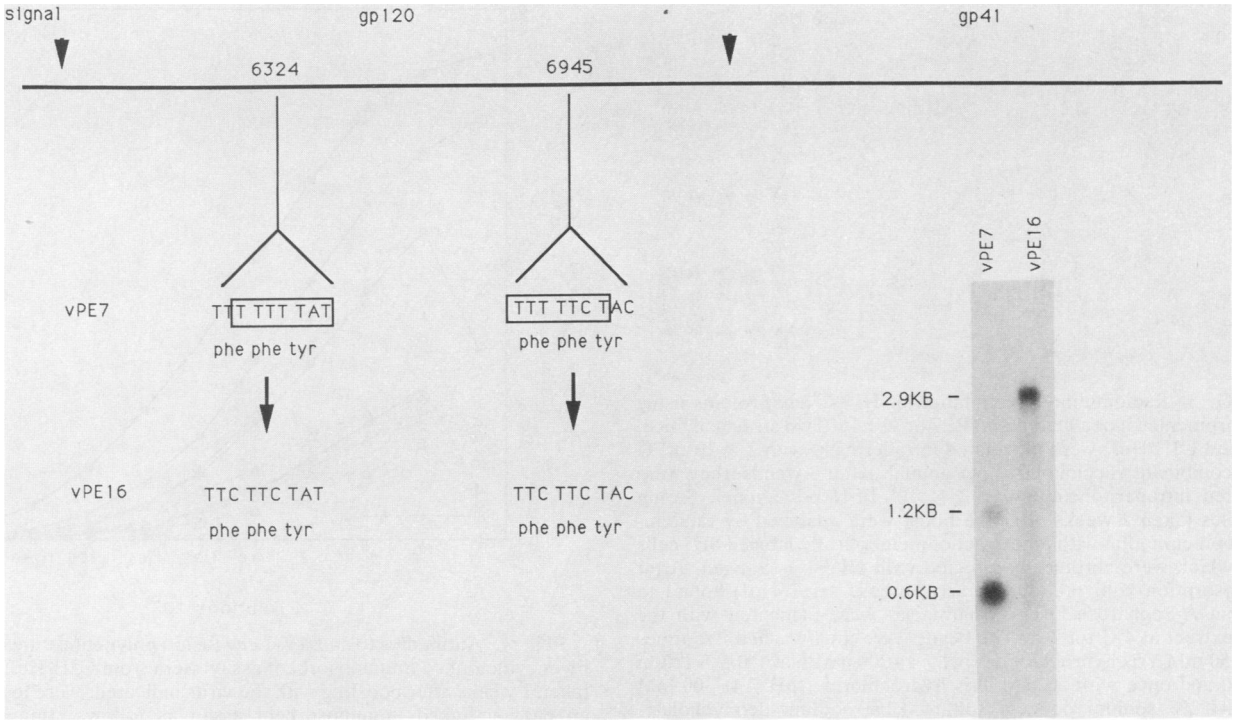

FIG. 1. Effect of T_5NT sequences on the size of HIV-1 *env* transcripts. The structure of the HIV-1 *env* gene, the sites of proteolytic cleavage (arrowheads), and the locations of the two sets of putative vaccinia virus early transcription termination signals, TTTTTTTAT and TTTTTCT, are indicated. Nucleotide numbers are from Ratner et al. (21). The 2.5-kilobase-pair *Eco*RV fragment containing the coding sequence of the *env* gene that was used to make pPE7 (29) was inserted into the *Sma*I site of m13Mp18. This bacteriophage was mutagenized with oligonucleotides GAATATGCATTCTTCTATAAACTT and GAGGGGAATTCTTCTACTGTA to alter the two copies of T_5NT within the body of the gp160 gene. The DNA was digested with *Sst*I, and the ends were blunted with bacteriophage T4 DNA polymerase. After digestion with *Sal*I, this 2.5-kilobase-pair fragment was ligated into the *Sal*I-*Stu*I site of pSC11-ss, a derivative of pSC11 (2) containing additional cloning sites. The recombinant virus vPE16 was made by homologous recombination (17). The autoradiogram is a Northern blot of the HIV-1 *env* transcripts made in cells infected with vPE7 and vPE16 in the presence of 100 μg of cycloheximide per ml. RNA was prepared as described by Fuerst et al. (12), and 20-μg samples were separated by 1% agarose gel electrophoresis in 6% formaldehyde–20 mM MOPS (morpholinepropanesulfonic acid)–50 mM sodium acetate–10 mM EDTA, pH 7.0. The RNA was blotted to nitrocellulose and probed with a ^{32}P-labeled DNA fragment containing 600 bases from the 5′ end of the gene. The sizes of the RNAs, indicated on the side of the gel, were determined with RNA markers (Bethesda Research Laboratories) in an adjacent lane. KB, kilobases.

early mRNAs is not 100% efficient (1). Confirmation that the T_5NT sequences were responsible for premature termination was obtained by making point mutations in the gene. As shown in Fig. 1, a single major 2,900-nucleotide band was detected in RNA extracted from cells infected with vPE16. These data are consistent with previous conclusions, obtained from studies with a soluble template-dependent transcription system, that the T_5NT sequence is both necessary and sufficient for transcription termination.

Because the P7.5 promoter, which is used to express the *env* gene, is a compound promoter composed of tandem individual early and late promoters, we could assess the role of T_5NT on both early and late protein syntheses. The time course of the appearance of gp160 in cells infected with vPE7 and vPE16 was determined by immunoblotting (Fig. 2). At 4 to 6 h postinfection, gp160 was detectable in cells infected with vPE16 but not in cells infected with vPE7. By 8 h after infection, however, gp160 was clearly made in cells infected with vPE7, and by 24 h comparable amounts were induced by vPE7 and vPE16. In other experiments, less gp160 was present in vPE7-infected cells than in vPE16-infected cells, even at 24 h. The results suggested that gp160 is expressed by both early and late functions of the P7.5 promoter in cells infected with vPE16 but only from the late function of P7.5 in cells infected with vPE7. In accordance with this inter-

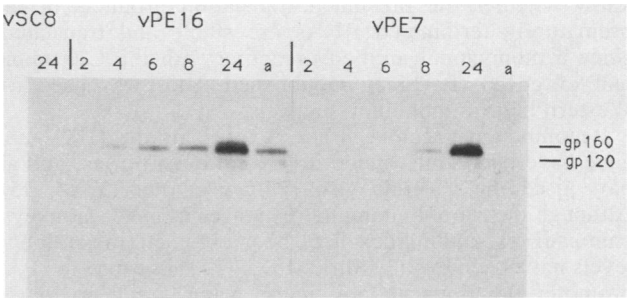

FIG. 2. Immunoblot of HIV-1 *env* proteins. BSC1 cells were infected at 30 PFU per cell. Cytosine arabinoside at 40 μg/ml was added where indicated (lane a) to cell monolayers 10 min before infection and maintained in the medium throughout the experiment. At 2, 4, 6, 8, and 24 h postinfection in the absence of cytosine arabinoside and at 24 h postinfection in the presence of cytosine arabinoside (lane a), the cells were harvested and lysed by suspension in 100 mM Tris hydrochloride (pH 8.0)–100 mM NaCl–0.5% Nonidet P-40. After 10 min on ice, cell debris was removed by centrifugation. Proteins were separated by 10% polyacrylamide gel electrophoresis, transferred to nitrocellulose, and incubated with monoclonal antibody 902 (4) and subsequently with ^{125}I-labeled protein A (Amersham Corp.) vSC8 (2) was a negative control.

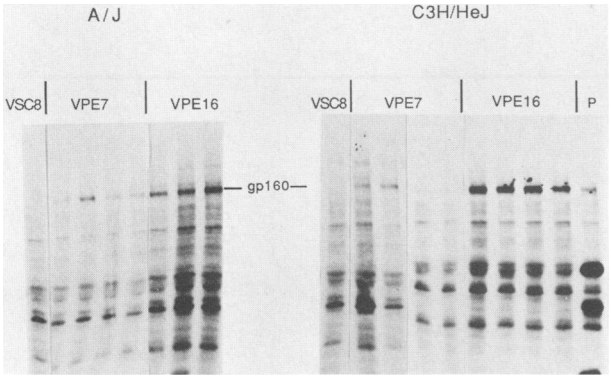

FIG. 3. Radioimmunoprecipitation of HIV-1 *env* proteins using sera from mice inoculated with vPE7 or vPE16. Two strains of mice, A/J and C3H/HeJ, were inoculated intradermally with 2×10^8 PFU of recombinant vaccinia virus per animal. After 3 weeks, they were boosted intraperitoneally with 2×10^8 PFU per animal. Serum samples taken 2 weeks after the boost were analyzed by radioimmunoprecipitation with an extract of metabolically labeled 8E5 cells (11) which were chronically infected with HIV-1 as antigen. After preabsorption with preimmune sera, mouse sera (4 μl) bound to protein A-Sepharose beads (Pharmacia) were incubated with the 8E5 extract at 4°C for 1 h. The beads were then washed five times with 50 mM Tris hydrochloride (pH 7.4)–300 mM NaCl–0.5% Triton X-100 and once with 50 mM Tris hydrochloride (pH 7.4)–300 mM NaCl–0.1% sodium dodecyl sulfate–0.1% sodium deoxycholate. Bound proteins were eluted in sample buffer and separated by 10% polyacrylamide gel electrophoresis. Control animals were inoculated with vSC8, which expresses *Escherichia coli* beta-galactosidase. HIV-1-positive human serum (P) was used as a marker.

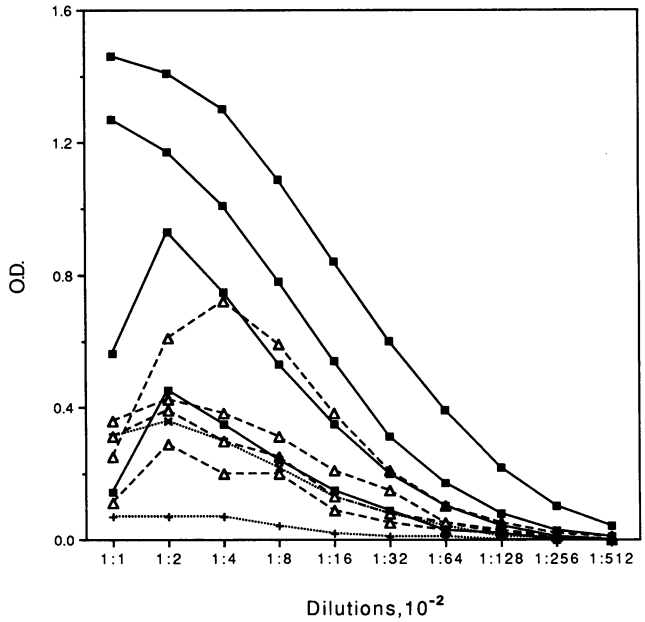

FIG. 4. Antibodies to an HIV-1 *env* fusion polypeptide measured by enzyme-linked immunosorbent assay. Sera from C3H/HeJ mice, taken 2 weeks after boosting with the virus indicated, were tested in an enzyme-linked immunosorbent assay as follows. Immulon I plates (Dynatech Laboratories, Inc.) were coated overnight with 1 μg of PB1-IIIB (20) per well in 1.34 M urea–phosphate-buffered saline. After the plate was thoroughly washed, twofold serum dilutions in phosphate-buffered saline containing 1% bovine serum albumin and 0.05% Tween 20 were incubated for 2 h at 37°C. The plates were then incubated for 1 h at room temperature with horseradish peroxidase-labeled goat anti-mouse immunoglobulin G serum in dilution buffer supplemented with 10% fetal bovine serum. Plates were developed with 2,2′-azino-di-[3-ethyl-benzthiazoline sulfonate] substrate (Kirkegaard & Perry Laboratories Inc.), and absorbance was read with a kinetic microplate reader (Molecular Devices). – – – –, vPE7; ———, vPE16; +·····+, preimmune sera; ×····· ×, vSC8. O.D., Optical density.

pretation, under conditions in which late protein synthesis was blocked with cytosine arabinoside, an inhibitor of DNA replication, *env* protein was not detected in cells infected with vPE7 but was abundant in cells infected with vPE16 (Fig. 2, lane a). This difference was consistent with the relative amounts of full-length transcripts made by the two viruses in the presence of cycloheximide (Fig. 1). We do not know whether the presumed translation products of the prematurely terminated RNAs are stable and translated, since a monoclonal antibody reactive with the C-terminal half of gp120 (P. Earl, unpublished data) was used for Western blot (immunoblot) analysis.

Recombinant vaccinia viruses expressing the HIV-1 *env* gene have been constructed in several laboratories, and all have used the vaccinia virus P7.5 promoter (3, 14, 15). Although measurable immune responses in mice, monkeys, chimpanzees, and humans have been obtained, the antibody levels have been low (3, 10, 13–15, 32–35). Because the P7.5 promoter functions at late times, when T_5NT no longer causes transcription termination, a considerable amount of gp160 was produced in tissue culture cells. The situation in animals, however, may be less favorable, in view of the thymidine kinase-negative phenotype of the recombinant viruses. It is thought that the vaccinia virus thymidine kinase might be required to produce precursors for DNA replication in resting cells. Since DNA replication is a prerequisite for late protein synthesis, the effect of T_5NT in animals might be worse than in rapidly growing tissue culture cells.

To compare the effects of the removal of the two T_5NT sequences on immunogenicity, mice were inoculated with vPE7 or vPE16. Sera taken 2 weeks after an intraperitoneal boost were analyzed by radioimmunoprecipitation (Fig. 3). Although sera from most of the animals immunoprecipitated some gp160, those infected with vPE16 consistently precipitated more than those infected with vPE7 did. This difference was most marked with C3H/HeJ mice. The results with C3H/HeJ mice were confirmed by enzyme-linked immunosorbent assay (Fig. 4). It may be significant that the PB1 peptide (20) used in the enzyme-linked immunosorbent assay would not be present in translation products of the prematurely terminated mRNAs. We are uncertain as to whether the better overall immune response is due to synthesis of more *env* protein by vPE16 or to earlier expression. On the basis of studies of Coupar et al. (9) and Townsend et al. (26) on the importance of early expression for good and consistent priming for influenza virus-specific cytotoxic T lymphocytes, we suspect that elimination of T_5NT also would enhance this cell-mediated immune response. It seems desirable on the basis of our present knowledge to use early or compound promoters and to alter the T_5NT sequences of the HIV-1 *env* (or any other) genes of recombinant vaccinia viruses that are being considered as candidate vaccines or for immunotherapy.

The original BH8 clone was obtained from the laboratory of R. Gallo, National Cancer Institute. PB1 was a generous gift of S.

Putney and J. Rusche, Repligen Corp. We thank A. Kinter and T. Folks, National Institute of Allergy and Infectious Diseases, for metabolically labeled 8E5 extracts.

LITERATURE CITED

1. **Bajszar, G., R. Wittek, J. P. Weir, and B. Moss.** 1983. Vaccinia virus thymidine kinase and neighboring genes: mRNAs and polypeptides of wild-type virus and putative nonsense mutants. J. Virol. **45**:62–72.
2. **Chakrabarti, S., K. Brechling, and B. Moss.** 1985. Vaccinia virus expression vector: coexpression of β-galactosidase provides visual screening of recombinant virus plaques. Mol. Cell. Biol. **5**:3403–3409.
3. **Chakrabarti, S., M. Robert-Guroff, F. Wong-Staal, R. C. Gallo, and B. Moss.** 1986. Expression of the HTLV-III envelope gene by a recombinant vaccinia virus. Nature (London) **320**:535–537.
4. **Chesebro, B., and K. Wehrly.** 1988. Development of a sensitive quantitative focal assay for human immunodeficiency virus infectivity. J. Virol. **62**:3779–3788.
5. **Cochran, M. A., C. Puckett, and B. Moss.** 1985. In vitro mutagenesis of the promoter region for a vaccinia virus gene: evidence for tandem early and late regulatory signals. J. Virol. **54**:30–37.
6. **Collins, P. L., K. Anderson, S. J. Langer, and G. W. Wertz.** 1985. Correct sequence for the major nucleocapsid protein mRNA of respiratory syncytial virus. Virology **146**:69–77.
7. **Collins, P. L., Y. T. Huang, and G. W. Wertz.** 1984. Nucleotide sequence of the gene encoding the fusion (F) glycoprotein of human respiratory syncytial virus. Proc. Natl. Acad. Sci. USA **81**:7683–7687.
8. **Cooper, J. A., R. Wittek, and B. Moss.** 1981. Extension of the transcriptional and translational map of the left end of the vaccinia virus genome to 21 kilobase pairs. J. Virol. **39**:733–745.
9. **Coupar, B. E. H., M. E. Andrew, G. W. Both, and D. B. Boyle.** 1986. Temporal regulation of influenza hemagglutinin expression in vaccinia virus recombinants and effects on the immune response. Eur. J. Immunol. **16**:1479–1487.
10. **Earl, P. L., M. Robert-Guroff, T. J. Matthews, K. Krohn, W. T. London, and B. Moss.** 1989. Isolate- and group-specific immune responses to the envelope protein of human immunodeficiency virus induced by a live recombinant vaccinia virus in macaques. AIDS Res. Hum. Retroviruses **5**:23–32.
11. **Folks, T. M., D. Powell, M. Lightfoote, S. Koenig, A. S. Fauci, S. Benn, A. Rabson, D. Daugherty, H. E. Gendelman, M. D. Hoggan, S. Venkatesen, and M. A. Martin.** 1986. Biological and biochemical characterization of a cloned leu-3$^-$ cell surviving infection with the acquired immune deficiency syndrome retrovirus. J. Exp. Med. **164**:280–290.
12. **Fuerst, T. R., P. L. Earl, and B. Moss.** 1987. Use of a hybrid vaccinia virus-T7 RNA polymerase system for expression of target genes. Mol. Cell. Biol. **7**:2538–2544.
13. **Hu, S.-L., P. N. Fultz, H. M. McClure, J. W. Eichberg, E. K. Thomas, J. Zarling, M. C. Singhal, S. G. Kosowski, R. B. Swenson, D. C. Anderson, and G. Todaro.** 1987. Effect of immunization with a vaccinia-HIV env recombinant on HIV infection of chimpanzees. Nature (London) **328**:721–723.
14. **Hu, S.-L., S. G. Kosowski, and J. M. Dalrymple.** 1986. Expression of AIDS virus envelope gene in recombinant vaccinia viruses. Nature (London) **320**:537–540.
15. **Kieny, M. P., G. Rautmann, D. Schmitt, K. Dott, S. Wain-Hobson, M. Alizon, M. Girard, S. Chamaret, A. Laurent, L. Montagnier, and J.-P. Lecocq.** 1986. AIDS virus env protein expressed from a recombinant vaccinia virus. Bio/Technology **4**:790–795.
16. **Lockyer, M. J., and R. T. Schwartz.** 1987. Strain variation in the circumsporozoite protein gene of Plasmodium falciparum. Mol. Biochem. Parasitol. **22**:101–108.
17. **Mackett, M., G. L. Smith, and B. Moss.** 1984. General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes. J. Virol. **49**:857–864.
18. **Mahr, A., and B. E. Roberts.** 1984. Arrangement of late RNAs transcribed from a 7.1-kilobase *Eco*RI vaccinia virus DNA fragment. J. Virol. **49**:510–520.
19. **Myers, G., S. F. Josephs, J. A. Berzofsky, A. B. Rabson, T. F. Smith, and F. Wong-Staal (ed.).** 1989. Human retroviruses and AIDS. Los Alamos National Laboratory, Los Alamos, N. Mex.
20. **Putney, S. D., T. J. Matthews, W. G. Robey, D. L. Lynn, M. Robert-Guroff, W. T. Mueller, A. J. Langlois, J. Ghrayeb, S. R. Petteway, Jr., K. J. Weinhold, P. J. Fischinger, F. Wong-Staal, R. C. Gallo, and D. P. Bolognesi.** 1986. HTLV-III/LAV-neutralizing antibodies to an *E. coli*-produced fragment of the virus envelope. Science **234**:1392–1395.
21. **Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. 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. T. Chang, R. C. Gallo, and F. Wong-Staal.** 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature (London) **313**:277–284.
22. **Rohrmann, G., L. Yuen, and B. Moss.** 1986. Transcription of vaccinia virus early genes by enzymes isolated from vaccinia virions terminates downstream of a regulatory sequence. Cell **46**:1029–1035.
23. **Schubert, M., G. G. Harmison, and E. Meier.** 1984. Primary structure of the vesicular stomatitis virus polymerase (*L*) gene: evidence for a high frequency of mutations. J. Virol. **51**: 505–514.
24. **Shuman, S., S. S. Broyles, and B. Moss.** 1987. Purification and characterization of a transcription termination factor from vaccinia virions. J. Biol. Chem. **262**:12372–12380.
25. **Shuman, S., and B. Moss.** 1988. Factor-dependent transcription termination by vaccinia virus RNA polymerase. J. Biol. Chem. **263**:6220–6225.
26. **Townsend, A., J. Bastin, K. Gould, G. Brownlee, M. Andrew, B. Coupar, D. Boyle, S. Chan, and G. Smith.** 1988. Defective presentation to class I-restricted cytotoxic T lymphocytes in vaccinia-infected cells is overcome by enhanced degradation of antigen. J. Exp. Med. **168**:1211–1224.
27. **Valenzuela, P., P. Gray, M. Quiroga, J. Zaldivar, H. M. Goodman, and W. J. Rutter.** 1979. Nucleotide sequence of the gene coding for the major protein of hepatitis B virus surface antigen. Nature (London) **280**:815–819.
28. **Vandepol, S. B., and J. J. Holland.** 1986. Evolution of vesicular stomatitis virus in athymic nude mice: mutations associated with natural killer cell selection. J. Gen. Virol. **67**:441–451.
29. **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.
30. **Yuen, L., and B. Moss.** 1986. Multiple 3′ ends of mRNA encoding vaccinia virus growth factor occur within a series of repeated sequences downstream of T clusters. J. Virol. **60**: 320–323.
31. **Yuen, L., and B. Moss.** 1987. Oligonucleotide sequence signaling transcriptional termination of vaccinia virus early genes. Proc. Natl. Acad. Sci. USA **84**:6417–6421.
32. **Zagury, D., J. Bernard, R. Cheynier, I. Desportes, R. Leonard, M. Fouchard, B. Reveil, D. Ittele, Z. Lurhuma, K. Mbayo, J. Wane, J. J. Salaun, B. Goussard, L. Dechazal, A. Burny, P. Nara, and R. C. Gallo.** 1988. A group specific anamnestic immune reaction against HIV-1 induced by a candidate vaccine against AIDS. Nature (London) **332**:728–731.
33. **Zagury, D., R. Leonard, M. Fouchard, B. Reveil, J. Bernard, D. Ittele, A. Cattan, L. Zirimwabagabo, M. Kalumbu, W. Justin, J. J. Salaun, and B. Goussard.** 1987. Immunization against AIDS in humans. Nature (London) **326**:249–250.
34. **Zarling, J. M., J. W. Eichberg, P. A. Moran, J. McClure, P. Sridhar, and S.-L. Hu.** 1987. Proliferative and cytotoxic T cells to AIDS virus glycoproteins in chimpanzees immunized with a recombinant vaccinia virus expressing AIDS virus envelope glycoproteins. J. Immunol. **139**:988–990.
35. **Zarling, J. M., W. Morton, P. A. Moran, J. McClure, S. G. Kosowski, and S.-L. Hu.** 1986. T-cell responses to human AIDS virus in macaques immunized with recombinant vaccinia viruses. Nature (London) **323**:344–346.