JOURNAL OF VIROLOGY, Mar. 1991, p. 1653–1657
0022-538X/91/031653-05$02.00/0

Vol. 65, No. 3

Rev Activates Expression of the Human Immunodeficiency Virus Type 1 *vif* and *vpr* Gene Products

ELIZABETH D. GARRETT, LAURENCE S. TILEY, AND BRYAN R. CULLEN*

Howard Hughes Medical Institute and Department of Microbiology and Immunology, Duke University Medical Center, Durham, North Carolina 27710

Received 28 September 1990/Accepted 3 December 1990

The proteins encoded by human immunodeficiency virus type 1 (HIV-1) can be divided into two temporally regulated classes. Early gene products are encoded by multiply spliced mRNA species and are expressed constitutively. In contrast, late proteins are encoded by a class of unspliced or singly spliced viral transcripts whose cytoplasmic expression is induced by the viral Rev *trans* activator. Here, we demonstrate that the viral Vif and Vpr proteins are encoded by singly spliced viral mRNAs whose expression is activated by Rev. This activation is shown to result from the reduced utilization of splice sites adjacent to or within the *vif* and *vpr* coding sequences. Vif and Vpr therefore belong to the class of late HIV-1 gene products.

The pathogenic retrovirus human immunodeficiency virus type 1 (HIV-1) displays a high level of genetic complexity (Fig. 1). In addition to carrying the *gag*, *pol*, and *env* genes characteristic of all known replication-competent retroviruses, HIV-1 encodes at least six auxiliary proteins. Two of these, termed Tat and Rev, are essential *trans* regulators of viral gene expression, while two others, termed Vpu and Vif, have been shown to be important for the morphogenesis and release of infectious virions (reviewed in reference 5). The roles of the viral *nef* and *vpr* gene products remain less clear, as both are dispensable for efficient HIV-1 replication in culture (3, 10, 16). However, recent data suggest that Vpr may be a virion structural protein whose expression modestly enhances the rate of viral replication (2, 3, 16).

The nine HIV-1 proteins enumerated or alluded to above are encoded by more than 20 distinct mRNA species that are derived from the posttranscriptional processing of the initial, genome-length viral transcript (7, 14, 19–21). The pattern of expression of these HIV-1 mRNAs displays a marked temporal regulation (9). In the early stages of infection, viral gene expression is limited to the small, multiply spliced mRNA species that are known to express the viral regulatory proteins Tat and Rev as well as the *nef* gene product (Fig. 1) (6, 7, 9). It is hypothesized (9, 17) that accumulation of the Rev protein to a critical level activates the expression of the unspliced and singly spliced mRNA species that encode the viral structural proteins Gag, Pol, and Env. HIV-1 proviruses lacking a functional *rev* gene product or bearing a defective copy of the *cis*-acting RNA target sequence for Rev, the Rev response element (RRE), are unable to progress to the late, structural phase of the viral replication cycle and remain locked in the early, regulatory phase (6, 7, 12).

Recently, it was demonstrated that Vpu is also a late gene product that is encoded by the same singly spliced bicistronic mRNA species as Env (21). However, the temporal regulation of the HIV-1 *vif* and *vpr* gene products has remained unclear. Schwartz et al. (20) have proposed that Vif and Vpr are encoded by two doubly spliced viral mRNA species containing the hypothetical coding exons designated E2A and E3A, respectively (Fig. 1D). These multiply spliced mRNAs, which would be structurally comparable to the known multiply spliced *tat* mRNAs (Fig. 1C), would lack an RRE and would therefore have to be expressed independently of Rev. Alternatively, Vif and Vpr could be encoded either predominantly or exclusively by mRNAs structurally comparable to the singly spliced viral mRNAs that encode Env and the truncated form of Tat (Fig. 1D) (18). In this case, the Vif and Vpr proteins would fall into the class of late viral gene products whose expression is activated by the Rev protein.

To examine the Rev dependence of HIV-1 *vif* gene expression, we transfected (4) cultures of the HIV-1 replication-permissive monkey cell line COS (11) with an expression plasmid containing a full-length, replication-competent HIV-1 provirus (pHIV-1) or with a similar HIV-1 provirus bearing a defective viral *rev* gene (pHIV-1ΔRev) (13). These expression plasmids are based on the HXB-3 proviral isolate and are similar to those previously described (13), except that the single-base-pair frame-shift mutation present within the *vpr* gene of the HXB-3 isolate (3, 15, 16) has been corrected in order to permit expression of the full-length *vpr* gene product. At 72 h after transfection, total cytoplasmic mRNA was harvested and subjected to Northern (RNA) analysis (12) with probes specific for the *tat* gene or the *vif* gene (Fig. 2). The HIV-1 *tat* gene is known to be expressed as a set of multiply spliced, ~2-kb transcripts in the absence of Rev and as a singly spliced, ~4-kb transcript in the presence of Rev (Fig. 1C) (7, 14, 20). This result is confirmed in Fig. 2, which shows a marked accumulation of exclusively multiply spliced *tat* mRNAs in the absence of Rev (Fig. 2, lane 2). In the presence of Rev, expression of the 2-kb *tat* mRNAs is reduced whereas the singly spliced *tat* mRNA, as well as the genomic transcript, is readily detected. In contrast, the probe specific for the *vif* open reading frame failed to detect any specific viral mRNA species in cells transfected with the pHIV-1ΔRev construct (Fig. 2, lane 5). Even with prolonged exposure, no specific mRNA species could be detected in this lane (data not shown). However, both a singly spliced and a genome-length viral RNA species were detected by the *vif*-specific probe in cells transfected with the wild-type HIV-1 proviral expression plasmid (Fig. 2, lane 6). Of note, the singly spliced mRNA detected by the *vif*-specific probe was observed to migrate more slowly than the predominant singly spliced species detected by the *tat*

* Corresponding author.

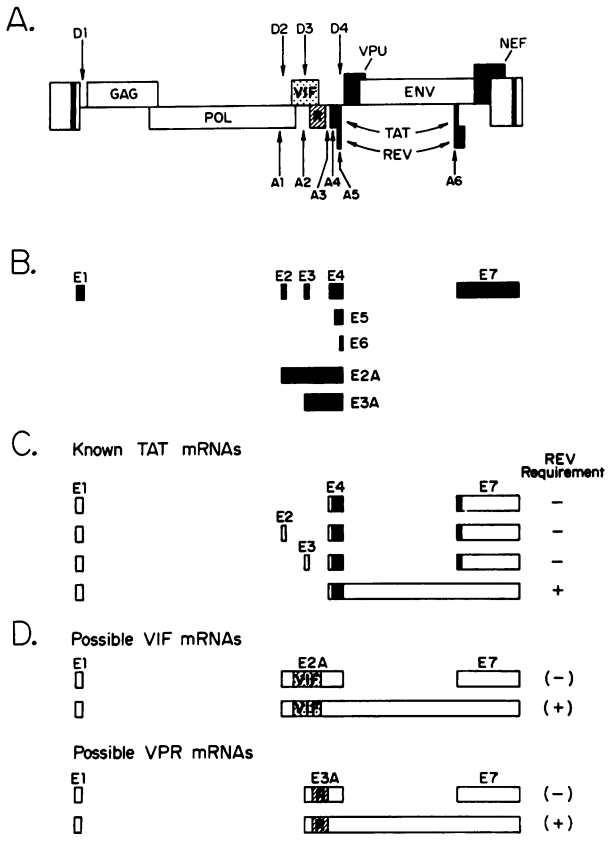

FIG. 1. RNA expression strategies in HIV-1. (A) Genetic organization of the HIV-1 provirus, showing the location of major splice donors (D1 to D4) and splice acceptors (A1 to A6). R, *vpr*. (B) HIV-1 exons observed in the multiply spliced viral mRNA species. E1 to E7 represent exons that have been cloned and characterized. E2A and E3A represent hypothetical exons proposed by Schwartz et al. (20). The exon nomenclature used here is similar to that previously proposed (14, 20). (C) HIV-1 Tat protein occurs in two forms (7, 12). A full-length, two-exon form of Tat is encoded by a set of at least three different multiply spliced mRNAs and is expressed independently of Rev (12, 19, 20). In the presence of Rev, a singly spliced Tat mRNA that encodes a truncated, one-exon form of Tat (7, 12) is observed. (D) By analogy with the Tat protein, the HIV-1 *vif* and *vpr* gene products could be expressed from doubly spliced mRNA species containing the hypothetical E2A and E3A exons (20). Expression of these mRNAs, which would lack the *env* gene-specific RRE signal, would therefore be predicted to be Rev independent. In contrast, Vif and Vpr might be translated entirely from singly spliced mRNAs (18) whose cytoplasmic expression would be dependent on Rev.

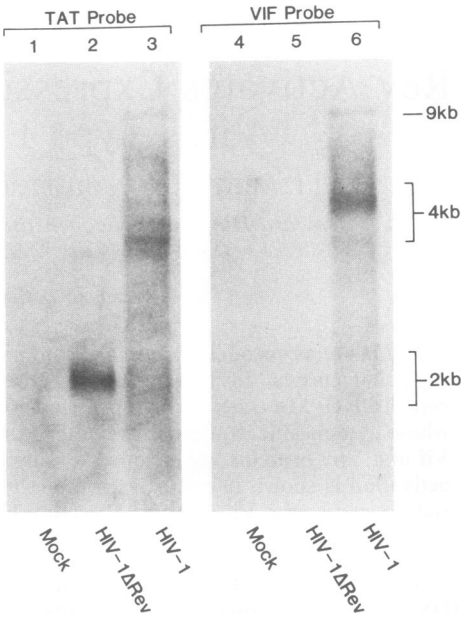

FIG. 2. Northern analysis of HIV-1 *tat* and *vif* mRNAs. COS cell cultures were transfected (4) with an intact HIV-1 provirus, with a provirus bearing a defective Rev gene (pHIV-1ΔRev), or with a negative control vector (mock transfected). Total cytoplasmic RNA was isolated (12) 72 h after transfection, and Northern analysis was performed as described previously (12), with 10 μg of RNA per lane. The nitrocellulose filter was initially analyzed with a probe specific for the first (E4) exon of *tat* (proviral coordinates 5794 to 5955; see reference 15) (lanes 1 to 3). Subsequently, the filter was stripped and rehybridized with a probe specific for the viral *vif* gene (proviral coordinates 5077 to 5332) (lanes 4 to 6). The approximate sizes of the unspliced (~9 kb), singly spliced (~4 kb), and multiply spliced (~2 kb) viral mRNAs, as determined by their mobilities, are indicated.

probe, which was predicted by their projected sizes of ~5.0 and ~4.1 kb, respectively (Fig. 1). Results obtained with a probe specific for the HIV-1 *vpr* gene were similar to those obtained with the *vif*-specific probe (data not shown).

The Northern analysis whose results are presented in Fig. 2 suggests that *vif* is detectably expressed only as a singly spliced, ~5-kb transcript and only in the presence of Rev. One possible explanation for this result is that Rev induces the utilization of the *vif* gene-specific A1 splice acceptor (Fig. 1). Alternatively, Rev might activate Vif expression by reducing utilization of the D2 splice donor in a population of RNA that has already completed the D1-A1 splice. It is of interest that such a splicing event would generate the small noncoding exon, designated E2 in Fig. 1, that has previously been detected in the multiply spliced HIV-1 mRNA species (14, 19, 20). To distinguish between these possible explanations, we subjected the cytoplasmic mRNA samples described above to quantitative S1 nuclease analysis (Fig. 3).

Figure 3a illustrates a probe, end labeled in the first exon of the *tat* gene, that spans the D4 splice site. This probe can therefore detect all cytoplasmic viral mRNAs containing exon E4 and will quantitate the utilization of the D4 splice donor in this population. In cells transfected with pHIV-1ΔRev (Fig. 3A, lane 2), essentially all of the cytoplasmic HIV-1 transcripts detected by this probe were spliced at the D4 site. In contrast, a large majority of the viral RNAs detected in the culture transfected with a wild-type HIV-1 provirus were unspliced at the D4 site (Fig. 3A, lane 3). This result therefore confirms those of a previous study (12) showing that coexpression of Rev reduces the utilization of the D4 splice site.

Figure 3b illustrates a second end-labeled probe that is designed to detect only those mRNAs that have already made the splice from the D1 splice donor to the A1 splice acceptor, i.e., mRNAs which could present the *vif* gene as the first open reading frame. This probe also spans the D2 splice site. It is therefore possible to quantitate the level of utilization of the D2 splice donor in a population of mRNAs that has already made the D1-A1 splice. Only mRNAs not spliced at D2 could encode the HIV-1 Vif protein. In fact, very little RNA not spliced at the D2 splice site (i.e., *vif*

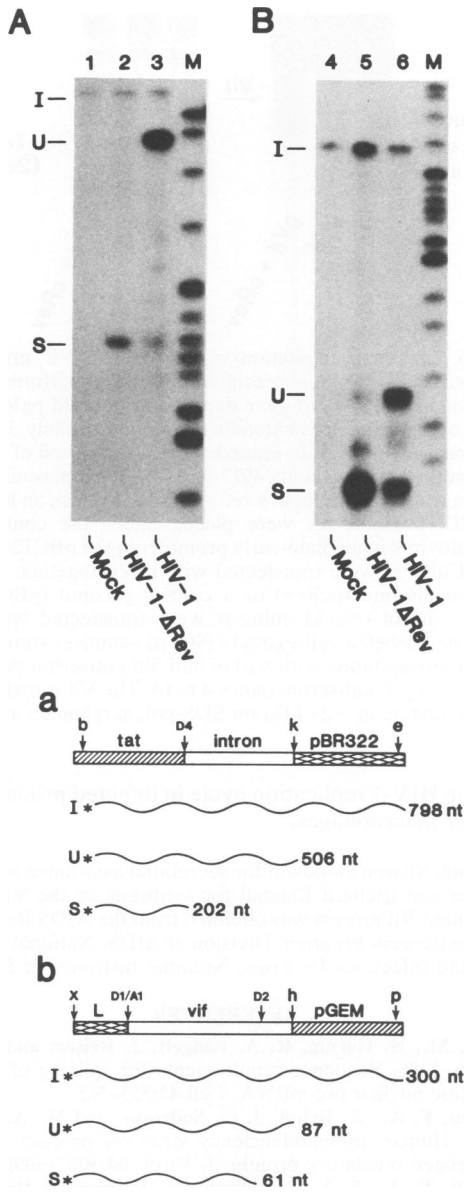

FIG. 3. S1 nuclease analysis of HIV-1 *tat* and *vif* mRNAs. The RNA samples used in this experiment were identical to those used for Northern analysis (Fig. 2). Quantitative S1 nuclease analyses were performed as described elsewhere (4, 12), with 5 μg of total cytoplasmic RNA per lane. (A, a) The probe strategy used in this experiment has been described previously (12). The probe used was end labeled at a *Bst*Y1 (b) site within the first coding exon of *tat* and extends through splice donor D4 into the *env* gene-specific intron. A pBR322 DNA tag was attached at an intron *Kpn*I (k) site to allow us to distinguish the full-length input (I) probe from probe fragments rescued by viral transcripts that were unspliced (U) or spliced (S) at the D4 splice donor. Size markers (M) were obtained by end labeling an *Msp*I digest of pBR322 DNA. e, *Eco*RI. (B, b) To quantitate the level of splicing at the D2 splice acceptor in HIV-1 transcripts already spliced at the A1 and D1 splice sites, we used the polymerase chain reaction to generate a molecular clone that contained the D1-A1 splice junction. The 5′ primer used was homologous to HIV-1 sequences located 3′ to the A1 splice acceptor (positions 4913 to 4927), and it also contained a 12-bp 5′ extension homologous to HIV-1 leader sequences located 5′ to the D1 splice donor (positions 732 to 743). The 3′ primer spanned a *Hin*dIII (h) site located 26 nucleotides (nt) 3′ to the D2 splice acceptor. This polymerase chain reaction fragment was cloned into the pGem3zf(+) polylinker (Promega). The probe used was end labeled at an *Xma*I (X) site introduced into the HIV-1 leader (L) sequence and extends through the D2 splice donor into the *vif* open reading frame. A pGem DNA tag was attached at a *vif* gene *Hin*dIII site to allow us to distinguish the full-length input (I) probe from probe fragments rescued by RNA species that were unspliced (U) or spliced (S) at the D2 splice donor. p, *Pvu*II.

mRNA) was detected in cells transfected with pHIV-1ΔRev (Fig. 3B, lane 5). Instead, we detected a high level of RNA spliced at the known D2 splice site as well as a lower level of a slightly larger RNA spliced at an immediately adjacent splice donor that we term D2*. Utilization of this second 5′ splice site was detected in several independent experiments, although always at a lower level than that of the known D2 site, and it therefore appears probable that this represents a novel minor splice donor present in the HIV-1 provirus. S1 nuclease analysis maps the location of this site to 14 nucleotides 3′ to the known D2 site. The D2* sequence is therefore 5′-A/GUAAU-3′, which is similar to the splice donor consensus sequence (5′-G/GUAAG-3′) (1). However, the significance of this novel 5′ splice site, which should result in a slightly larger noncoding E2 exon, is unclear.

In cells transfected with a wild-type HIV-1 provirus, a marked increase in the level of mRNA unspliced at the D2 site (i.e., *vif* mRNA) was detected, as was a concomitant drop in the level of mRNA spliced at either D2 or D2* (Fig. 3B, lane 6). This result therefore supports the hypothesis that Rev activates the expression of *vif* mRNA by reducing the utilization of the D2 splice donor.

The experiments presented thus far have examined Vif and Vpr expression entirely at the RNA level. To more directly test whether the expression of the Vif and Vpr proteins depends on Rev coexpression, we raised rabbit antisera specific for these viral proteins by using as an immunogen either a peptide (N-CEQAPEDQGPQREPHNE WTLE-C) identical to amino acids 2 to 21 of the predicted *vpr* open reading frame or a full-length recombinant Vif protein obtained from the National Institutes of Health AIDS Reagent Program. We then used these antisera, together with a previously described (12) rabbit antiserum specific for the HIV-1 Tat protein, to analyze the expression of Vpr, Vif, and Tat proteins in transfected cells. For this purpose, cultures transfected with pHIV-1, pHIV-1ΔRev, or a control plasmid were metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine (12). The labeled proteins were then extracted (12), and equal aliquots were subjected to immunoprecipitation analysis with anti-Tat, anti-Vpr, or anti-Vif rabbit antisera. Precipitated proteins were resolved by electrophoresis through a denaturing sodium dodecyl sulfate (SDS)–14% polyacrylamide gel and were then visualized by autoradiography (Fig. 4).

As predicted, the culture transfected with pHIV-1ΔRev gave rise to a high level of the two-exon form of Tat protein (Fig. 4, lane 5) encoded by the various multiply spliced *tat* mRNA species (Fig. 1B) (20). Coexpression of Rev resulted in a marked reduction in the expression of the full-length Tat protein and an induction of the one-exon form of Tat (Fig. 4, lane 6) encoded by the singly spliced *tat* mRNA (Fig. 1C) (12, 20). The culture transfected with the wild-type HIV-1 proviral clone also gave rise to a specific band (~14 kDa) with the mobility predicted previously (2) for the HIV-1 Vpr protein (Fig. 4, lane 3). However, no Vpr protein was detected in the absence of Rev coexpression (Fig. 4, lane 2).

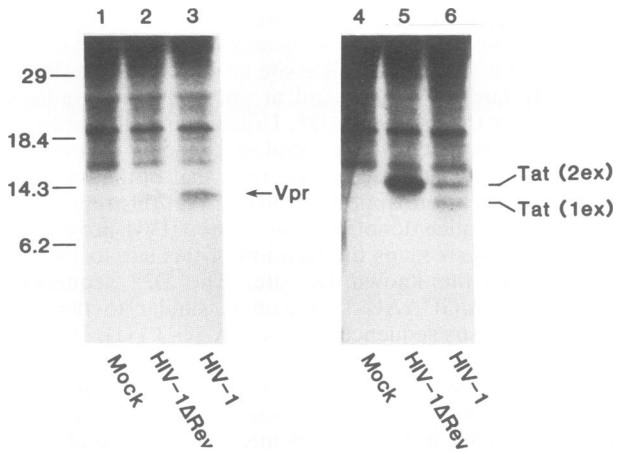

FIG. 4. Immunoprecipitation analysis of COS cell cultures transfected with proviral clones. COS cell cultures were transfected with the wild-type HIV-1 provirus or with a provirus bearing a defective *rev* gene (pHIV-1ΔRev) or were mock transfected with a control plasmid, pBC12/CMV, as indicated. At 72 h posttransfection, cultures were radiolabeled and lysed (12), and 800-μl aliquots of the solubilized proteins were subjected to immunoprecipitation (12) with 8 μl of anti-Vpr antiserum (lanes 1 to 3) or 5 μl of anti-Tat antiserum (lanes 4 to 6). The precipitated proteins were resolved by electrophoresis. The relative mobilities of ^{14}C-labeled protein molecular size standards (Bethesda Research Labs, Inc.) are indicated by their molecular masses in kilodaltons on the left. Vpr has been shown to migrate at ~14 kDa (2), while the two-exon (2ex) and one-exon (1ex) forms of Tat migrate at ~15.5 and ~14 kDa, respectively (12).

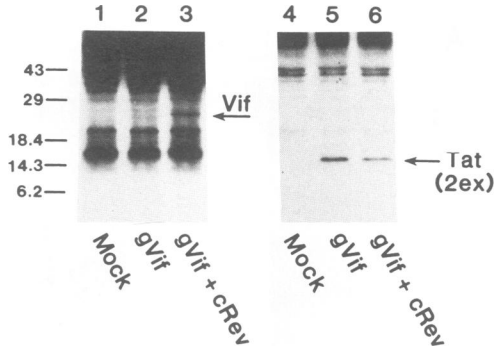

FIG. 5. Immunoprecipitation of the HIV-1 Vif protein from transfected cells. Immunoprecipitations were performed as described for Fig. 4. The *vif* gene expression plasmid pgVif contains HIV-1 proviral sequences extending from immediately 3′ to the A1 splice acceptor (Fig. 1A) to immediately 3′ to the end of the second coding exon of *tat* (positions 4917 to 8475). This plasmid therefore contains a truncated, defective *rev* gene but includes an intact RRE. These HIV-1 sequences were placed under the control of the cytomegalovirus immediate-early promoter in the pBC12/CMV plasmid (4). Cultures were transfected with pgVif together with a Rev expression plasmid (pcRev) or a control plasmid (pBC12/CMV). Negative control (mock) cultures were transfected with pBC12/CMV alone. Labeled cell extracts (800-μl samples) were subjected to immunoprecipitation with 8 μl of anti-Vif antiserum (lanes 1 to 3) or 5 μl of anti-Tat antiserum (lanes 4 to 6). The Vif protein has been shown to migrate at ~23 kDa on SDS-polyacrylamide gels (8).

Therefore, Vpr expression, like expression of the one-exon form of Tat, is dependent on Rev.

Unfortunately, it proved impossible to detect Vif protein in cultures transfected with HIV-1 proviral clones by using the rabbit anti-Vif antibody at our disposal (data not shown). In an attempt to increase expression to a detectable level, we constructed an expression plasmid, pgVif, in which a segment of the HIV-1 genome extending from immediately 3′ to the A1 splice acceptor to immediately 3′ to the end of the second exon of Tat was inserted into the expression plasmid pBC12/CMV (4). This segment of the HIV-1 provirus contains an intact viral RRE but lacks a functional *rev* gene. Unspliced mRNAs transcribed from this plasmid are predicted to be structurally comparable to the singly spliced *vif* mRNA and should, therefore, direct the synthesis of Vif protein. In contrast, fully spliced mRNAs derived from pgVif are predicted to encode predominantly the two-exon form of the HIV-1 Tat protein. In fact, a culture transfected with pgVif alone produced no detectable Vif protein (Fig. 5, lane 2) but yielded a high level of two-exon Tat (Fig. 5, lane 5). In contrast, coexpression of Rev in *trans* resulted in the induction of the 23-kDa Vif protein (Fig. 5, lane 3) (8, 22) and reduced synthesis of the 2-exon form of viral Tat protein (Fig. 5, lane 6).

In this report, we have demonstrated that expression of the HIV-1 Vif and Vpr proteins is activated by and dependent upon coexpression of the HIV-1 Rev protein. These results were obtained by the transfection of full-length HIV-1 proviral clones into the monkey cell line COS. Although COS cells are permissive for HIV-1 replication (11), they do not represent a physiologically relevant host cell for the HIV-1 virus. Nevertheless, these results clearly predict that Vif and Vpr expression is likely to be activated late in the HIV-1 replication cycle in infected primary human T cells or macrophages.

We thank Sharon Goodwin for secretarial assistance and Mildred McAdams and Richard Randall for synthesis of the Vpr peptide. Recombinant Vif protein was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

REFERENCES

1. **Aebi, M., H. Hornig, R. A. Padgett, J. Reiser, and C. Weissmann.** 1986. Sequence requirements for splicing of higher eukaryotic nuclear pre-mRNA. Cell **47**:555–565.
2. **Cohen, E. A., G. Dehni, J. G. Sodroski, and W. A. Haseltine.** 1990. Human immunodeficiency virus *vpr* product is a virion-associated regulatory protein. J. Virol. **64**:3097–3099.
3. **Cohen, E. A., E. F. Terwilliger, Y. Jalinoos, J. Proulx, J. G. Sodroski, and W. A. Haseltine.** 1990. Identification of HIV-1 *vpr* product and function. J. Acquired Immune Defic. Syndr. **3**:11–18.
4. **Cullen, B. R.** 1986. *trans*-activation of human immunodeficiency virus occurs via a bimodal mechanism. Cell **46**:973–982.
5. **Cullen, B. R., and W. C. Greene.** 1990. Functions of the auxiliary gene products of the human immunodeficiency virus type 1. Virology **178**:1–5.
6. **Feinberg, M. B., R. F. Jarrett, A. Aldovini, R. C. Gallo, and F. Wong-Staal.** 1986. HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA. Cell **46**:807–817.
7. **Felber, B. K., C. M. Drysdale, and G. N. Pavlakis.** 1990. Feedback regulation of human immunodeficiency virus type 1 expression by the Rev protein. J. Virol. **64**:3734–3741.
8. **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.
9. **Kim, S., R. Byrn, J. Groopman, and D. Baltimore.** 1989. Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential gene

expression. J. Virol. **63**:3708–3713.
10. **Kim, S., K. Ikeuchi, R. Byrn, J. Groopman, and D. Baltimore.** 1989. Lack of a negative influence on viral growth by the *nef* gene of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **86**:9544–9548.
11. **Levy, J. A., C. Cheng-Mayer, D. Dina, and P. Luciw.** 1986. AIDS retrovirus (ARV-2) clone replicates in transfected human and animal fibroblasts. Science **232**:998–1001.
12. **Malim, M. H., J. Hauber, R. Fenrick, and B. R. Cullen.** 1988. Immunodeficiency virus *rev trans*-activator modulates the expression of the viral regulatory genes. Nature (London) **335**:181–183.
13. **Malim, M. H., L. S. Tiley, D. F. McCarn, J. R. Rusche, J. Hauber, and B. R. Cullen.** 1990. HIV-1 structural gene expression requires binding of the Rev *trans*-activator to its RNA target sequence. Cell **60**:675–683.
14. **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/lymphadenopathy retrovirus. Nature (London) **313**:450–458.
15. **Myers, G., A. B. Rabson, J. A. Berzofsky, T. F. Smith, and F. Wong-Staal.** 1990. Human retroviruses and AIDS: a compilation and analysis of nucleic acid and amino acid sequences. Los Alamos National Laboratory, Los Alamos, N.Mex.
16. **Ogawa, K., R. Shibata, T. Kiyomasu, I. Higuchi, Y. Kishida, A. Ishimoto, and A. Adachi.** 1989. Mutational analysis of the human immunodeficiency virus *vpr* open reading frame. J. Virol. **63**: 4110–4114.
17. **Pomerantz, R. J., D. Trono, M. B. Feinberg, and D. Baltimore.** 1990. Cells nonproductively infected with HIV-1 exhibit an aberrant pattern of viral RNA expression: a molecular model for latency. Cell **61**:1271–1276.
18. **Rabson, A. B., D. F. Daugherty, S. Venkatesan, K. E. Boulukos, S. I. Benn, T. M. Folks, P. Feorino, and M. A. Martin.** 1985. Transcription of novel open reading frames of AIDS retrovirus during infection of lymphocytes. Science **229**:1388–1390.
19. **Robert-Guroff, M., M. Popovic, S. Gartner, P. Markham, R. C. Gallo, and M. S. Reitz.** 1990. Structure and expression of *tat*-, *rev*-, and *nef*-specific transcripts of human immunodeficiency virus type 1 in infected lymphocytes and macrophages. J. Virol. **64**:3391–3398.
20. **Schwartz, S., B. K. Felber, D. M. Benko, E.-M. Fenyö, and G. N. Pavlakis.** 1990. Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. J. Virol. **64**:2519–2529.
21. **Schwartz, S., B. K. Felber, E.-M. Fenyö, and G. N. Pavlakis.** 1990. Env and Vpu proteins of human immunodeficiency virus type 1 are produced from multiple bicistronic mRNAs. J. Virol. **64**:5448–5456.
22. **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.