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Identification of a Protein Encoded by the *trans* Activator Gene *tat*III of Human T-Cell Lymphotropic Retrovirus Type III

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The human T-cell lymphotropic virus type III (HTLV-III/LAV) is a retrovirus associated with acquired immune deficiency syndrome. The region on the viral genome that is necessary for *trans*-activation of the HTLV-III/LAV long terminal repeat called *tat*III has previously been determined to lie between nucleotides 5365 and 5607. Here we report that a bacterial fusion protein containing amino acid sequences specified by the first coding exon of the *tat*III gene is recognized by some patient antisera. We also demonstrate that lymphoid and epithelial cells that express the *trans* activator function express a 14-kilodalton (kDa) protein recognized by a patient antiserum that reacts with the bacterial *tat*III fusion protein. Cells transiently transfected with a deletion mutant of the *trans* activator protein produce a 12-kDa protein rather than the 14-kDa protein. These observations indicate that the *tat*III region contains a functional gene and is capable of expressing a protein that migrates with an apparent molecular size of 14 kDa in some lymphoid and epithelial cells transfected with plasmids containing the *tat*III region. We propose that the product of the *trans* activator gene be designated p14$^{tat\text{-}III}$.

Human T-cell leukemia virus type III (HTLV-III/LAV) is the etiological agent of acquired immune deficiency syndrome (AIDS). Infection with the virus may result in AIDS-related complex and other virus-related disorders, including degeneration of the central nervous system, lymphoid interstitial pneumonitis, an increased incidence of Kaposi sarcoma, B-cell lymphoma of a Burkitt type, Hodgkin's lymphoma, and thrombocytopenic purpura, collectively called HTLV-III/LAV-related disorders (2, 3, 5, 7–9, 15–17, 21). AIDS is clinically typified by a depletion of T cells of the $T4^+$ class in vitro (6). Large-scale production of the virus was made possible by the development of $T4^+$ cell lines that were susceptible to virus infection but were partially resistant to its cytopathic effects (11). Such cell lines permit the production of high virus titers. The prolific replication of HTLV-III/LAV can be attributed in part to the presence of a viral *trans* activator gene called *tat*III that positively regulates gene expression directed by HTLV-III/LAV long terminal repeat (LTR) sequences (1, 18, 19). The rate of HTLV-III/LAV LTR-directed gene expression, as measured by chloramphenicol acetyltransferase (CAT) activity, increases approximately 1,000-fold relative to the activity in uninfected cells.

Although the region of the HTLV-III/LAV genome necessary for the *trans*-activation of the HTLV-III/LAV LTR has been determined and shown to contain an open reading frame capable of encoding a protein of 86 amino acids in two exons (Fig. 1), the *trans* activator protein itself has not been identified. Here we report that a protein that migrates with an apparent molecular size of 14 kilodaltons (kDa) is synthesized from the HTLV-III/LAV *trans* activator gene and that this protein can be immunoreactive in some individuals infected with HTLV-III/LAV.

The predicted first coding exon of the HTLV-III/LAV *trans* activator gene is shown in Fig. 1. It lies 5′ to the envelope gene (from nucleotide 5357 to 5625). A single ATG codon at position 5411 could potentially initiate the *trans* activator protein *tat*III. The second coding exon is present in an alternative reading frame within the *env* gene. Doubly spliced RNA molecules containing these exons have been detected in virus-producing cell lines (1, 10).

To determine whether the predicted open reading frame encodes a functional protein product that is antigenic in patients, 221 nucleotides of the predicted first coding exon from nucleotide 5391 to 5611 were inserted into an open reading frame vector containing the bacterial *beta-galactosidase* gene (20). Both promoter and translation initiation signals are provided by the *ompF* gene, an *Escherichia coli* gene encoding an abundant outer membrane protein. A plasmid containing the *tat*III region was constructed to create a continuous open reading frame between the *ompF* and *beta-galactosidase* genes (*ompF-tat*III-*beta-galactosidase*, pBGT-III) (Fig. 2). A plasmid containing an in-frame fusion product between the *ompF* and *beta-galactosidase* genes without an insert was also constructed (*ompF-beta-galactosidase*, pBG).

To analyze the proteins expressed by the bacterial open reading frame vectors, TK1046 cells containing pBGT-III or pBG were grown in L broth at room temperature until the optical density at 550 nm was approximately 0.3 to 0.4. They were then labeled in M63 glucose medium with 100 μCi of [^{35}S]cysteine per ml for 15 min. Labeled proteins were processed for immunoprecipitation as previously described (20). Cells containing the pBGT-III plasmid express an approximately 120-kDa protein that can be detected by immunoprecipitation with rabbit anti-beta-galactosidase serum (Fig. 3A). The rabbit anti-beta-galactosidase serum also immunoprecipitates a smaller protein of the expected 116-kDa size from cells transformed by the pBG plasmid alone. To determine if the fusion protein is immunogenic in patients, a panel of serum from people seropositive for HTLV-III/LAV was used to immunoprecipitate extracts

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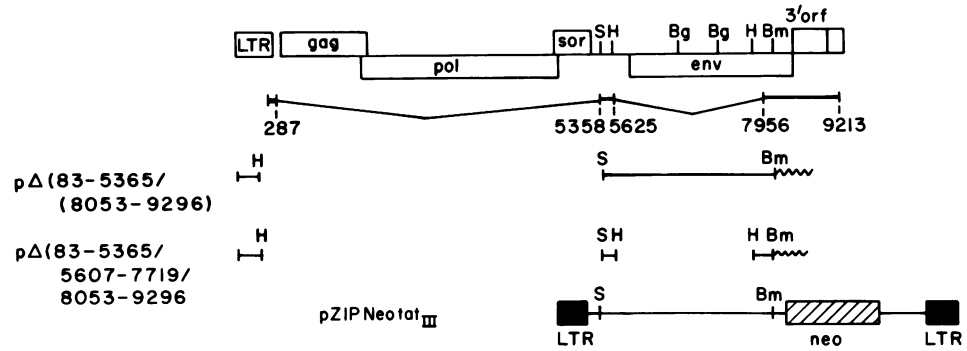

FIG. 1. Structure of HTLV-III/LAV deletion mutants that retain *trans*-activating ability. The restriction map of the complete HTLV-III/LAV provirus in plasmid pHXBc2 is shown on the topmost line. The second line drawing depicts the splicing pattern of the message coding for the potential *tat*III gene product based on the sequence of cDNA messages from HTLV-III/LAV-infected cells (1, 10). The coordinates for the *tat*III gene-coding domain are 5411 to 5625 [exon 1] and 7956 to 8001 [exon 2]. Construction of the deletion mutants and the pZIPNeotatIII plasmid have been described elsewhere (14, 18). Numbers corresponding to the deletion endpoints are based on the sequence of Ratner et al. (13) where the RNA cap site is designated as +1. Zigzag lines mark the polyadenylation and splice signals from the simian virus 40 small t-antigen coding region. Abbreviations: H, *Hin*dIII, S, *Sal*I; Bg, *Bgl*II; Bm, *Bam*HI.

derived from bacteria containing plasmids pBG or pBGT-III. Of 20 tested serum samples (5 of which are shown in Fig. 3A), 1 was positive (38-1). This serum specifically immunoprecipitated the pBGT-III fusion protein, but did not react with the product of the pBG plasmid, indicating that the antigenic determinants recognized by the serum were probably provided by the HTLV-III/LAV insert. We conclude that the *tat*III gene can encode an immunogenic product.

To determine if the positive antiserum 38-1 could identify the native viral protein product encoded by the *tat*III gene, cells that express only the *tat*III gene were used in immunoprecipitation analysis. For this study, lymphocytic cell lines were established by infecting Raji, a human B-cell line, and Jurkat, a human T-cell line, cells with a retroviral vector, pZIPNeotatIII, that was constructed to allow expression of the *tat*III gene (Fig. 1) (14). The retroviral vector does not contain the *gag*, *pol*, *sor*, or 3′ *orf* open reading frames. These cell lines stably express the HTLV-III/LAV *trans* activator gene as judged by an enhanced rate of HTLV-III/LAV LTR-directed expression of the CAT gene (Table 1). For immunoprecipitation studies, about 2×10^6 cells were labeled with 100 μCi of [^{35}S]cysteine per ml for 12 h. Cells were lysed in 500 μl of RIPA lysis buffer (0.15 M NaCl, 0.05 M Tris hydrochloride [pH 7.2], 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate) and clarified at 100,000 × *g* for 1 h at 4°C. Total cellular lysates were then incubated with 5 μl of patient serum and 80 μl of 10% protein A-Sepharose beads (Pharmacia, Inc., Piscataway, N.J.) overnight. To reduce the nonspecific binding of cellular proteins, the serum-bound beads were precleared with cold uninfected Raji or Jurkat cellular lysates. Precipitated proteins bound to the beads were washed five to six times with RIPA lysis buffer minus sodium deoxycholate and two times with 0.15 M NaCl–0.05 M Tris hydrochloride (pH 7.2) before loading on a sodium dodecyl sulfate-polyacrylamide gel. In both the Raji ZIP*tat*III and Jurkat ZIP*tat*III cell lines, a 14-kDa protein was immunoprecipitated by patient serum 38-1 (Fig. 3B). This protein was absent in the uninfected parental cell lines.

Similary, HeLa cells were transfected with a deletion mutant of the HTLV-III/LAV genome that had previously been designated pΔ(83-5365/8053-9296) (18). Cell lines were established by cotransfection with a plasmid that encodes a gene for resistance to the antibiotic G418. Immunoprecipitation of the transfected cells with the patient 38-1 serum showed a 14-kDa protein that was absent in the untransfected HeLa cells (Fig. 3C). Serum 4-3 that does not immunoprecipitate the *tat*III-beta-galactosidase fusion protein also failed to recognize the 14-kDa protein.

To examine further whether the epitopes on the 14-kDa protein recognized by patient serum 38-1 are those provided by the *tat*III region on the *tat*III-beta-galactosidase fusion protein, we analyzed the expression of a deletion mutant of the *tat*III gene, pΔ(83-5365/5607-7719/8053-9296), that contains only the first 69 amino acids of the first coding exon and deletes most of the envelope gene. Raji cells (10^7) were transiently transfected with 10 μg of either this plasmid or pΔ(83-5365/8053-9296) by using DEAE-dextran as previously described (12). Cells were harvested after 48 h and labeled with 100 μCi of [^{35}S]cysteine per ml overnight.

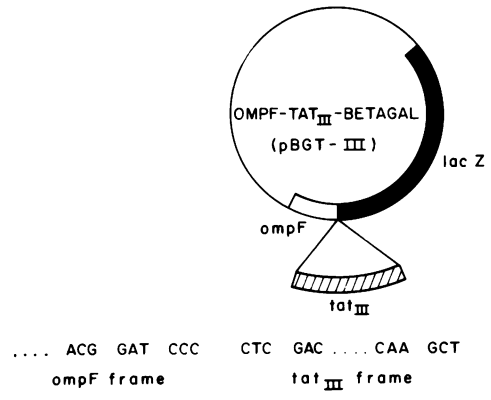

FIG. 2. Construction of a *tat*III fusion product in a bacterial open reading frame vector. About 221 nucleotides (*Taq*I-*Hin*dIII fragment) of the first coding exon of *tat*III (nucleotides 5391 to 5611) from a proviral DNA clone, pHXBc2 (kindly provided by G. M. Shaw, F. Wong-Staal, and R. Gallo), were inserted into the *Sma*I site of pORF1 (20). Shown below are the frames of the *ompF* gene, the *tat*III gene, and the *lacZ* gene. Plasmids were initially selected in *E. coli* MH3000 by scoring for blue lacZ$^+$ colonies on L broth-ampicillin plates containing 5-bromo-4-chloro-3-indoxyl-beta-D-galactoside (20). Plasmids were then transferred to *E. coli* TK1046, a strain that permits higher expression of the *ompF* promoter.

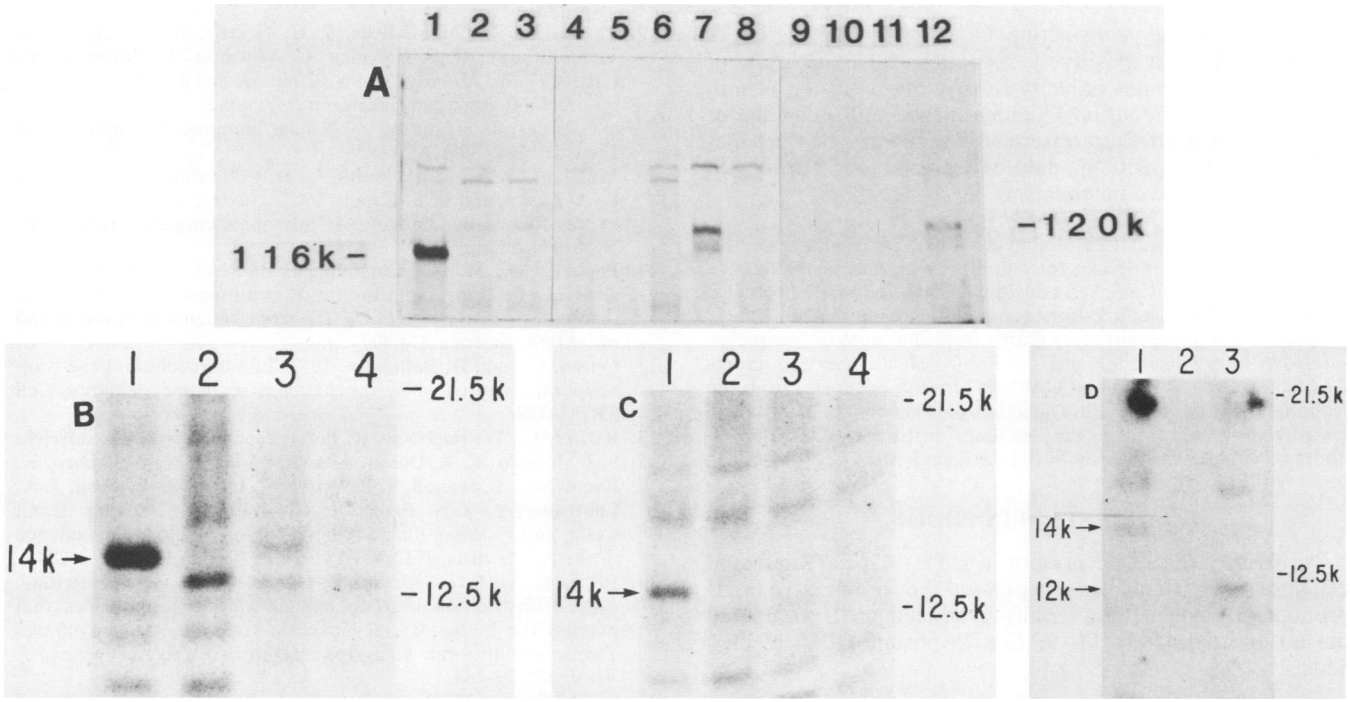

FIG. 3. (A) Expression of the fusion *ompF-tat*III-*beta-galactosidase* protein. Lanes 1 through 6 are immunoprecipitates of the *ompF-beta-galactosidase* product from pBG with rabbit anti-beta-galactosidase serum and patient antisera 4-3, 11-2, 12-4, 13-4, and 38-1, respectively. Lanes 7 through 12 are immunoprecipitates of the *ompF-tat*III-*beta-galactosidase* protein from pBGT-III with rabbit anti-beta-galactosidase serum and patient serum 4-3, 11-2, 12-4, 13-4, and 38-1, respectively. (B) Immunoprecipitation studies of cells infected with pZIPNeo*tat*III. Patient serum 38-1 was used to react with lysates from Raji ZIPtatIII (lane 1), uninfected Raji (lane 2), Jurkat ZIPtatIII (lane 3), and uninfected Jurkat cells (lane 4). (C) Immunoprecipitation of HeLa/pΔ(83-5365/8053-9296). Cells were labeled overnight with 100 μCi of [^{35}S]cysteine per ml, and total lysates were used for immunoprecipitation with either patient antiserum 38-1 (lane 1) or antiserum 4-3 (lane 2) as described in the legend to Fig. 3B. Lanes 3 and 4 are immunoprecipitates of untransfected HeLa cells with patient antisera 38-1 and 4-3, respectively. (D) Protein expression in Raji cells transiently transfected with HTLV-III/LAV deletion mutants. Total cellular lysates were prepared as in the legend to Fig. 3B and reacted with patient antiserum 38-1. Cold Raji lysates were used as a competitor to reduce background during immunoprecipitation. Lanes 1 through 3 show immunoprecipitation of cells transfected with pΔ(83-5365/8053-929), untransfected Raji cells, and pΔ(83-5365/5607-7719/8053-9296), respectively. Indicated to the right of each panel are positions and molecular sizes of marker proteins (Amersham Corp., Arlington Heights, Ill.). Abbreviation: k, kDa.

TABLE 1. Effect of HTLV-III/LAV *tat* gene expression on HTLV-III/LAV LTR-directed gene expression

Cell lines[a]	Relative CAT activity with plasmid[b]:	
	pSV2 CAT	pU3 R-III
HeLa	1.0	0.10
HeLa III extat	1.0	67 (670)
Raji	1.0	0.24
Raji ZIPNeotatIII	1.0	650 (2,708)
Jurkat	1.0	0.60
Jurkat ZIPNeotatIII	1.0	330 (550)

[a] The Raji ZIPNeo*tat*III and Jurkat ZIPNeo*tat*III cell lines were established by infection with the retroviral *tat*III expression vector pZIPNeotat_{III} as previously described (14). The HeLa III-extat line was established by cotransfection with plasmids III-extat (formerly pΔ83-5365/8053-9296) and pU3R-IIINEO.

[b] Plasmid pSV2CAT contains the bacterial CAT gene under control of the simian virus 40 early region promoter sequence (4). Plasmid pU3R-III contains HTLV-III/LAV LTR sequences directing expression of the CAT gene (18). Cells were transfected with 2 μg of plasmid DNA, and CAT assays were performed 48 h posttransfection. CAT activity is normalized to the activity obtained with pSV2CAT in the same cell. Numbers in parentheses represent the fold stimulation in the infected compared with uninfected cells.

Immunoprecipitation analysis revealed that whereas pΔ(83-5365/8053-9296) produces a 14-kDa protein, the 38-1 serum recognizes a smaller protein of approximately 12 kDa in cells transfected with pΔ(83-5365/5607-7719/8053-9296), a shift in size consistent with the expected loss of 17 amino acids (Fig. 3D). The results indicate that the 14-kDa protein recognized by serum 38-1 must be encoded by the *tat*III gene.

In summary, the 14-kDa protein is present in all the cells we tested that demonstrate *trans*-activation of HTLV-III/LAV LTR-directed gene expression. The cell lines contain plasmids constructed so as to express specifically those proteins encoded by the *tat*III gene. The cells contain portions of the HTLV-III/LAV genome in which the entire *gag*, *pol*, *sor*, and 3′ *orf* genes have been deleted. For these reasons, we conclude that the *tat*III gene encodes the protein that migrates with an apparent molecular size of 14 kDa. The immunoprecipitated 14-kDa protein in the cell lines tested is somewhat larger than its predicted size (10 kDa). This may be caused by posttranslational modification or, alternatively, by anomalous migration associated with highly basic and proline-rich polypeptides.

The 14-kDa protein identified in this study is probably the factor responsible for the greatly increased expression of genes under the control of the HTLV-III/LAV LTR. Pro-

duction of a viral *trans*-acting factor in HTLV-III/LAV-infected cells that positively regulates the expression of virally encoded genes is likely to play an important role in the life cycle of the virus. The identification of the mediator of *trans*-activation and antisera that recognize this product may lead to therapeutic modalities designed to interfere with this viral replicative mechanism.

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ADDENDUM IN PROOF

To identify the *tat*III protein in HTLV-III/LAV-infected cell lines, we have also overexpressed the entire native *tat*III product in bacteria. Bacterially produced *tat*III competed out an approximately 14- to 15-kDa protein from infected cells.

LITERATURE CITED

1. **Arya, S. K., C. Guo, S. F. Josephs, and F. Wong-Staal.** 1985. *Trans*-activator gene of human T-lymphotropic virus type III (HTLV-III). Science **229**:69–73.
2. **Barre-Sinoussi, F., J. C. Chermann, R. Rey, M. T. Nugeyre, S. Chanaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Brun-Vezinet, C. Rouzioux, W. Rozenbaum, and L. Montagnier.** 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immunodeficiency syndrome (AIDS). Science **220**:868–871.
3. **Gallo, R. C., S. Z. Salahuddin, M. Popovic, G. M. Shearer, M. Kaplan, B. F. Haynes, T. J. Palker, R. Redfield, J. Oleske, B. Safai, G. White, P. Foster, and P. D. Markham.** 1984. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science **224**: 500–503.
4. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
5. **Gottlieb, M. S., R. Schroff, H. M. Schenker, J. D. Weisman, P. T. Fan, R. A. Wolf, and A. Saxon.** 1981. *Pneumocystis carinii* pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. N. Engl. J. Med. **305**:1425–1431.
6. **Klatzmann, D., F. Barre-Sinoussi, M. T. Nugeyre, C. Dauguet, E. Vilmer, C. Griscelli, F. Brun-Vezinet, C. Rouzioux, J. C. Gluckman, J. C. Chermann, and L. Montagnier.** 1984. Selective tropism of lymphadenopathy associated virus (LAV) for helper-inducer T-lymphocytes. Science **225**:59–62.
7. **Lane, H. C., H. Masur, L. C. Edgar, G. Whalen, A. H. Rook, and A. S. Fauci.** 1984. Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome. N. Engl. J. Med. **309**:453–458.
8. **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.
9. **Masur, H., M. A. Michelis, J. G. Greene, I. Onorato, R. A. Vande Stouwe, R. S. Holzman, G. Wormser, L. Brettman, M. Lange, H. W. Murray, and S. Cunningham-Rundles.** 1981. An outbreak of community-acquired *Pneumocystis carinii* pneumonia: initial manifestation of cellular immune dysfunction. N. Engl. J. Med. **305**:1431–1438.
10. **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.
11. **Popovic, M., M. G. Sarngadharn, E. Read, and R. C. Gallo.** 1984. Detection, isolation and continuous production of cytopathic retrovirus (HTLV-III) from patients with AIDS and pre-AIDS. Science **224**:497–500.
12. **Queen, C., and D. Baltimore.** 1983. Immunoglobulin gene transcription is activated by downstream sequence elements. Cell **33**:741–748.
13. **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.
14. **Rosen, C. A., J. G. Sodroski, K. Campbell, and W. A. Haseltine.** 1986. Construction of recombinant murine retroviruses that express the human T-cell leukemia virus type II and human T-cell lymphotropic virus type III *trans* activator genes. J. Virol. **57**:379–384.
15. **Sarngadharan, M. D., M. Popovic, L. Bruch, J. Schupbach, and R. C. Gallo.** 1984. Antibodies reactive with human T-lymphotropic retroviruses (HTLV-III) in the serum of patients with AIDS. Science **224**:506–508.
16. **Shaw, G. M., M. E. Harper, B. H. Hahn, L. G. Epstein, D. C. Gajdusek, R. W. Price, B. A. Navia, 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**:177–181.
17. **Siegal, F. P., C. Lopez, G. S. Hammer, A. E. Brown, S. J. Kornfeld, J. Gold, J. Hassett, S. Z. Hirshman, C. Cunningham-Rundles, B. R. Adelsberg, D. M. Parham, M. Siegal, S. Cunningham-Rundles, and D. Armstrong.** 1981. Severe acquired immunodeficiency in male homosexuals, manifested by chronic perianal ulcerative herpes simplex lesions. N. Engl. J. Med. **305**:1439–1444.
18. **Sodroski, J., R. Patarca, C. Rosen, F. Wong-Staal, and W. Haseltine.** 1985. Location of the *trans*-activating region on the genome of human T-cell lymphotropic virus type III. Science **229**:74–77.
19. **Sodroski, J., C. Rosen, F. Wong-Staal, S. Z. Salahuddin, M. Popovic, S. Arya, R. C. Gallo, and W. A. Haseltine.** 1985. *Trans*-acting transcriptional regulation of human T-cell leukemia virus type III long terminal repeat. Science **227**:171–173.
20. **Weinstock, G. M., C. A. Rhys, M. L. Berman, B. Hampar, D. Jackson, T. J. Silhavy, J. Weiserman, and M. Zweig.** 1983. Open reading frame expression vectors: a general method for antigen production in *E. coli* using protein fusions to beta-galactosidase. Proc. Natl. Acad. Sci. USA **80**:4432–4436.
21. **Ziegler, J. L., J. A. Beckstead, P. A. Volberding, D. I. Abrams, A. M. Levine, R. J. Lukes, P. S. Gill, R. L. Burkes, P. R. Meyer, C. E. Metroka, J. Mouradian, A. Moore, S. A. Riggs, J. J. Butler, F. C. Cabanillas, E. Hersh, G. R. Newell, L. J. Laubenstein, D. Knowles, C. Odajnyk, B. Raphael, B. Koziner, C. Urmacher, and B. D. Clarkson.** 1984. Non-Hodgkin's lymphoma in 90 homosexual men: relation to generalized lymphadenopathy and the acquired immunodeficiency syndrome. N. Engl. J. Med. **311**:565–570.