Disturbance of Nuclear Transport of Proteins in CD4⁺ Cells Expressing gp160 of Human Immunodeficiency Virus

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An extensive cytopathic effect occurred in the CD4⁺ cells expressing gp160 of human immunodeficiency virus. A protein capable of nuclear location that was microinjected into such cells was not transported into the nuclei at an early stage when little cytopathic effect had yet to occur.

The pathophysiology of AIDS is characterized by an extensive decrease in the number of CD4⁺ helper-inducer T cells (6). There is evidence that both the CD4 molecule and the virus envelope glycoprotein play a role in the cytopathic effect seen in infected CD4⁺ cells. For instance, T-cell lines expressing a high level of CD4 are sensitive to the cytopathic effects of human immunodeficiency virus (HIV). However, monocytoid cells, which express a relatively low level of CD4, are resistant to such cytopathic effects (1, 8). In addition, a study with recombinant HIV suggested that the *env* region contains the determinant of viral cytopathogenicity (13, 20).

In a previous study (11), human CD4⁺ and CD4⁻ cell lines were transfected with a constructed plasmid which had the *env* gene of HIV under the transcriptional control of the human metallothionein-IIA promoter; the transfected cells were then cloned. The CD4⁺ and CD4⁻ transfectant cell clones, both of which expressed almost the same amount of gp160 after induction with metal ions, were selected and named U2ME7 and UPME1, respectively. Thereafter, they were used for an ultrastructural analysis of the distribution of the envelope glycoprotein within the cells. A notable finding in this study was the fact that the sites corresponding to the nuclear pores were occupied with aggregates of gp160 in U2ME7 cells. Electron microscopy also showed abnormal morphology around the nuclear pores and their perinuclear space. In UPME1 cells, however, no aggregates of gp160 or any abnormal structures of the nuclear membrane region were observed. These results support the possibility that such gp160 complexes, which accumulate around the nuclear pores in CD4⁺ cells, primarily disturb the transportation of molecules across the nuclear membranes and result in a cytopathic effect in CD4⁺ cells.

In the present study, U2ME7 and UPME1 cells were used to further examine whether the nuclear transport system is disturbed and, if so, to establish a possible relationship with the cytopathic effect induced by gp160 in U2ME7 cells. Fluorescein diacetate is taken up by all cells but is hydrolyzed enzymatically only in live cells to reveal fluorescence (16). To determine cell viability, cells were loaded with fluorescein diacetate and their fluorescence levels were assessed by fluorescence microscopy (Fig. 1). U2ME7 cells exhibited bright fluorescence both before (Fig. 1A) and 4 h after (Fig. 1E) induction; however, there was barely any

detectable level of fluorescence at 24 h (Fig. 1I) after induction. This means that U2ME7 cells were still viable at 4 h but dead at 24 h. On the other hand, UPME1 cells showed a slightly decreased but significant level of fluorescence even 24 h (Fig. 1J) after induction as well as before (Fig. 1B) and 4 h after (Fig. 1F) induction. Although such a moderate decrease in fluorescence intensity 24 h after induction in UPME1 cells may indicate a decrease in cell viability, on the other hand, UPME1 cells were still growing almost normally 3 days after induction in the previous study (11).

When U937-2 and U937-P, which are the parental cell lines of U2ME7 and UPME1, respectively, were treated with 20 μM CdCl₂ (Fig. 2), the level of fluorescence from incorporated fluorescein diacetate did not change significantly between U937-2 and U937-P cells either before (Fig. 2A and B) or 24 h after (Fig. 2E and F) CdCl₂ treatment. This excludes the possibility that the injury to U2ME7 cells seen after CdCl₂ induction is due to the extraordinary sensitivity of their parental cells to CdCl₂ toxicity.

The nuclear inner and outer membranes are continuous with each other around the nuclear pores through which proteins synthesized in the cytoplasm are transported into the nucleus (7). Small proteins with molecular sizes of less than 15 kDa diffuse passively across the nuclear membrane, whereas the transport of larger proteins through nuclear pores is regulated by a selective mechanism (4, 5). Although bovine serum albumin (BSA) (68 kDa) is not easily transported into the nucleus when it is microinjected into cells, BSA conjugated with a 13-mer synthetic peptide which contains an amino acid sequence homologous to the nuclear transport signal of simian virus 40 T antigen is capable of migrating into the nucleus (14).

To examine the competence of the nuclear transport system of U2ME7 cells, BSA conjugated with such a synthetic peptide (BSA-S) was prepared and microinjected into the cells by the erythrocyte ghost fusion method (Fig. 3). The migration of proteins was examined by immunofluorescence microscopy with sheep anti-BSA antibody and fluorescein isothiocyanate-conjugated anti-sheep immunoglobulin G antibody. BSA carrier proteins themselves were not transported into the nucleus when they were microinjected into U2ME7 cells without induction (Fig. 3M). Figure 3N shows the fluoresence microscopy of U2ME7 cells stained with fluorescein isothiocyanate–anti-sheep immunoglobulin G antibody only. When BSA-S was microinjected into U2ME7 cells without induction (Fig. 3A), the protein was detected predominantly in the nuclei. However, at both 4 h
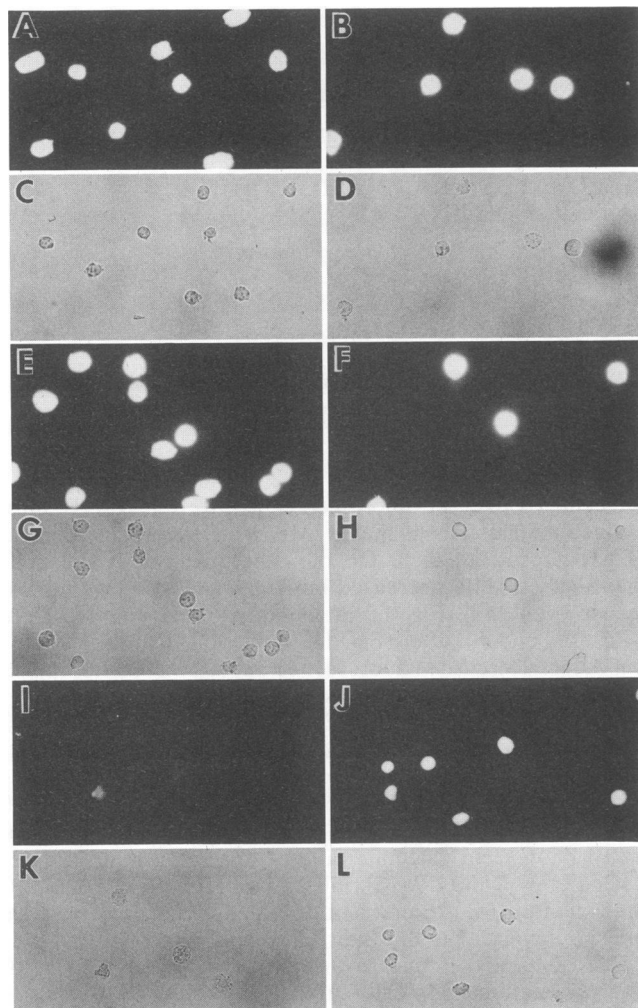
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FIG. 1. Determination of viability with fluorescein diacetate. Cell numbers were adjusted to 2×10^5/ml in RPMI 1640 medium containing 10% fetal calf serum. Each of the U2ME7 cell suspensions before induction (A and C) and 4 h (E and G) and 24 h (I and K) after induction with 20 μM CdCl$_2$ were treated with fluorescein diacetate at 5 μg/ml. After incubation at room temperature for 10 min, the fluorescence levels of the cells were examined by fluorescence microscopy (A, E, and I). By the same procedure, the viability of UPME1 cells was determined before induction (B and D) as well as 4 h (F and H) and 24 h (J and L) after induction. Panels C, G, K, D, H, and L show the phase-contrast microscopy of panels A, E, I, B, F, and J, respectively.

FIG. 2. Effect of CdCl$_2$ on viability of parental cell lines. Cell numbers were adjusted to 2×10^5/ml. U937-2 cells before (A) or 24 h after (E) the addition of 20 μM CdCl$_2$ were examined for their levels of fluorescence intensity after treatment with fluorescein diacetate as described in the legend to Fig. 1. U937-P cells before (B) or 24 h after (F) the addition of 20 μM CdCl$_2$ were also examined by the same method. Panels C, D, G, and H are the visual light images of panels A, B, E, and F, respectively.

(Fig. 3E) and 8 h (Fig. 3I) after induction, when little cytopathic effect had yet to occur in the cells (Fig. 1E), the migration of BSA-S into the nuclei had already been extensively inhibited in U2ME7 cells. In UPME1 cells, which do not exhibit either any abnormal morphology of the perinuclear area or cell killing after induction (11), such a disturbance of protein transport was not found either before induction (Fig. 3B) or at 4 h (Fig. 4F) and 8 h (Fig. 4J) after induction. When a protein of low molecular weight, a 24-mer synthetic peptide consisting of a sequence irrelevant to the nuclear localization signal, was microinjected into U2ME7 (Fig. 4A) or UPME1 (Fig. 4B) cells at 4 h after induction, the passive diffusion of the peptide into the nucleus was easily detected in both of the cells. These results indicate that in
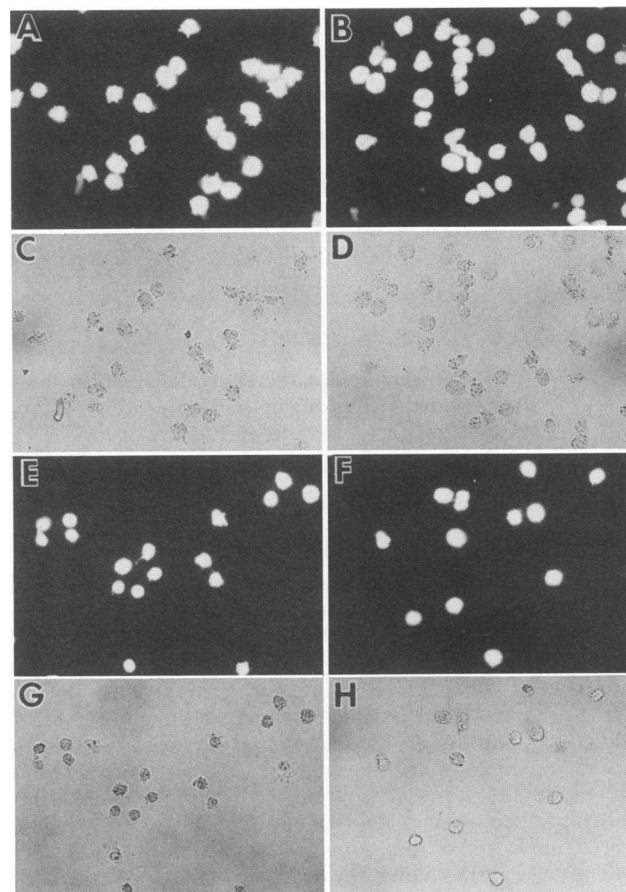
U2ME7 cells at a very early stage after induction when no evident cytopathic effect had yet occurred, the nuclear transport of large proteins, in which an active regulatory mechanism plays an important role, is selectively inhibited.

The envelope glycoprotein of HIV is synthesized as a precursor glycoprotein, gp160, and some of the gp160 is cleaved into gp120 and gp41 in cells infected with HIV (17). gp120 is secreted outside the cells. However, gp160, which is produced inside the cells, is inefficiently transported to the cell surface. The majority of gp160 is also retained in the endoplasmic reticulum or degraded in lysosomes (18). The retention of CD4 in the endoplasmic reticulum as a result of binding with gp160 has been reported by other investigators (2, 3, 9). Also, in our U2ME7 cells (CD4$^+$), such intracellular accumulation of gp160 bound with CD4 was found, whereas in UPME1 cells (CD4$^-$), no aggregation of gp160 was observed (12). Therefore, in U2ME7 cells, either gp160-CD4 or gp160 complexes accumulating in the perinuclear area may disturb the active transport of proteins through nuclear pores. The contribution of CD4 molecules to the inhibition of nuclear transport appears to be a critical factor because UPME1 cells not expressing CD4 do not exhibit such a
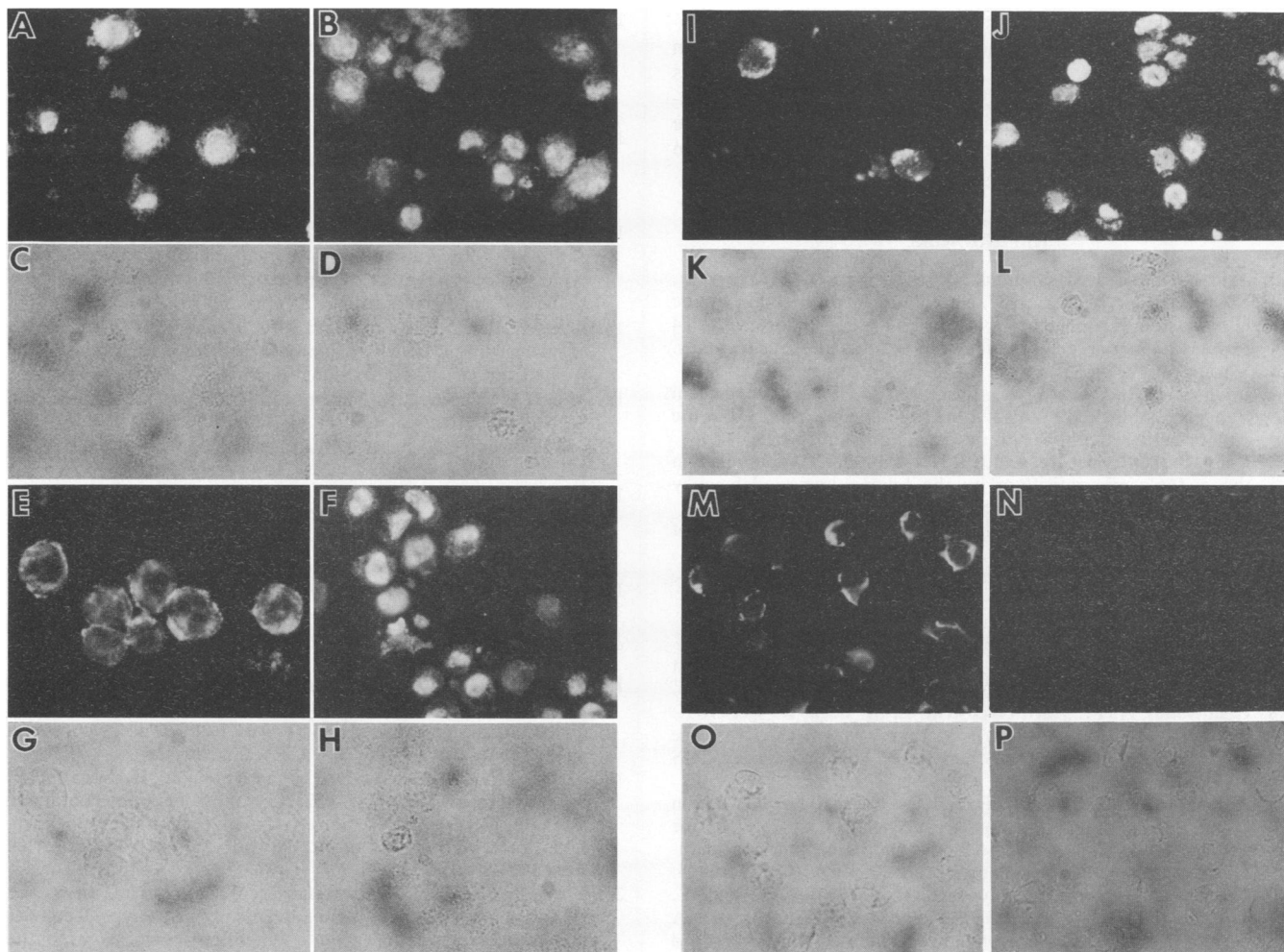
FIG. 3. Microinjection of BSA-S into cells. A 13-mer synthetic peptide, CGYGPKKKKRKVGG, was synthesized by the method of Lanford et al. (14). The seven amino acids underlined represent the core sequence for nuclear transport of simian virus 40 T antigen. The peptides were coupled to a BSA carrier protein as reported by Lanford et al. (14). The coupling ratio was 15 to 17 synthetic peptides per BSA molecule according to this method (14). BSA proteins conjugated with such synthetic peptides (BSA-S) were trapped in a human erythrocyte ghost by a method described elsewhere (19). The resulting ghosts (3×10^8) containing BSA-S were fused with cells (3×10^6) by the addition of 50% polyethylene glycol 4000, 5 µg of poly-L-arginine per ml, and 10% dimethyl sulfoxide in RPMI 1640 medium at 37°C for 10 min. The cells were then washed, resuspended in RPMI 1640 medium, and incubated at 37°C for 2 h to allow the BSA-S to migrate into the nucleus. Using ^{125}I-labeled BSA-S, the amount of BSA-S incorporated into U2ME7 or U2PME1 cells was estimated to be 20 to 30 pg per cell. For detection of BSA-S inside cells, the cells were spread on a glass slide and treated with 3.7% formaldehyde for 10 min and 0.1% Nonidet 40 for 4 min at room temperature. After being washed with phosphate-buffered saline, the cell specimens were incubated with sheep anti-BSA antibody (800×; The Binding Site, Ltd., Birmingham, England) and fluorescein isothiocyanate-conjugated anti-sheep immunoglobulin G antibody (400×; Cappel, West Chester, Pa.) at 37°C for 1 h each. The following abbreviations are used to describe the panels: the mode of image (f, fluorescent light image; v, visual light image); cells used (U2, U2ME7; UP, UPME1), and hours after induction. (A) f, U2, 0; (B) f, UP, 0; (C) v, U2, 0; (D) v, UP, 0; (E) f, U2, 4; (F) f, UP, 4; (G) v, U2, 4; (H) v, UP, 4; (I) f, U2, 8; (J) f, UP, 8; (K) v, U2, 8; (L) v, UP, 8; (M) f, U2, 0, BSA instead of BSA-S was injected; (N) same as panel M except that the cells were stained with fluorescein isothiocyanate–anti-sheep immunoglobulin G only; (O and P) visual images of panels M and N, respectively.
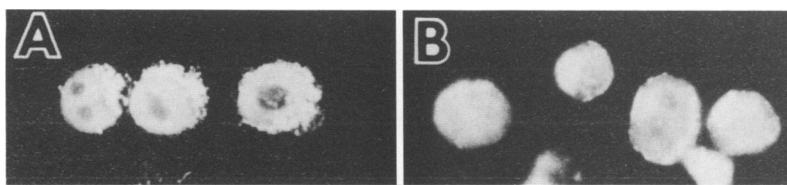

FIG. 4. Microinjection of a 24-mer synthetic peptide. A 24-mer synthetic peptide, SYEPSHDGDLGFEKGEQLRILEQS, deduced from the N region of the *lck* cDNA amino acid sequence (10) was microinjected into the cells and examined with anti-Lck monoclonal antibody (15) by the same method as that described in the legend to Fig. 3. Some small defects of fluorescence in the cells can be seen in the nucleoli. (A) U2ME7. (B) UPME1.

disturbance of nuclear transport, although these cells produce almost the same amount of gp160 as is produced in U2ME7 cells. The binding of CD4 to gp160 may thus facilitate the retention of gp160 in the endoplasmic reticulum, or inhibit the degradation of gp160, or induce some interaction with other cellular components, thus resulting in a cytotoxic effect to the cells.

REFERENCES

1. **Asjo, B., I. Ivhed, M. Gidlund, S. Fuerstenberg, E. M. Fenyo, K. Nilson, and H. Wigzell.** 1987. Susceptibility to infection by the human immunodeficiency virus (HIV) correlates with T4 expression in a parental monocytoid cell line and its subclones. Virology **157**:359–365.

2. **Buonocore, L., and J. K. Rose.** 1990. HIV-1 glycoprotein transport is blocked by a soluble CD4 molecule retained in the endoplasmic reticulum. Nature (London) **345**:625–628.

3. **Crise, B., L. Buonocore, and J. K. Rose.** 1990. CD4 is retained in the endoplasmic reticulum by the human immunodeficiency virus type 1 glycoprotein precursor. J. Virol. **64**:5585–5593.

4. **De Robertis, E. M.** 1983. Nucleocytoplasmic segregation of proteins and RNAs. Cell **32**:1021–1025.

5. **Dingwall, C.** 1985. The accumulation of proteins in the nucleus. Trends Biochem. Sci. **10**:64–66.

6. **Fauci, A. S.** 1988. The human immunodeficiency virus infectivity and mechanisms of pathogenesis. Science **239**:617–622.

7. **Fawcett, D. W.** 1981. Nucleus, p. 195–302. *In* D. W. Fawcett (ed.), The cell, 2nd ed. The W. B. Saunders Co., Philadelphia.

8. **Gendelman, H. E., J. M. Ovenstein, L. M. Baca, B. Weiser, H. Burger, D. C. Kalter, and M. S. Meltzer.** 1989. The macrophage in the persistence and pathogenesis of HIV infection. AIDS **3**:475–495.

9. **Jabbar, M. A., and D. P. Nayak.** 1990. Intracellular interaction of human immunodeficiency virus type 1 (ARV-2) envelope glycoprotein gp160 with CD4 blocks the movement and maturation of CD4 to the plasma membrane. J. Virol. **64**:6297–6304.

10. **Koga, Y., N. Caccia, B. Toyonaga, R. Spolski, Y. Yanagi, Y. Yoshikai, and T. W. Mak.** 1986. A human T cell-specific cDNA clone (YT16) encodes a protein with extensive homology to a family of protein-tyrosine kinases. Eur. J. Immunol. **16**:1643–1646.

11. **Koga, Y., M. Sasaki, K. Nakamura, G. Kimura, and K. Nomoto.** 1990. Intracellular distribution of the envelope glycoprotein of human immunodeficiency virus and its role in the production of cytopathic effect in $CD4^+$ and $CD4^-$ human cell lines. J. Virol. **64**:4661–4671.

12. **Koga, Y., M. Sasaki, H. Yoshida, H. Wigzell, G. Kimura, and K. Nomoto.** 1990. Cytopathic effect determined by the amount of CD4 molecules in human cell lines expressing envelope glycoprotein of HIV. J. Immunol. **144**:94–102.

13. **Kowalski, M., L. Bergeron, T. Dorfman, W. Haseltine, and J. Sodroski.** 1991. Attenuation of human immunodeficiency virus type 1 cytopathic effect by a mutation affecting the transmembrane envelope glycoprotein. J. Virol. **65**:281–291.

14. **Lanford, R. E., P. Kanda, and R. C. Kennedy.** 1986. Induction of nuclear transport with a synthetic peptide homologous to the SV40 T antigen transport signal. Cell **46**:575–582.

15. **Moroi, Y., Y. Koga, K. Nakamura, M. Oh-tsu, G. Kimura, and K. Nomoto.** Accumulation of p60^{lck} in HTLV-I-transformed T cell lines detected by an anti-Lck monoclonal antibody, MOL 171. Jpn. J. Cancer Res., in press.

16. **Rotman, B., and B. W. Papermaster.** 1966. The membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. Proc. Natl. Acad. Sci. USA **55**:134–137.

17. **Veronese, F. D., A. L. De Vico, T. D. Copeland, S. Oroszlan, R. C. Gallo, and M. G. Sarngadharan.** 1985. Characterization of gp41 as the transmembrane protein coded by the HTLV-III/LAV envelope gene. Science **229**:1402–1405.

18. **Willey, R. L., J. S. Bonifacino, B. J. Potts, M. A. Martin, and R. D. Klausner.** 1988. Biosynthesis, cleavage, and degradation of the human immunodeficiency virus 1 envelope glycoprotein gp160. Proc. Natl. Acad. Sci. USA **85**:9580–9584.

19. **Yoneda, Y., T. Arioka, N. Imamoto-Sonoda, H. Sugawa, Y. Shimonishi, and T. Uchida.** 1987. Synthetic peptides containing a region of SV40 large T-antigens involved in nuclear localization direct the transport of proteins into the nucleus. Exp. Cell Res. **170**:439–452.

20. **York-Higgins, D., C. Cheng-Maryer, D. Bauer, J. A. Levy, and D. Dina.** 1990. Human immunodeficiency virus type 1 cellular host range, replication, and cytopathicity are linked to the envelope region of the viral genome. J. Virol. **64**:4016–4020.