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CD4 Down-Modulation during Infection of Human T Cells with Human Immunodeficiency Virus Type 1 Involves Independent Activities of *vpu*, *env*, and *nef*

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The human immunodeficiency virus type 1 (HIV-1) genes *vpu*, *env*, and *nef* have all been implicated in modulating the levels of cell surface CD4 on infected cells. To quantitatively assess the relative contribution of each gene product to the regulation of CD4 during HIV infection of Jurkat T cells and peripheral blood mononuclear cells, we have developed an infectious HIV reporter system which expresses different combinations of these genes. To distinguish infected cells in the early or late stages of infection from uninfected cells, these viruses were designed to express human placental alkaline phosphatase with the kinetics of either early or late viral genes. Flow cytometry to detect placental alkaline phosphatase and CD4 in infected cells showed that *vpu*, *env*, and *nef* are independently capable of down-modulation of CD4. As predicted by their respective expression patterns, *nef* down-modulated CD4 rapidly during the early phase of virus infection whereas *vpu* and *env* functioned late in the infection. In both Jurkat cells and peripheral blood mononuclear cells, a combination of the three genes was more efficient than any one or two genes, demonstrating that all three genes are required to achieve maximal CD4 down-modulation. In primary cells, down-modulation of CD4 was less efficient than in Jurkat cells and there was a stronger dependence on *nef* function for reducing cell surface CD4. HIV therefore has three genes that are able to independently down-modulate CD4; together, they can eliminate the bulk of cell surface CD4.

The CD4 molecule plays a central role in the pathogenesis of AIDS (reviewed by Bour et al. [4]). Depletion of the T cells which carry this marker is the clinical hallmark of the immune system dysfunction characteristic of AIDS. In humans infected with human immunodeficiency virus (HIV), the CD4 cell surface antigen serves as the major receptor for the virus (17, 23). CD4 is also of pivotal importance in the development and maintenance of normal immune function. The binding of CD4 to major histocompatibility complex type II on antigen-presenting cells plays an essential role in the process of T-cell antigen recognition and subsequent T-cell activation (reviewed by Weiss and Littman [39]).

Early studies of HIV infection in vitro suggested that infection of cells could down-modulate cell surface CD4 (9, 17). Since then, numerous experiments based largely upon stable or transient transfection have implicated the actions of the viral genes *env*, *vpu*, and *nef* in this process. These genes appear to work through different mechanisms; however, the independent activities or potential cooperativity of their gene products during HIV infection of human T cells has not been investigated.

env and *vpu* are coordinately expressed on a dicistronic, singly spliced mRNA (32). Because efficient expression is dependent upon prior expression of the early viral protein, Rev, *vpu* and *env* are referred to as late genes. The *env* gene product is a glycoprotein, gp160, which mediates virus binding and fusion to the host cell. Stable expression of *env* in $CD4^+$ cells can down-modulate CD4 (35). Analysis of stable cell lines expressing gp160 shows that it can bind to CD4 in the endoplasmic reticulum to form aggregates that are retained in the endoplasmic reticulum (8, 15).

Vpu is a viral integral membrane phosphoprotein that was originally characterized as a 16-kDa protein which increases viral particle release from infected cells (36). Subsequent studies demonstrated that expression of Vpu could decrease the half-life of CD4 in experiments on transfected HeLa epithelial carcinoma cells (40). In this system, the effect of *vpu* on the stability of CD4 was dependent upon the presence of coexpressed gp160 capable of interaction with CD4. Although the mechanism of the effect of *vpu* on CD4 is still unclear, recent studies have demonstrated direct in vitro binding of *vpu* to the cytoplasmic tail of CD4 (5).

The HIV regulatory protein Nef is a myristylated 27-kDa protein expressed early during virus infection from a multiply spliced transcript (16, 18). Nef can also down-modulate CD4 as one of its functions (10, 12). Expression of *nef* in a T-cell line appears to induce cell surface CD4 endocytosis which is dependent upon a dileucine motif in the cytoplasmic tail of CD4 (1). A second function of *nef* is to increase viral replication rates (25, 34); however, this activity and its ability to down-modulate CD4 can be separated by specific point mutations in *nef* (29). The mechanisms by which *nef* affects cell surface CD4 levels and viral replication rates remain unclear.

Many reports concerning the mechanisms of CD4 modulation by each of these viral gene products have appeared; however, none has provided a quantitative picture of the efficiency of action of each during infection of $CD4^+$ T cells. It is unclear whether each gene can function independently and to what extent CD4 is removed from the cell surface during infection. Furthermore, no data exist regarding the potential cooperativity with which these genes act during infection of T-cell lines or primary human T cells by HIV. In this study, we systematically demonstrate the individual and combinatorial effects of *vpu*, *env*, and *nef* on cell surface CD4 during infection of hu-

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man T cells. To assess the changes in cell surface CD4 during the different stages of virus infection, we have developed an infectious HIV reporter system which permits quantitative examination of infected cells at various stages of infection via flow cytometry. Using such a system, we find that a virus lacking *nef*, *vpu*, and *env* does not down-modulate CD4. Each of these three genes is then able to independently down-modulate CD4. These effects are present with a greater efficiency in Jurkat cells than in primary human T cells. Together, these three viral genes are able to eliminate the preponderance of surface CD4 from infected cells.

MATERIALS AND METHODS

Cells. Jurkat clone E6-1 was obtained from the AIDS Reagent Repository, donated by Arthur Weiss. Peripheral blood mononuclear cells (PBMC) were isolated from HIV- and hepatitis B virus-seronegative donors. PBMC from buffy coats were purified by centrifugation over a Ficoll-Hypaque (Pharmacia) gradient and incubated overnight in RPMI 1640 containing 2 μg of phytohemagglutinin (PHA) per ml and 10% fetal calf serum. Subsequently, PBMC were maintained in RPMI–10% fetal calf serum with 20 U of recombinant interleukin-2 (Genzyme) per ml.

Viruses. HIV proviral constructs are based upon R7, a modified clone of HXB-2D which contains a repaired *nef* open reading frame (kindly provided by Mark Feinberg). Virus was produced by calcium phosphate transfection of 293 cells by standard methods (27). Virus from the transfection was quantitated by p24 enzyme-linked immunosorbent assay prior to infection (26). Infections were performed for 8 to 12 h in 8 μg of Polybrene per ml. Viral constructs were produced with standard recombinant DNA methods as described below.

To create the virus designated HXBnPLAP, we inserted a placental alkaline phosphatase (PLAP) gene amplified by PCR in the *nef* site, replacing the luciferase gene in HXB-Luc (7). The PLAP gene was amplified with the primers 5′-ACT CAC AGA GCG GCC GCA CTG CTG CTG CTG CTG CTG CTG GGC-3′ and 5′-CGC TAT ACG CGT CTC GAG TCA GGG AGC AGT GGC CGT CTC-3′, *Pfu* polymerase (Stratagene), and 100 ng of template PLAP cDNA, using 20 amplification cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 3 min) under buffer conditions recommended by the manufacturer. To repair the translation initiation codon of *vpu*, which is defective in HXB-2D, PCR-mediated mutagenesis was used to generate a *Bam*HI-*Sal*I fragment isogenic with the normal HXB-2D sequence except for the creation of an ACG-to-ATG point mutation at the initiation codon of *vpu*. Constructs were confirmed along the entire PCR-amplified region by sequence analysis (ABI Sequenator; Applied Biosystems). A frameshift in the *env* reading frame was introduced by partial digestion with *Nde*I and filling in with the Klenow fragment of DNA polymerase. To insert the internal ribosomal entry site (IRES) element of encephalomyocarditis virus at the 5′ end of the *nef* reading frame and to reintroduce the 5′ end of *nef*, a PCR-mediated method was used. Briefly, a PCR fragment of the IRES element was generated with the primers 5′-GGT ACG TAC GTC GAC GAA TTC CGC CCC TCT CCC TCC-3′ and 5′-ACT TTT TGA CCA CTT GCC ACC CAT ATT ATC ATC GTG TTT TTC AAA-3′. The resulting fragment produces a fusion gene with the IRES sequence immediately followed by 24 nucleotides of the 5′ end of *nef*. A second PCR fragment of the 5′ end of *nef* extending past the unique *Xho*I site was also generated. A fusion PCR product was generated with these two PCR products as a template in a third PCR reaction. The resulting fragment was cloned into pBluescript, and its sequence was confirmed before transfer into the unique *Xho*I site in HXBnPLAP.

To insert PLAP into the 5′ end of the *env* gene, a polylinker was first inserted to replace the 5′ end of the *env* gene, deleting the initial 1,035 nucleotides of *env* coding sequence up to an *Nhe*I site. A PLAP gene was inserted into the resulting virus to create HXBePLAP. To create HXBePLAP N$^-$, a frameshift mutation was introduced into the *nef* open reading frame by cutting with *Xho*I, filling in with Klenow, and religating the blunt-ended fragment.

Murine leukemia virus (MLV) vector expressing PLAP, DAP, was a gift from Connie Cepko (6). Helper-free amphotropic retrovirus was produced by transient transfection of the amphotropic MLV-packaging cell line, BING, as previously described (27).

Flow cytometry and Western blot analysis. Cells were stained with a 1:400 dilution of rabbit anti-human PLAP (DAKO) and a 1:100 dilution of anti-human CD4-biotin (Caltag). Goat anti-rabbit fluorescein isothiocyanate, human and mouse serum adsorbed (BioSource International) at 1:250, and streptavidin PE (Caltag) at 1:250 were used as secondary stains. Samples were fixed in 1% paraformaldehyde prior to analysis. Flow cytometry was performed on a FACSCAN (Becton Dickinson) with CellQuest (Becton Dickinson) data acquisition and analysis software. The data shown are gated on living cells on the basis of forward-scatter and side-scatter characteristics. Fluorescence compensation was adjusted on control samples labeled with single fluorochromes to minimize the effects of spectral overlap. The following antibodies for Western blotting (immunoblotting) of Vpu, Env, Nef, and HIV proteins were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases: anti-HIV-1 Vpu rabbit serum from Frank Maldarelli and Klaus Strebel, anti-Env sheep serum from Michael Phelan, anti-HIV-1 Nef rabbit serum from BioTechnology General, and pooled human HIV immune globulin from Alfred Prince.

RESULTS

PLAP reporter system. We engineered an infectious HIV strain to express a cell surface marker that could be monitored by flow cytometry by introducing the human PLAP gene (13) into the molecular clone of HIV-1, HXB-2D. The PLAP gene encodes a cell surface, glycosylphosphatidylinositol-anchored protein that is not normally present on cells of the lymphoid lineage. The PLAP gene product can be easily stained by indirect immunofluorescence to allow quantitative detection by flow cytometry. Furthermore, because glycosylphosphatidylinositol-anchored proteins generally have a low turnover rate (37), accumulation of PLAP may also serve as an indicator of the stage of virus infection and efficiency of virus gene expression.

As a preliminary test of the system, we examined whether PLAP itself may affect levels of cell surface CD4 or whether it might have toxic effects upon cell growth outside the context of HIV infection. We infected Jurkat cells with amphotropic-enveloped, helper-free MLV expressing the PLAP gene. In a population of Jurkat cells infected with this nonreplicating vector, levels of PLAP detected on PLAP-positive cells did not change appreciably over 28 days of propagation, suggesting that the expression of PLAP has little or no detrimental effect upon the growth of Jurkat T cells (Fig. 1A). High-level PLAP expression was maintained for over one month in a fixed percentage of cells in the absence of selection (Fig. 1B). The percentage of PLAP-positive cells remained stable in both the CD4$^+$ and CD4$^-$ compartments. Fluorescence intensity values for CD4 and PLAP in these cells did not vary appreciably over this period, demonstrating that the expression of PLAP itself does not affect the levels of cell surface CD4 (data not shown).

Placement of the PLAP gene in two distinct coding regions of the HIV genome allowed us to selectively mark cells from the early or late stages of virus infection (Fig. 2). The resulting viruses, HXBnPLAP or HXBePLAP, do not express *nef* or *env*, respectively, but instead produce PLAP in place of one of these gene products. Replacing *nef* with PLAP in HXBnPLAP produced a replication-competent HIV strain that expressed PLAP with early gene kinetics. Replacement of the *env* gene with PLAP in HXBePLAP produced a virus which expressed PLAP with late gene kinetics. Infection with HXBePLAP required *trans* complementation of the missing *env* gene during virus production. This could be accomplished efficiently by cotransfecting 293 cells with an expression vector for the MLV amphotropic envelope with the *env*-deficient proviral DNA to give rise to a pseudotyped HIV [HXBePLAP(ampho)] competent for a single round of infection (7, 20, 22).

Infection in the absence of *vpu*, *env*, and *nef*. We first analyzed the effects of infection of the CD4$^+$ Jurkat T-cell line with a viral construct which expressed none of the genes implicated in CD4 regulation (Fig. 3A). The virus, HXBePLAP N$^-$(ampho), expressed PLAP in place of *env* and contained a frameshift mutation in the *nef* reading frame. In addition, the HXB-2D molecular clone contains a point mutation in the initiation codon of *vpu* which abrogates expression of this gene. Western blot analysis showed that HXBePLAP N$^-$ did not express Vpu, Env, or Nef (Fig. 4, lane 1). Infection with HXBePLAP N$^-$(ampho) gave rise to a population of cells that stained positive for PLAP and could be monitored over time by flow cytometry. Examination of the infection on day 3, at a time when near-maximal numbers of PLAP$^+$ cells were detected, revealed no apparent changes in the level of CD4 ex-

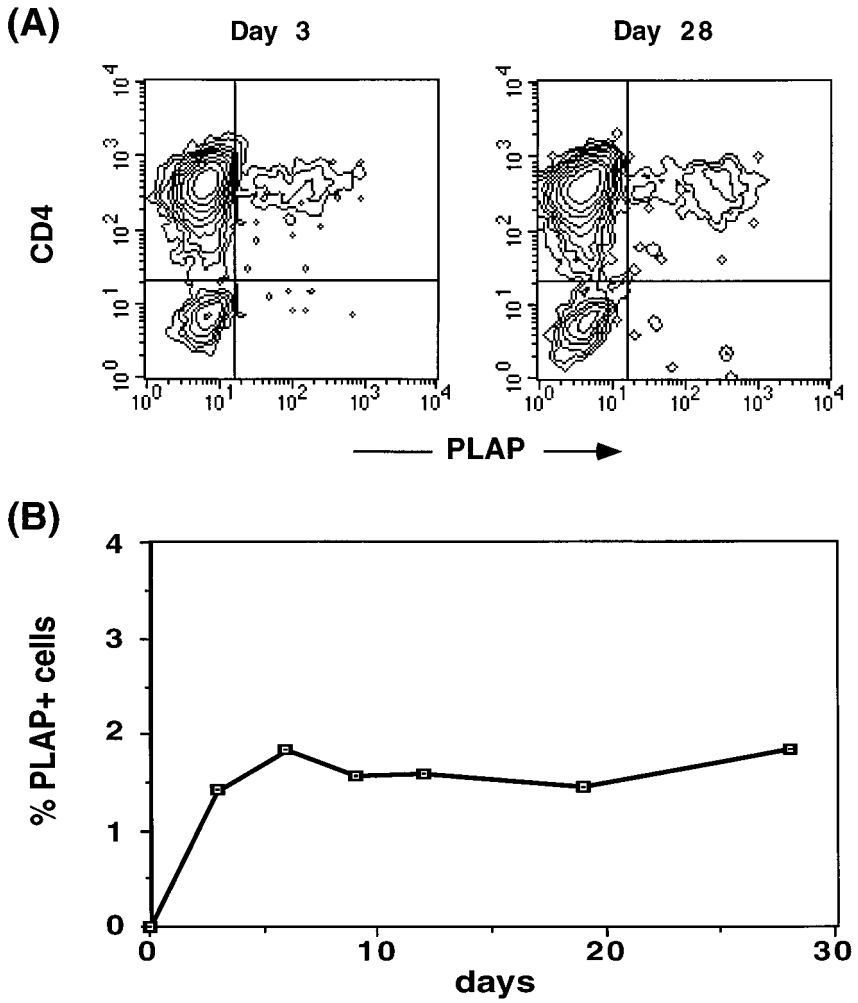

FIG. 1. Stable expression of PLAP in Jurkat cells by helper-free MLV infection. Jurkat cells were infected with an amphotropic-enveloped, replication-deficient MLV vector expressing PLAP, DAP(ampho). (A) Flow-cytometric analysis of infected cells stained for CD4 and PLAP 3 and 28 days after infection. (B) Graph illustrating the percentage of cells in the infected culture staining positive for PLAP over time, as determined by FACSCAN analysis.

pression on $PLAP^+$ cells compared with $PLAP^-$ cells (Fig. 5B). After day 3, the percentage of $PLAP^+$ cells decreased over time (data not shown), suggesting that in the absence of new rounds of viral infection, infected cells were at a competitive disadvantage with respect to uninfected cells. Small numbers of $CD4^-$ cells were found to be $PLAP^+$. These $PLAP^+$ $CD4^-$ cells were probably due to the infection of $CD4^-$ Jurkat cells by amphotropic envelope-pseudotyped HIV because the small fraction of $CD4^-$ cells in the $PLAP^+$ population reflected the fraction of $CD4^-$ cells normally found in Jurkat cells. As a comparison, mock-infected Jurkat cells were largely CD4 positive with only minimal background PLAP staining (Fig. 5A). This result demonstrates that inactivation of *nef*, *vpu*, and *env* is sufficient to eliminate all CD4-modulatory function during viral infection.

Effect of *nef* alone on CD4 down-modulation. We next determined the effect on CD4 of a virus expressing *nef* as the sole potential CD4-modulatory factor, HXBePLAP (Fig. 3A shows the structure and genotype). In contrast to HXBePLAP N^-, HXBePLAP produced a functional Nef (Fig. 4, lane 2). When infected with HXBePLAP(ampho), virtually all cells expressing PLAP were CD4 negative (Fig. 5C), in contrast to infection with HXBePLAP N^- (Fig. 5B). In the infection with HXBe PLAP(ampho), PLAP is predicted to be expressed with late-gene kinetics and consequently marks only those cells in the late phase of HIV infection. The nearly complete absence of $PLAP^+$ $CD4^+$ cells in this infection suggests that all $PLAP^+$ cells had produced sufficient quantities of the early protein, Nef, to down-modulate most of their cell surface CD4 before the expression of significant levels of surface PLAP. This result suggests that *nef* has a strong down-modulatory effect on surface CD4 which is evident before the expression of late genes such as *env*.

Effect of *env* alone on CD4 down-modulation. We next constructed and examined a virus expressing *env* but not *vpu* or *nef*, designated HXBnPLAP (Fig. 3B shows the structure and genotype; Fig. 4, lane 3, shows the Western blot). When infected with HXBnPLAP, Jurkat cells with low levels of PLAP (1- to 50-fold above background staining [$PLAP^{lo}$]) maintained high levels of CD4 (Fig. 5D). However, cells expressing the higher levels of PLAP (greater than 50-fold over background [$PLAP^{hi}$]) down-regulated CD4 to a level close to that on cells expressing no CD4. This virus expresses PLAP with early-gene kinetics, so that the protein may accumulate on the cell surface during both the early and late stages of infection. Unlike HXBePLAP, HXBnPLAP is replication competent and in 7 to 10 days can spread to infect the majority of cells in a culture (data not shown). At such late times in infection, virtually all

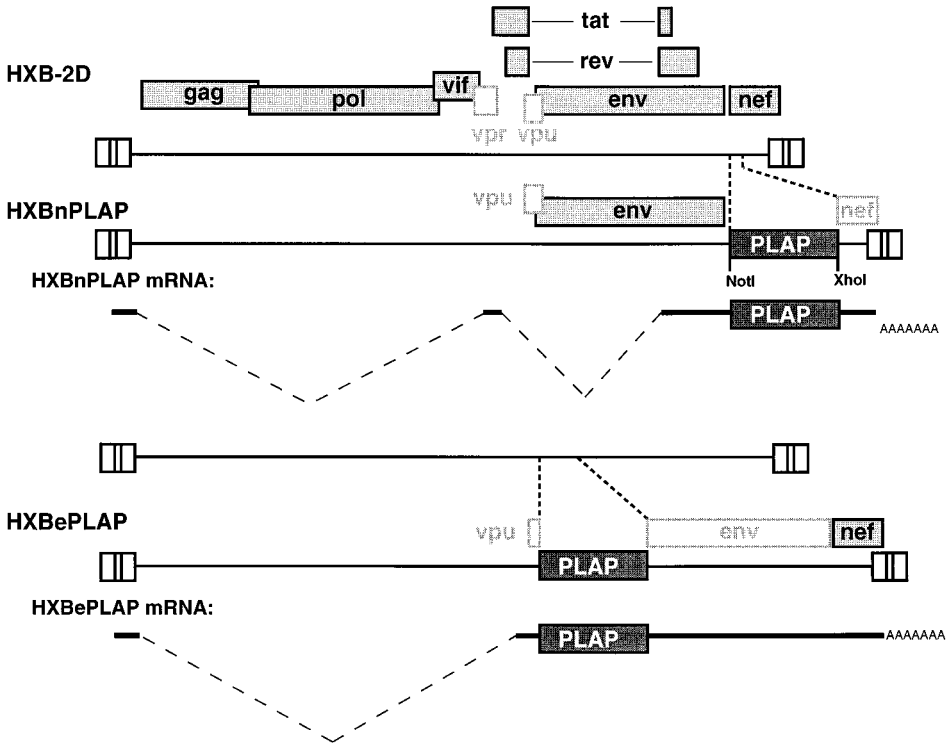

FIG. 2. Genomic organization and pattern of mRNA splicing of recombinant HIV constructs made to express PLAP. Parental virus, HXB-2D, is shown at the top, and the insertion of PLAP into the *nef* and *env* reading frames is illustrated below the sequence. HXBnPLAP contains an inserted PLAP gene at the 5′ end of *nef*. PLAP is expressed with early-gene kinetics on a multiply spliced mRNA. HXBePLAP contains an inserted PLAP gene at the 5′ end of *env*. PLAP is expressed with late-gene kinetics on a singly spliced mRNA. Genes that are shaded indicate open reading frames expressed in the virus construct. Unshaded genes are not expressed by that virus.

cells eventually progress to become $PLAP^{hi}$, suggesting that levels of PLAP allow the discrimination between early events in $PLAP^{lo}$ cells and late events in $PLAP^{hi}$ cells. The observation that only $PLAP^{hi}$ cells have down-modulated CD4 demonstrates that *env* can independently and efficiently down-modulate CD4 in Jurkat cells in the late phase of the HIV life cycle.

Effect of *vpu* alone on CD4 down-modulation. To test whether viruses expressing *vpu* in the absence of *nef* and *env* could also down-modulate CD4, we restored the *vpu* reading frame by repairing the *vpu* translation initiation codon of HXB-2D in the virus HXBnPLAP. When the ATG was repaired, the Vpu protein could be readily detected by Western blotting of cells transfected with the proviral DNA (Fig. 4, lane 4). So that we might observe the effects of *vpu* on CD4 without the coexpression of *env*, we inactivated the envelope reading frame by introducing a frameshift near the 5′ end of the *env* gene. This *env*-deficient, *nef*-deficient virus, HXBnPLAP U^+E^- (Fig. 3B shows the structure and genotype) was noninfectious unless pseudotyped with MLV amphotropic envelope (data not shown). When pseudotyped, the resulting virus, HXBnPLAP U^+E^-(ampho), was competent for only a single round of infection. A virus expressing *vpu* but not *env* or *nef* could induce significant CD4 down-modulation in $PLAP^{hi}$ cells (Fig. 5E), demonstrating that like *env*, *vpu* can independently regulate CD4 late during infection. In addition, a chimeric HXB-2D-based virus with an identical CD4 modulatory genotype ($U^+E^-N^-$) containing the *vpu* and *env* sequence from another molecular clone of HIV-1, NL4-3, was made. This virus produced an identical CD4 modulation phenotype to that of HXBnPLAP U^+E^- (data not shown), confirming that this effect could be observed with two different alleles of *vpu*.

Effect of combinations of CD4 modulatory genes. To evaluate how different combinations of the CD4-modulatory genes cooperate in their effects on CD4, we first analyzed the combination of *env* and *vpu* expression during virus infection of Jurkat cells. The virus HXBnPLAP U^+ expresses PLAP with early-gene kinetics in addition to both *env* and *vpu* (Fig. 3B shows the genotype; Fig. 4, lane 5, shows the Western blot). This virus induced $PLAP^{hi}$ cells to down-modulate CD4 similarly to virus which expressed either *env* or *vpu* alone (Fig. 5F; compare with Fig. 5D and E). The levels of CD4 down-modulation were subtly but reproducibly more efficient with the combination of the two than with either gene alone (see the quantitative analysis described below).

We next designed a virus capable of expressing PLAP along with all three CD4-regulatory genes. To accomplish this, we introduced an IRES from encephalomyocarditis virus (11, 28) into the region immediately upstream of the *nef* gene in the HXBnPLAP virus. When inserted into an RNA molecule, this picornavirus element allows for cap-independent initiation of translation at a downstream AUG (24). We simultaneously repaired the *nef* reading frame so that full-length Nef could be translated under the control of the IRES. The resulting virus, HXBnPLAP IRES-N^+ (Fig. 3C shows the structure and genotype), should produce Nef from the early stage of the virus life cycle. Western blot analysis showed that Nef protein was present at high levels in 293 cells transfected with HXBnPLAP IRES-N^+ (Fig. 4).

When Nef was produced in addition to Env during viral infection by HXBnPLAP IRES-N^+, a larger fraction of PLAP-positive cells was CD4 negative, as a result of an increase in the numbers of $PLAP^{lo}$ cells with decreased levels of CD4 (Fig. 5G; compare with Fig. 5D). This finding suggests that during infection by a nef^+ virus, CD4 down-modulation occurs efficiently in the early phase of the virus life cycle, when Nef is expressed.

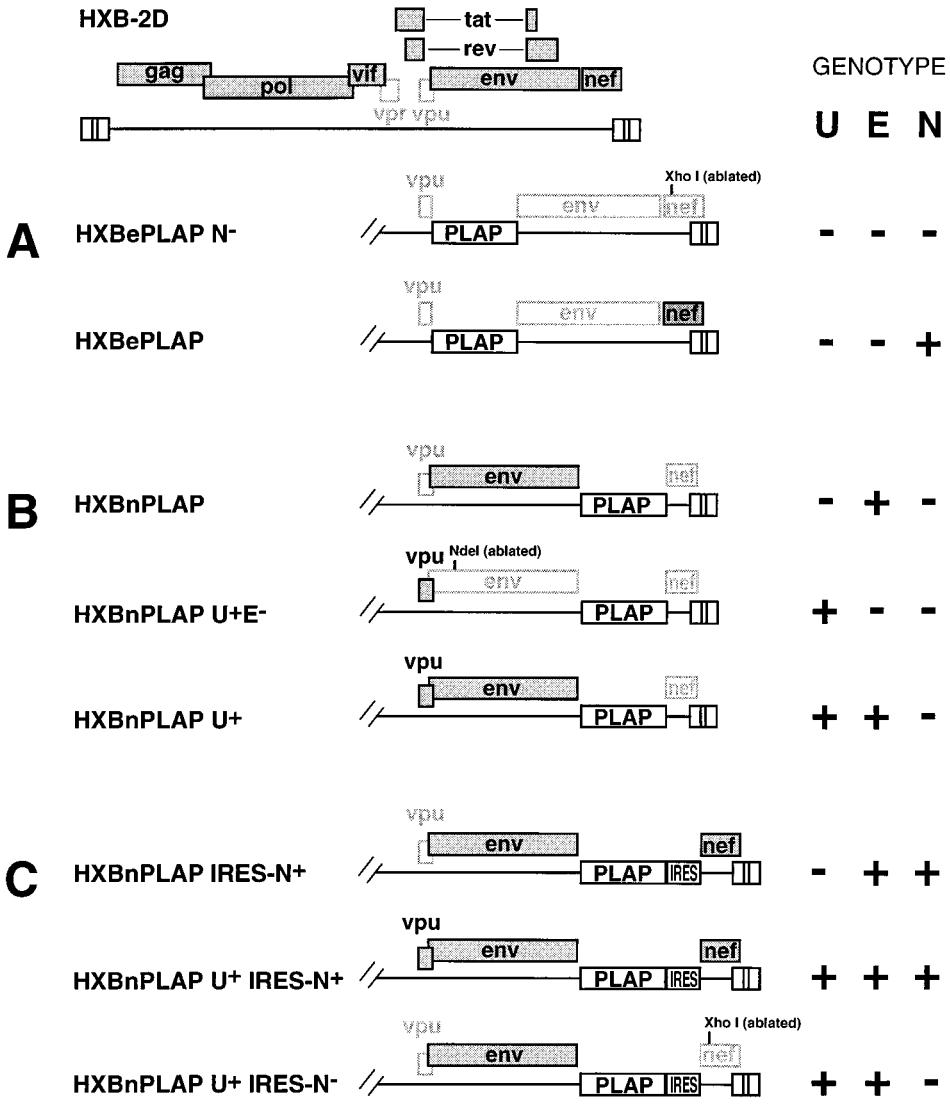

FIG. 3. Genomic organization and CD4-modulatory genotype of HIV constructs fall into three major subtypes: vectors expressing PLAP in place of *env*, designated HXBePLAP (A); vectors expressing PLAP in place of *nef*, designated HXBnPLAP (B); and vectors expressing PLAP in place of *nef* with a restored *nef* open reading frame driven by an IRES, designated HXBnPLAP IRES (C). All virus constructs are identical in the 5′ half to the left of the parallel slash marks. Viral genes that are shaded indicate open reading frames expressed in the virus construct. Unshaded genes are not expressed by that virus. The code for CD4-modulatory genes is as follows: U, *vpu*; E, *env*; N, *nef*.

Infection of Jurkat cells with virus containing all three regulatory genes, HXBnPLAP U^+-IRES N^+ (Fig. 3C shows the structure and genotype) displayed a pattern generally similar to that with a virus carrying *nef* and *env* (Fig. 5H). A quantitative analysis described below demonstrates that the addition of *vpu* to a virus carrying *env* and *nef* did induce an additional decrease in the levels of CD4. As a control to determine whether the insertion of an IRES may alter the intrinsic CD4-modulatory effects of a virus, we compared a virus which contained an IRES element with one with the same CD4-modulatory genotype without an IRES. A disruption of the *nef* open reading frame in HXBnPLAP IRES-N^+ by a frameshift mutation creates the virus HXBnPLAP-IRES N^- ($U^+E^+N^-$), which has the same CD4-modulatory genotype as HXBnPLAP U^+ (also $U^+E^+N^-$ [Fig. 3C]). When these viruses were used to infect Jurkat cells, the addition of an IRES did not alter the pattern of CD4 down-modulation (Fig. 5I; compare Fig. 5F).

Infection of primary cells and comparison with Jurkat cells. Infection of PBMC with PLAP-expressing HIV gave rise to a percentage of $PLAP^+$ cells similar to that seen with Jurkat cells. As in Jurkat cells, in the absence of all three CD4-modulatory genes, the virus had no effect upon cell surface CD4 (Fig. 6B). When viruses carrying *nef*, *env*, and *vpu* genes individually were used to infect PBMC, they again showed independent CD4 down-modulatory effects (Fig. 6C through E). The extent to which each of these genes acted in primary cells, however, was significantly weaker. In particular, CD4 down-modulation with *env* or with *vpu* as the sole CD4-modulatory factor was less efficient in PBMC than in Jurkat cells. For example, a smaller fraction of total $PLAP^{hi}$ cells had down-modulated CD4 when PBMC were infected with a virus expressing *env* alone, HXBnPLAP, compared with that in a similar analysis with Jurkat cells (compare Fig. 6D with Fig. 5D). While infection with HXBnPLAP down-modulated CD4 in more than 65% of $PLAP^{hi}$ Jurkat cells at 3 days postinfection, only 20% of such a population down-modulated CD4 in

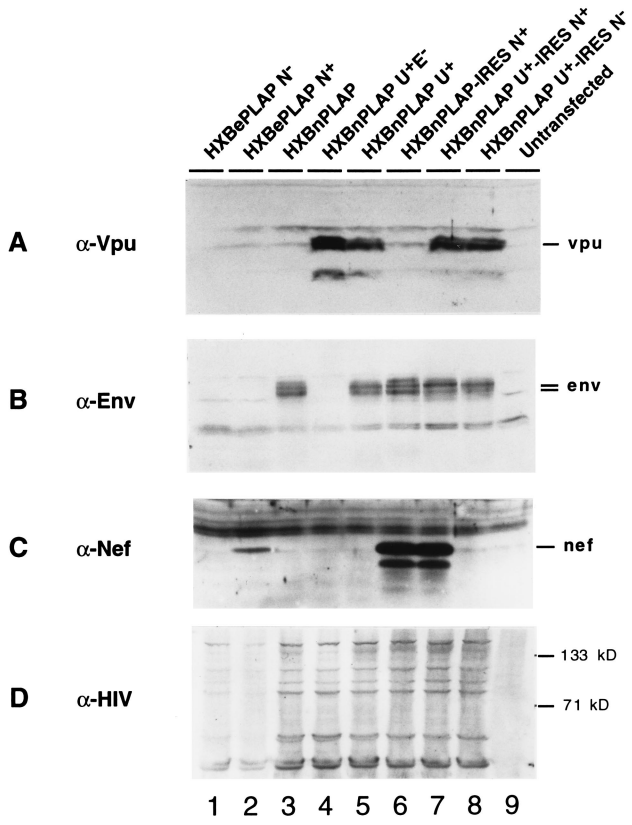

FIG. 4. Western blot analyses of 293 cells transfected with proviral DNAs to produce PLAP viruses. Blots were developed with anti-*vpu* (A), anti-*env* (B), anti-*nef* (C), and anti-HIV (D) antisera. The anti-HIV blot serves as a control for the amount of total viral proteins produced in these total-cell lysates.

PBMC (Table 1). A similar phenomenon was evident when PBMC were infected with a virus which expresses *vpu* alone (HXBnPLAP U^+E^-). This case illustrated that the down-modulation function of *vpu* is less efficient in PBMC than in Jurkat cells (compare Fig. 6E with Fig. 5E). Interestingly, *vpu* alone performed slightly better in PBMC than did *env* alone, whereas the reverse occurred in Jurkat cells (Table 1).

Because the down-modulation was less efficient by *env* or *vpu* alone in PBMC, the overall importance of *nef* in CD4 modulation is greater in PBMC than in Jurkat cells. In particular, a greater fraction of maximal CD4 down-modulation can be attributed to Nef function in PBMC. On day 3 of an infection, only in the presence of *nef* did down-modulation of CD4 increase to above 33% of primary cells, whereas in Jurkat cells, over 75% of the cells had low CD4 levels when infected with a virus expressing both *env* and *vpu* but not *nef* (Table 1).

Quantitative analysis of CD4 down-modulation. When we compared the average levels of CD4 fluorescence on cells which had achieved equivalent levels of PLAP fluorescence, more subtle differences in the efficiency of CD4 modulation were appreciated (Fig. 7). In Jurkat cells, *vpu* alone was less efficient than *env* alone in down-modulating CD4. The combination of the two genes was more efficient than was either one alone. In contrast, *vpu* alone was more efficient than *env* alone in PBMC, and the combination of the two late genes was only slightly better at down-regulating CD4 than was *vpu* alone. As discussed above, we observed a greater dependence on *nef* to achieve maximal CD4 down-modulation in PBMC. In both cell types, the lowest CD4 staining was observed after infection with virus that expresses all three regulatory genes.

The level of CD4 staining in Jurkat cells infected with a virus containing all three gene products was nearly equivalent to the intensity of staining found on a control sample stained with an isotype-matched monoclonal antibody, suggesting that depletion of CD4 in this case is nearly complete. We estimate that on average, 98% of the total CD4-associated fluorescence is removed from infected, $PLAP^{hi}$ cells. During the infection of PBMC, the efficiency of down-modulation at a similar 3-day time point was impressive but not as great as that seen in Jurkat cells. We found that on average, 92% of the original levels of CD4-associated fluorescence was removed from the surface of $PLAP^{hi}$ PBMC. While the average percentage of CD4 removed from the surface of Jurkat cells was greater than that removed from PBMC, it is important to consider that primary $CD4^+$ T cells have three- to fourfold-higher levels of CD4-associated fluorescence (Fig. 7). This observation suggests that the total amount of CD4 down-modulated from each T cell is potentially greater in PBMC.

DISCUSSION

We have used a sensitive, virus-based marker gene system to quantitatively determine the relative contributions of *vpu*, *env*, and *nef* to the regulation of the viral receptor, CD4, during the infection of both a T-cell line and primary $CD4^+$ T cells. We show that in the absence of these viral genes, no down-modulation of cell surface CD4 takes place during infection. We found that each of these three genes is capable of mediating CD4 down-modulation independently but with differing final efficiencies in T-cell lines versus primary T cells.

We demonstrate the cooperative effects of *vpu*, *env*, and *nef* on CD4 levels during infection of primary human $CD4^+$ cells. While all three genes are able to down-modulate CD4 independently, combinations of them are able to do so faster and more completely. Only when all three genes are expressed do we see maximal CD4 down-modulation in Jurkat cells or PBMC. The cooperative effects of these CD4 regulatory genes appear to be even more critical in primary cells, in which CD4 modulation is less efficient, perhaps as a result of higher initial levels of cell surface CD4 in primary T cells. We found that in PBMC, *nef* seems to account for a larger fraction of the total CD4 down-modulating activity than in the Jurkat cell line. Interestingly, *nef* is the only HIV gene which is thought to down-regulate CD4 molecules that are present on the cell surface before an infection. A heavy reliance on *nef* in these cells might suggest that primary cells have a lower intrinsic turnover rate of CD4 than do Jurkat cells.

Substantial evidence indicates that *nef* and *env*, when overexpressed in $CD4^+$ cells on their own, can down-modulate CD4. A previous study has suggested that the levels of transfected Nef expressed in cells may be important in determining whether *nef* can down-modulate CD4 (31). Our data support evidence that *nef* decreases CD4 levels during virus infection of transformed T cells (1) and further establish that these functions are temporally regulated, with *nef* functioning early and *env* and *vpu* functioning late in the viral life cycle during the infection of both Jurkat cells and primary human $CD4^+$ lymphocytes.

Previous metabolic labelling studies which determined the half-life of CD4 in transfected HeLa cells predicted that the CD4-modulatory function of *vpu* may be dependent upon *env* (41). Another study of transfected HeLa cells demonstrated that expression of large amounts of Vpu relative to CD4 could result in decreased levels of CD4 in the absence of other viral genes products including Env (19). Our data show that *env* is not essential for the CD4-modulatory function of *vpu* during

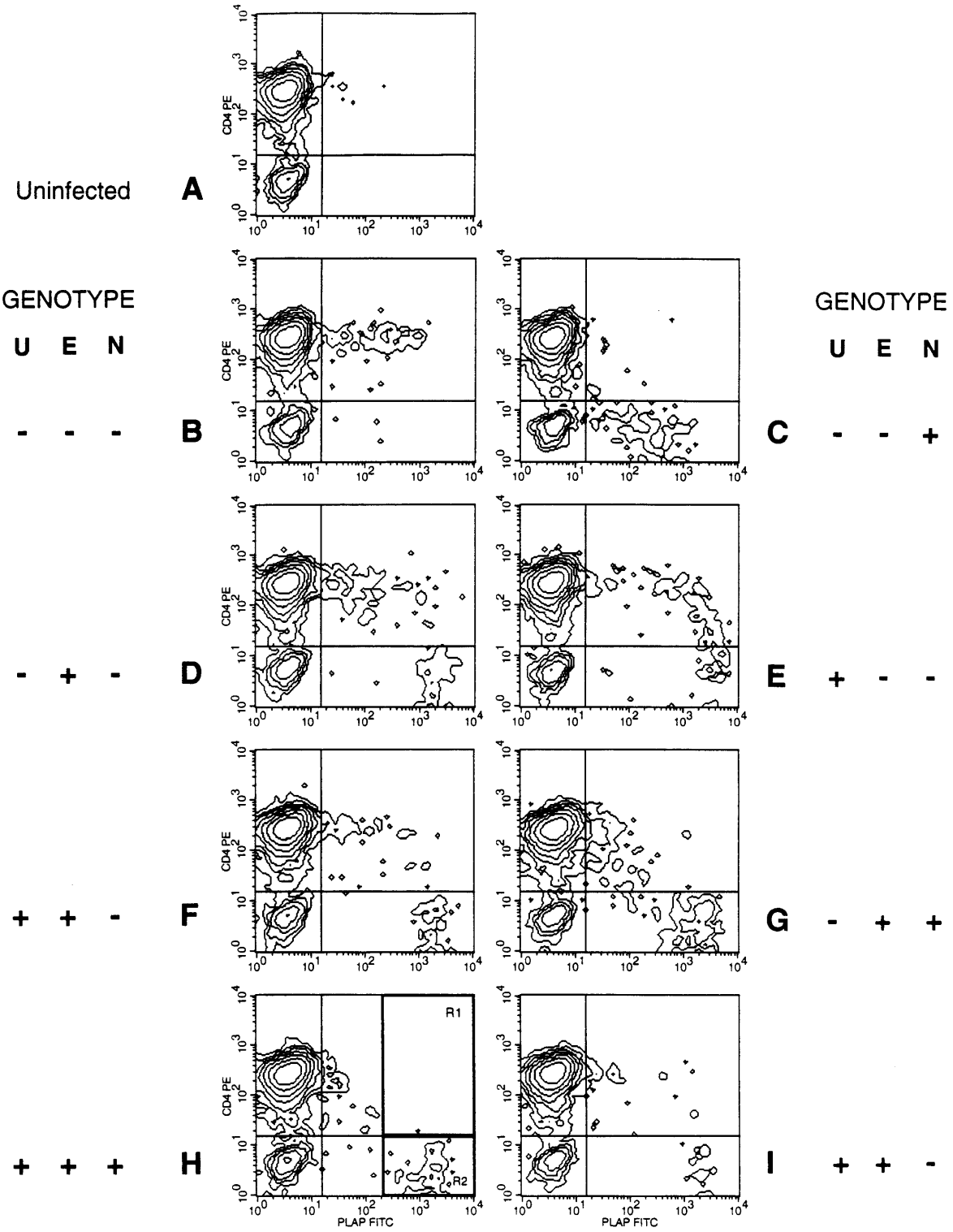

FIG. 5. Flow-cytometric analysis of Jurkat cells infected with different viral constructs analyzed 3 days after infection. Analysis of other time points, both earlier and later, showed similar trends of PLAP and CD4 staining (data not shown). Data from day 3 are illustrated because they show the maximum number of PLAP$^+$ cells for *env*$^-$ constructs, which generally begins to decrease after this time point. Contour plots of infected cells are presented in a logarithmic scale to clearly illustrate relatively low-frequency events. (A) Uninfected Jurkat cells; (B through I) infections of Jurkat cells by HIV constructs with the indicated genotype. All samples shown are stained by indirect immunofluorescence with antibodies against CD4 and PLAP. The single-letter code U (*vpu*), E (*env*), and N (*nef*) represents the CD4-modulatory genotype of the virus used in each experiment.

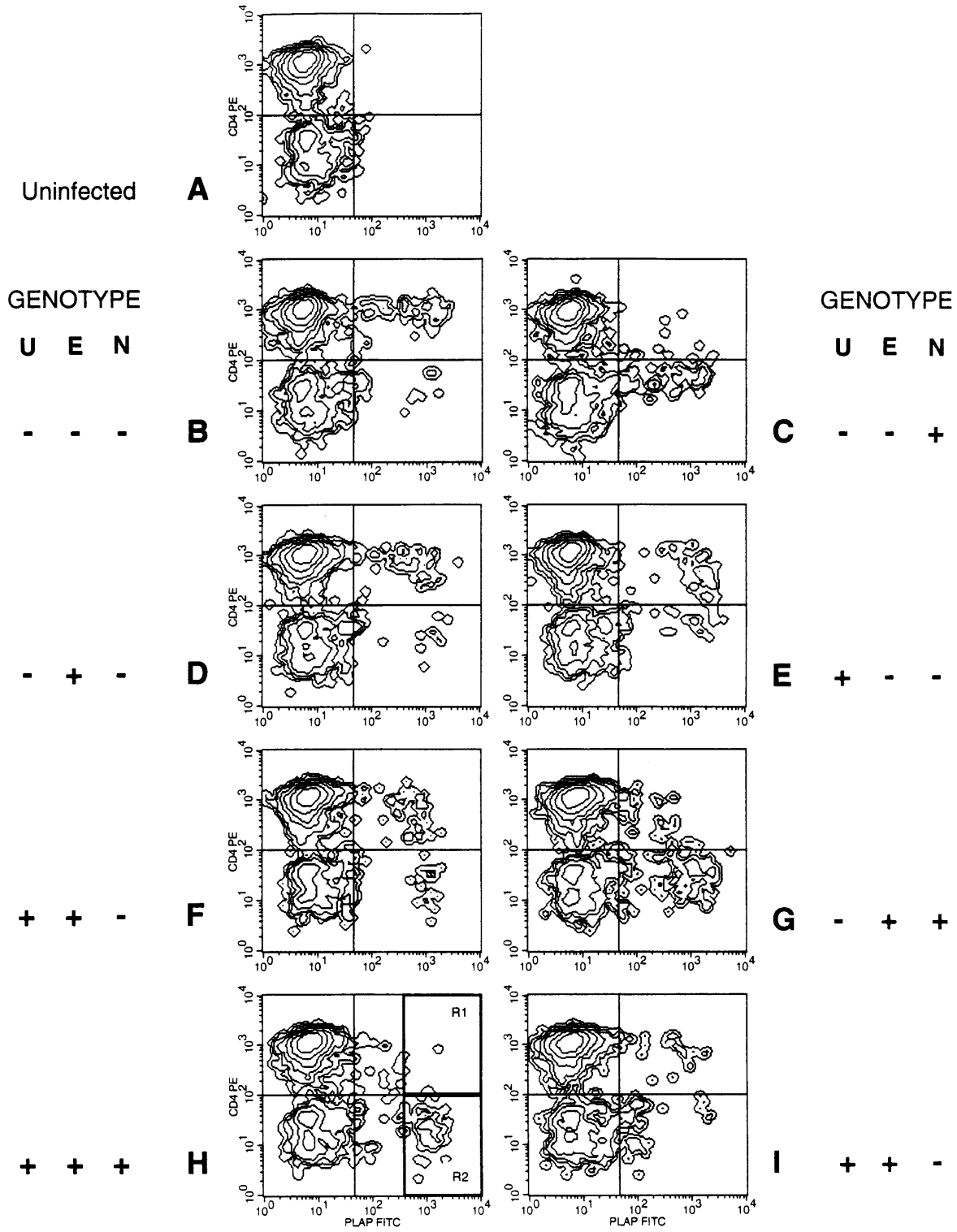

FIG. 6. Flow-cytometric analysis of PBMC infected with different viral constructs analyzed 3 days after infection. Analysis of other time points, both earlier and later, showed similar trends of PLAP and CD4 staining (data not shown). Data from day 3 are illustrated because they show the maximum number of $PLAP^+$ cells for env^- constructs, which generally begins to decrease after this time point. Contour plots of infected cells are presented in a logarithmic scale to clearly illustrate relatively low-frequency events. (A) Uninfected PBMC; (B through I) infections of PBMC by HIV constructs with the indicated genotype. All samples shown are stained by indirect immunofluorescence with antibodies against CD4 and PLAP. The single-letter code U (*vpu*), E (*env*), and N (*nef*) represents the CD4-modulatory genotype of the virus used in each experiment.

TABLE 1. CD4 down-modulation ratio on day 3 of infection[a]

Virus	Genotype	Jurkat			PBMC		
		% $CD4^{lo}$	% $CD4^{hi}$	Down-Mod ratio	% CD4lo	% CD4hi	Down-Mod ratio
HXBePLAP N^-	$U^-E^-N^-$	0	100	0.00	10	90	0.11
HXBePLAP	$U^-E^-N^+$	97	3	36.00	90	10	9.43
HXBnPLAP	$U^-E^+N^-$	67	33	2.00	20	80	0.25
HXBnPLAP U^+E^-	$U^+E^-N^-$	41	59	0.68	25	75	0.33
HXBnPLAP U^+	$U^+E^+N^-$	77	23	3.42	33	67	0.48
HXBnPLAP IRES-N^+	$U^-E^+N^+$	98	2	40.25	91	9	9.71
HXBnPLAP U^+IRES-N^+	$U^+E^+N^+$	100	0	>53	91	9	9.84
HXBnPLAP U^+IRES-N^-	$U^+E^+N^-$	71	29	2.50	21	79	0.27

[a] See flow cytometry plots in Fig. 5H and 6H for definition of region 1 (R1) and region 2 (R2) for Jurkat cells and PBMC, respectively. [R2/(R1 + R2)] × 100% = %$CD4^{lo}$, representing the percentage of all $PLAP^{hi}$ cells which have down-modulated CD4. [R1/(R1 + R2)] × 100% = %$CD4^{hi}$, representing the percentage of all $PLAP^{hi}$ cells which have not down-modulated CD4. %$CD4^{lo}$/%$CD4^{hi}$ = down-modulation (Down-Mod) ratio.

infection of human T cells, indicating that the levels of Vpu produced during infection are sufficient to influence endogenous levels of cell surface CD4. While the CD4-modulatory function of *vpu* is not dependent upon the expression of *env*, it is still likely that the ability of Env to reach the cell surface will depend upon the ability of Vpu to degrade nascent CD4 molecules and thereby inhibit the formation of CD4-gp160 aggregates.

These studies make it clear that HIV has evolved three independent mechanisms to ensure that CD4 levels in infected cells are efficiently reduced. When working together, these three viral genes achieve what we estimate is virtually complete depletion of cell surface CD4. This focus on CD4 regulation implies that the down-modulation of CD4 should be critical for the optimal fitness or perhaps the survival of the virus in vivo. Despite this presumed importance, the true function of this phenomenon remains speculative. It is possible that CD4 down-modulation enhances the fitness of newly emerging viruses. For example, receptor down-modulation may be important in enhancing virus particle release or preventing receptor incorporation into particles, which could result in aggregation of virions or functional inactivation of the viral envelope. These mechanisms have precedent in other viral systems, e.g., influenza virus, in which inactivation of the viral receptor by neuraminidase is critical for release of infectious virus particles (21, 33). Because the CD4 molecule is involved in T-cell signal transduction, down-modulation may have functional sequelae in the infected T cell. It has been suggested that down-modulation of CD4 may cause the release of the Src-like tyrosine kinase, Lck, which normally associates with the cytoplasmic tail of CD4 (1). Release of this molecule could lead to T-cell activation, which in turn might improve the cellular environment for viral replication. Another hypothesis suggests that receptor down-modulation could protect an infected cell from a CD4-mediated pathway toward apoptosis (2). Lastly, it has been suggested that CD4 down-modulation is a mechanism which the virus uses to avoid superinfection toxicity (3). These hypotheses are by no means inconsistent with one another; therefore, further studies must be done to test the validity of any one.

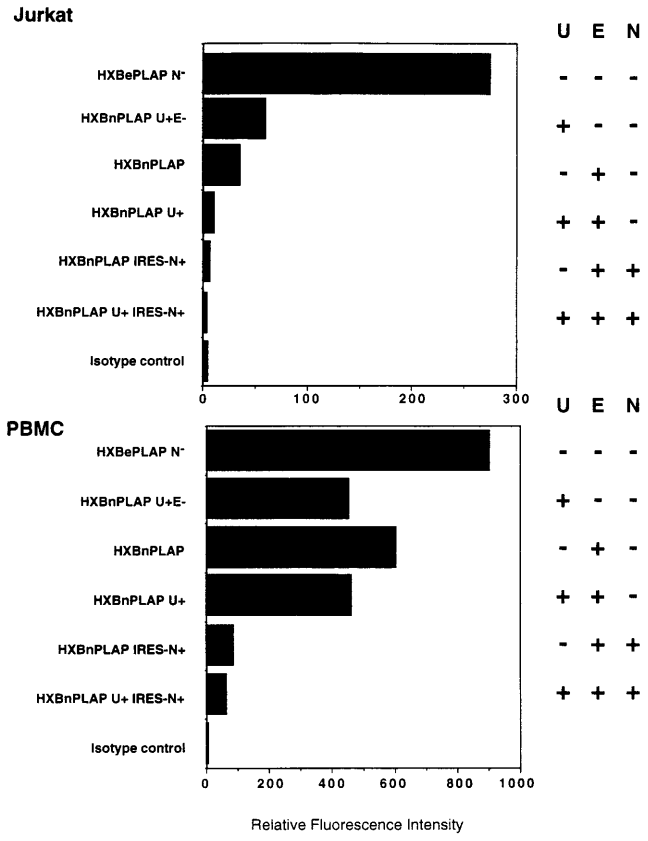

FIG. 7. Average CD4-associated fluorescence intensity of $PLAP^{hi}$ cells for cells infected with the designated viruses. $PLAP^{hi}$ cells are those that stain greater than 50-fold over background levels by flow cytometry and can be found within region R1 or R2, as illustrated in Fig. 5H and 6H. All cells which fall into either of these regions were included in this analysis. The isotype control analysis shown was performed with an anti-trinitrophenol mouse immunoglobulin G2a antibody to determine background fluorescence levels.

In apparent contradiction to in vitro studies, DNA PCR analysis on PBMC from HIV-infected patients finds that most proviral DNA is associated with $CD4^+$ cells (30). The reasons for this discrepancy are unclear; however, several explanations are possible. Recent studies have estimated that productively infected T lymphocytes survive only 2 days following infection in vivo (14, 38). The inability to find proviral DNA in $CD4^-$ cells may be a result of the short average lifetime of a productively infected cell. In contrast, cells infected with defective or nonproductive virus are likely to be more long-lived. Thus, DNA PCR of peripheral blood cells may provide a biased measure mainly of cells which have been infected with defective or nonproductive virus and, consequently, would maintain high levels of CD4. If the short half-life of infected cells is due largely to the effects of a highly efficient cytotoxic T-cell response, one would predict that $CD4^-$ HIV^+ cells could be detected at a higher frequency during acute infection before a specific anti-HIV response has developed. Such a hypothesis has yet to be tested.

An alternative explanation of why $CD4^-$ HIV^+ cells have not been found in patients is that CD4 down-modulation may be evident only during active infection of T cells in the peripheral lymphoid organs. Productively infected cells may preferentially home to lymphoid organs following cellular and viral activation. Since HIV does not replicate in the quiescent T cells and since the T cells found circulating in the peripheral blood are not normally in an activated state, perhaps the bulk of productive infection and consequent CD4 down-modulation are evident primarily in the peripheral lymphoid organs. This hypothesis would predict that $CD4^-$ HIV^+ cells may be found in lymph nodes. Future studies on viral regulation of CD4 should be directed toward understanding the functional consequences of CD4 down-modulation for the virus and T cells and understanding how this process may be important in the pathogenesis of AIDS.

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