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Oscillation of the Human Immunodeficiency Virus Surface Receptor Is Regulated by the State of Viral Activation in a CD4$^+$ Cell Model of Chronic Infection

SALVATORE T. BUTERA,[1]* VICTOR L. PEREZ,[1]† BEI-YU WU,[2] GARY J. NABEL,[2] AND THOMAS M. FOLKS[1]

Retrovirus Diseases Branch, Division of Viral and Rickettsial Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333,[1] and Departments of Internal Medicine and Biological Chemistry, Howard Hughes Medical Institute, University of Michigan Medical Center, Ann Arbor, Michigan 48109[2]

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We have developed a unique physiologic model of chronic human immunodeficiency virus type 1 (HIV-1) infection, OM-10.1, clonally derived from infected HL-60 promyelocytes and harboring a single integrated provirus. Unlike other models of chronic infection, OM-10.1 cultures remain CD4$^+$ under normal culture conditions, during which <10% of the cells constitutively express HIV-1 proteins. However, when treated with tumor necrosis factor alpha (TNF-α), OM-10.1 cultures dramatically increased (>35-fold) HIV-1 expression and rapidly down-modulated surface CD4, as >95% of the cells became HIV-1$^+$. The complete loss of surface CD4 following viral activation was neither associated with apparent cytopathicity nor due to a decline of available CD4 mRNA. There was, however, a temporal association between CD4 down-modulation and the accumulation of intracellular HIV-1 gp160/120; in addition, intracellular CD4-gp160 complexes were identifiable in OM-10.1 cell lysates at time points following TNF-α induction after surface CD4 was no longer detectable. Surface CD4 expression by OM-10.1 cells returned once viral activation ceased and could be repeatedly oscillated upon HIV-1 reactivation. Furthermore, inhibition of protein kinase activity following maximal TNF-α stimulation of OM-10.1 cells quickly returned activated HIV-1 to a state of latency, as evidenced by an accelerated return of surface CD4. These results with the new OM-10.1 cell line demonstrate that CD4 surface expression can be maintained during chronic infection and is critically dependent on the state of viral activation, that CD4-gp160 intracellular complexing is involved in CD4 down-modulation, and that protein kinase pathways not only function in the primary induction of latent HIV-1 but also are required for maintaining the state of viral activation.

There is normally an initial period of clinical latency during the course of AIDS which extends for several years after infection with human immunodeficiency virus type 1 (HIV-1) (10, 36). During this period, HIV-1 may exist as a dormant or nonexpressing provirus in a reservoir population of chronically infected cells (23). In vitro studies with chronically HIV-1 infected tumor cell lines have shown that activation of the dormant provirus occurs when the correct stimuli are encountered (4, 11–13). Theoretically, a similar activation occurs with chronically infected cells in vivo and results in CD4 down-modulation, production of viral progeny, and cytopathic sequelae (10).

Because the CD4$^+$ T lymphocyte is the in vivo reservoir for HIV-1 (35, 40), CD4 surface expression apparently can be maintained during viral latency and expression of the virus is necessary to cause CD4 down-modulation. During HIV-1 expression, intracellular HIV-1 gp160/120-CD4 complexing (7, 25, 27) and disruption of CD4 transcription (22, 39) or translation (16, 47) have been observed to explain this effect on surface CD4 levels. In addition, intracellular HIV-1 gp160/120-CD4 complexing has been implicated by some as a mechanism of viral cytopathicity (22, 27), while other investigators have shown the cytopathic effect to be dependent on the level of CD4 expression by the target cells (28, 38).

Although these facts are well established, it remains uncertain why CD4 expression is lost from the surface of chronically HIV-1 infected cell lines, since they constitutively express only minimal amounts of HIV-1 proteins (4, 13, 23, 42). Down-modulation of surface CD4 may be the mechanism by which these cell lines escaped the cytopathic effects of HIV-1 infection. Nonetheless, the inability of chronically infected cell lines to maintain surface CD4 expression has made them less than ideal models of HIV-1 latency and unusable systems for exploring the molecular mechanisms involved in CD4 modulation by HIV-1.

Here we describe a unique chronically infected promyelocytic clone, OM-10.1, which, unlike other chronically infected tumor cell lines, remains CD4$^+$ until HIV-1 activation. At this point, the cells undergo a time-dependent loss of surface CD4 concomitant with increasing viral expression. This loss of surface CD4 is not associated with a decrease of CD4-specific mRNA, and intracellular CD4 remains detectable as a complex with HIV-1 gp160. Furthermore, when the activating stimulus is removed from OM-10.1 cultures, HIV-1 returns to a state of latency and surface CD4 reappears. Continued HIV-1 expression by these cells appears to be critically dependent on protein kinase activity. Thus, HIV-1 activation in the chronically infected OM-10.1 cell line describes the first synchronized study of HIV-1 induced CD4 modulation and provides a controllable system with which to investigate additional virus-host interactions.

* Corresponding author.
† Present address: Department of Surgery, Boston University, Boston, MA 02118.

MATERIALS AND METHODS

Cell lines and culture conditions. The derivation of the OM-10.1 cell line has previously been described (3). Briefly, parental HL-60 promyelocytes (6) were first cocultured with HIV-1 (LAV)-infected, γ-irradiated A3.01 T lymphocytes. After several weeks, the remaining cells were cloned and an infected, HL-60-derived clone, designated OM, was identified and characterized (3). The OM-10.1 cell line was derived by further subcloning of the OM culture. All cell lines used in this study were propagated in RPMI 1640 basal medium (GIBCO, Grand Island, N.Y.) containing 10% fetal bovine serum, 2 mM glutamine, and 1% Pen-Strep (GIBCO) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Mycoplasma-free OM-10.1 cell cultures were established and, in general, showed no biologic differences compared with the original OM-10.1 cultures.

HIV-1 expression was induced from OM-10.1 cells by treatment with either 20 U of recombinant tumor necrosis factor alpha (TNF-α; Genzyme Corp., Boston, Mass.) per ml or 0.1 μM phorbol-12-myristate-13-acetate (PMA; Sigma Chemical Co., St. Louis, Mo.). All induction experiments were performed at a concentration of 5×10^5 cells per ml for a maximum of 36 h. In experiments in which protein kinase inhibitors were used, H7 (1-[5-isoquinolinesulfonyl]-2-methylpiperazine hydrochloride) and HA1004 (*N*-[2-guanidinoethyl]-5-isoquinolinesulfonamide hydrochloride) (Seikagaku America, Inc., Rockville, Md.) were added to a final culture concentration of 16.67 μM.

Quantitation of HIV-1 expression. HIV-1 expression was quantitated by reverse transcriptase (RT) enzymatic activity. As described previously (46), 5 μl of cell-free culture supernatant was added to 25 μl of RT cocktail containing polyadenylate, oligo(dT) (Pharmacia Fine Chemicals, Piscataway, N.J.), $MgCl_2$, and [α-^{32}P]dTTP (Amersham Corp., Arlington Heights, Ill.) in duplicate wells of a U-bottom 96-well plate and incubated at 37°C for 2 h. Five microliters of this mixture was then spotted onto DE81 ion-exchange chromatography paper (Whatman International, Maidstone, England), air dried, washed five times in 1.5× SSC (1× SSC is 15 mM sodium citrate plus 150 mM NaCl), and washed twice more with 95% ethanol. The paper spots were then dried, and the remaining radioactivity was quantitated in a Beckman LS7000 scintillation counter. HIV-1 expression was also quantitated by p24 antigen-specific enzyme-linked immunosorbent assay (ELISA) (Maryland Medical Laboratories, Inc., Baltimore, Md.) of cell culture supernatant as instructed by the manufacturer.

Cells were examined directly for HIV-1 expression by immunofluorescence assay, using a fluorescein isothiocyanate-conjugated, partially purified, polyclonal anti-HIV-1 antiserum (prepared from serum of AIDS patients and provided by S. McDougal, Centers for Disease Control) (3). Cells were first attached to AdhesioSlides (MM Developments, Ottawa, Ontario, Canada) and then fixed in 4°C acetone. Cell fields were blocked with 5% normal goat serum and then reacted with a 1:50 anti-HIV-1 antiserum solution; both were prepared with the buffer used for flow cytometric analysis (see below). After 45 min at room temperature, the slides were rinsed with phosphate-buffered saline (PBS; 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 150 mM NaCl, pH 7.4) and examined by UV microscopy.

Determination of cell surface antigen expression. In preparation for flow cytometry, cells were washed once in cold PBS and once in cold PBS containing 2% normal human AB^+ serum, 0.2% sodium azide, and 0.1% bovine serum albumin (FACS buffer). Cell surface expression of myeloid-specific antigens (CD13, CD4, MY8, CD33, and CD34), HLA class I/II, and transferrin receptor (CD71) was determined by flow cytometry as described previously (3), using commercially available reagents. For the determination of CD4 expression, cell pellets were resuspended in 75 μl of FACS buffer containing 12.5 μl of anti-CD4 monoclonal antibody (OKT4; Ortho, Raritan, N.J.) or, as a negative control, anti-CD8 monoclonal antibody (OKT8; Ortho). After 1 h at 4°C, cells were washed twice with FACS buffer and resuspended in 100 μl of FACS buffer containing 2 μl of phycoerythrin-conjugated goat-anti-mouse immunoglobulin G antibody (Tago, Burlingame, Calif.). After an additional 1 h at 4°C, cells were washed twice in FACS buffer and fixed at 4°C with PBS containing 1% paraformaldehyde in preparation for analysis on a Becton Dickinson FACScan system. Additionally, culture supernatants were tested by ELISA for soluble CD4 shed from the cell surface, according to the manufacturer's protocol (T-Cell Sciences, Cambridge, Mass.).

Southern blotting of OM-10.1 genomic DNA. A total of 5×10^7 cells were washed twice with cold PBS, lysed in 20 mM Tris buffer containing 5 mM EDTA and 5 mg of sodium dodecyl sulfate per ml, and digested with 100 μg of proteinase K per ml for 5 h (30). After phenol extraction, the final aqueous phase was precipitated with ethanol, resuspended in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.6), and quantitated by A_{260}. Approximately 25 μg of total genomic DNA was digested overnight with an excess of *Eco*RI and then separated through a 0.8% agarose gel. The digested DNA was transferred to a nylon membrane and then hybridized with a ^{32}P-labeled, *Eco*RI-*Kpn*I 0.5-kb HIV-1 fragment of pBenn-7 (17) for the 5′ viral genome and associated host flank. Bands of hybridization were detected by autoradiography.

Immunoblotting of cellular lysates. Cells were washed twice in PBS and lysed in a detergent buffer as described previously (45). Total protein of each lysate was quantitated by the bicinchoninic acid method as instructed by the manufacturer (Pierce, Rockford, Ill.). One hundred micrograms of total cellular protein was separated by polyacrylamide gel electrophoresis under reducing conditions and then electrotransferred to nitrocellulose as described previously (44). HIV-1-specific proteins were detected by using a 1:100 dilution of pooled sera from AIDS patients, and CD4 was detected by using a 1:1,000 dilution of rabbit anti-human CD4 polyclonal antibody (provided by S. McDougal, Centers for Disease Control). Proteins bound by these primary antibodies were resolved with ^{125}I-labeled protein G (10^5 cpm/ml) and autoradiography. Initial blocking, incubations, and washes were performed as described previously (45).

The same cellular lysates were also used for combined immunoprecipitation and immunoblot analysis. Precleared cellular protein (150 μg) was immunoprecipitated with either anti-CD4 (OKT4) or anti-gp120 (DuPont, Billerica, Mass.) monoclonal antibodies conjugated to protein A-Sepharose (Sigma). The precipitated proteins were eluted and separated by polyacrylamide gel electrophoresis under reducing conditions. After transfer to nitrocellulose, HIV-1 proteins specifically extracted by immunoprecipitation were detected by immunoblotting as described above.

Northern (RNA) blotting of total cellular RNA. Following the appropriate culture conditions, cells were washed twice with cold PBS and then lysed in a guanidine thiocyanate buffer as described previously (43). Total RNA was purified

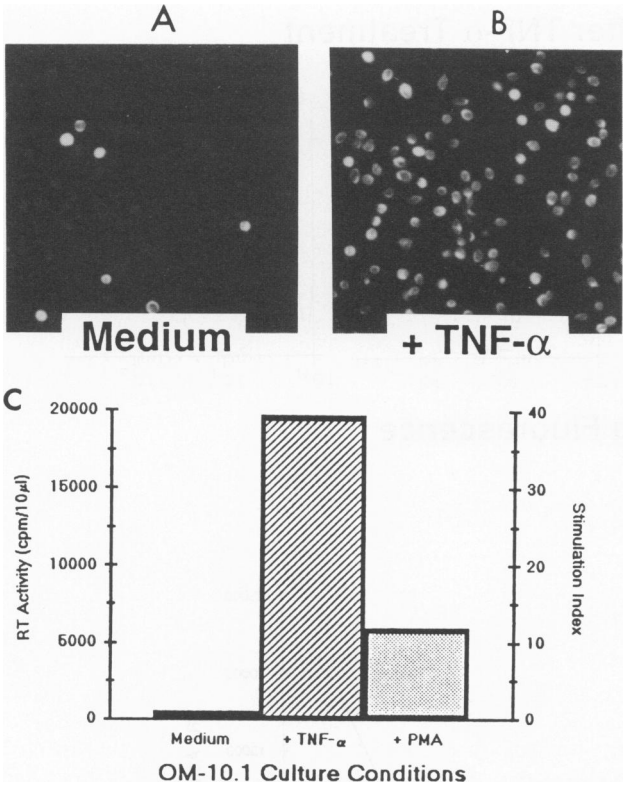

FIG. 1. Constitutive and induced HIV-1 expression by OM-10.1 cells. (A and B) Direct immunofluorescence assay of OM-10.1 cells maintained in medium (A) and treated with TNF-α for 36 h (B) for HIV-1-specific protein expression. OM-10.1 cells constitutively contain approximately 5% HIV-1$^+$ cells (A), which increase to virtually 100% HIV-1$^+$ upon treatment with TNF-α. (C) Induction of HIV-1 expression by OM-10.1 cells after 36-h treatment with TNF-α (20 U/ml) or PMA (0.1 μM), as determined by RT activity of culture supernatants. Data are presented both as actual RT activity and as a stimulation index [(RT counts per minute of treated culture)/(RT counts per minute of medium culture)]. Results are representative of more than 10 separate trials.

by cesium chloride gradient ultracentrifugation and quantitated by A_{260}.

Ten micrograms of total purified RNA was denatured and electrophoresed through a 0.8% agarose gel containing formaldehyde. The separated RNA was then transferred to a nylon membrane (Hybond; Amersham) and probed in a 50% formamide hybridization buffer overnight at 42°C. For CD4 mRNA detection, a 2-kb cDNA probe (29), labeled with [α-^{32}P]dCTP by random priming (Amersham), was used. After hybridization, the membranes were washed twice at 57°C in 2× SSC plus 1% sodium dodecyl sulfate in preparation for autoradiography.

RESULTS

Characterization and induction of the OM-10.1 clone. The OM-10.1 clone was derived by limit diluting the cells which survived an acute HIV-1 (LAV) infection of HL-60 promyelocytes (3). After clonal expansion, <10% of OM-10.1 cells were HIV-1$^+$ by direct immunofluorescence (Fig. 1A), and a low level of RT activity was detected in OM-10.1 culture supernatants (Fig. 1C). By flow cytometric analysis, OM-10.1 cells expressed levels of myeloid-specific surface antigens (CD13, CD14, MY8, CD33, and CD34), HLA-A/B/C, HLA-DR, and CD71 (transferrin receptor) similar to those expressed by the parental uninfected HL-60 cells (data not shown).

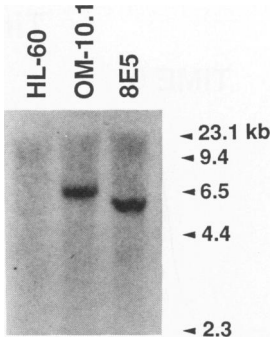

FIG. 2. Southern blot analysis of *Eco*RI-restricted total genomic DNA from HL-60, OM-10.1, and 8E5 cells. The restricted DNA was probed with a ^{32}P-labeled, *Eco*RI-*Kpn*I 0.5-kb HIV-1 fragment for the 5′ region of the HIV-1 genome and a variable region of host flank (dependent on the viral integration site). The molecular size markers are based on the migration of lambda phage DNA restricted by *Hin*dIII digestion.

Because of their low constitutive HIV-1 expression, OM-10.1 cultures were treated with either TNF-α or PMA and then evaluated for induced HIV-1 expression. As measured by RT activity of culture supernatants (Fig. 1C), TNF-α treatment of OM-10.1 cells increased virus expression almost 40-fold, while PMA treatment resulted in a 12-fold increase within 36 h. The induction of HIV-1 expression by OM-10.1 cultures was even more dramatic when quantitated by p24 ELISA (data not shown), in which HIV-1 levels after TNF-α treatment rose 1,000-fold over background in some experiments. Directly associated with the increased HIV-1 expression, virtually 100% of the cells from TNF-α-treated OM-10.1 cultures were HIV-1$^+$ by immunofluorescence (Fig. 1B).

The clonal origin of the OM-10.1 cell line was confirmed by Southern analysis. Total genomic DNA was restricted by *Eco*RI digestion and probed for the 5′ region of the HIV-1 provirus and associated host genomic flank (Fig. 2). The DNA from OM-10.1 cells produced a distinct single band of approximately 6.5 kb when analyzed in this manner, whereas no bands were visible from HL-60 DNA. The DNA from 8E5 cells, a cloned T-cell line harboring a single HIV-1 provirus (14), also produced a single band following hybridization. These results verified the clonal derivation of the OM-10.1 line and established that these cells harbor a single integrated HIV-1 provirus.

Pattern of surface CD4 expression by OM-10.1 cells. Unexpectedly, most (>90%) uninduced OM-10.1 cells maintained surface CD4 expression at a level similar to that of uninfected parental HL-60 cells (Fig. 3A). A small percentage of CD4$^-$ cells was evident in the uninduced OM-10.1 cultures, apparently due to the <10% HIV-1$^+$ population observed by direct immunofluorescence. However, upon TNF-α treatment, surface CD4 expression by OM-10.1 cultures began to decrease progressively until >95% of the cells were CD4$^-$ after 36 h. TNF-α treatment of uninfected parental HL-60 cells did not alter the level of surface CD4 expression (Fig. 3A).

During TNF-α induction of OM-10.1 cultures, an inverse

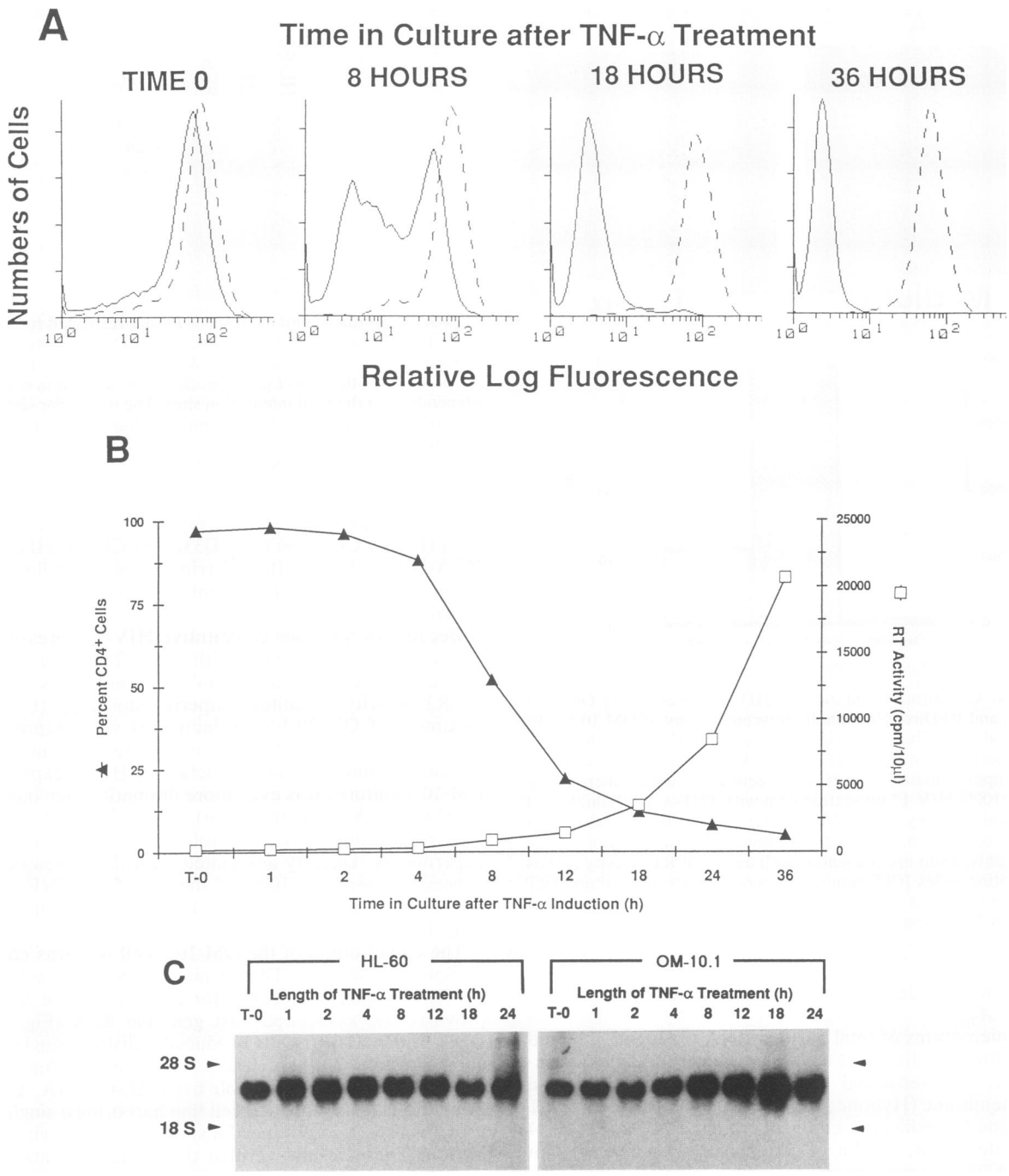

FIG. 3. Surface CD4 expression by uninduced and TNF-α-treated OM-10.1 and HL-60 cells. (A) Comparison of CD4 surface expression by OM-10.1 (——) and HL-60 (– – –) cells during the kinetic course of TNF-α treatment. Histogram profiles from flow cytometric analysis are presented, and the level of surface CD4 expression is determined by the relative degree of log fluorescence intensity (abscissa of each histogram). (B) The kinetic association between TNF-α-induced down-modulation of surface CD4 on OM-10.1 cells and the rise of RT activity in the culture supernatants. The percentage of $CD4^+$ cells was determined at each time point by gating in flow cytometry against a negative control (CD8) peak. RT activity is reported as counts per minute per 10 μl of assay reaction mixture. (C) Northern blot analysis of 10 μg of total RNA from OM-10.1 and HL-60 cells for CD4-specific mRNA during a time course of TNF-α treatments.

kinetic relationship was observed between the expression of surface CD4 and HIV-1-associated RT activity (Fig. 3B). A reduction in the percentage of $CD4^+$ OM-10.1 cells occurred within 4 h of TNF-α addition and declined rapidly to <5% by 24 to 36 h. Inversely, HIV-1 expression by TNF-α-treated OM-10.1 cells was detectable in culture supernatants at 8 h, and RT activity continued to rise for the remainder of the 36-h kinetic study (Fig. 3B). Because the anti-CD4 monoclonal antibody used in analysis, OKT4, defines a CD4 epitope not obstructed by HIV-1 gp120 binding (22), HIV-1 activa-

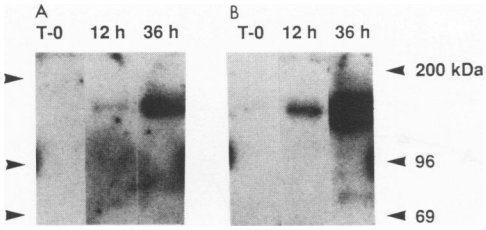

FIG. 4. Combined immunoprecipitation and immunoblot analysis of OM-10.1 cell lysates for the detection of intracellular HIV-1 gp160/120-CD4 complexes. Lysates were used from uninduced OM-10.1 cells (T-0) and after 12 h or 36 h of TNF-α treatment. These lysates were immunoprecipitated with either anti-CD4 (OKT4) (A) or anti-gp120 (B) monoclonal antibodies and then immunoblotted for HIV-1-specific proteins with pooled AIDS sera. Molecular weight markers are as indicated.

tion from OM-10.1 cells results in a true loss of surface CD4 expression.

The mechanism of CD4 down-modulation in TNF-α-treated OM-10.1 cultures was not due to a reduction in CD4 mRNA (Fig. 3C). OM-10.1 and HL-60 cells were treated with TNF-α for various lengths of time, and then 10 μg of total RNA from these cultures was analyzed for the level of CD4-specific mRNA. After ethidium bromide staining, confirmation of the RNA quantity and integrity was obtained by UV visualization of the ribosomal bands (data not shown). As shown in Fig. 3C, OM-10.1 cells transcribed as much CD4-specific mRNA as did HL-60 cells, and the levels did not decline during the course of TNF-α treatment. The possibility that HIV-1 expression caused a shedding of surface CD4 was ruled out by an ELISA for soluble CD4 in which both TNF-α-treated OM-10.1 and HL-60 culture supernatants were negative (data not shown). Furthermore, the continued presence of intracellular CD4 protein in OM-10.1 cells was verified by immunoblot analysis, even after 36 h of TNF-α treatment (data not shown). These results suggested that surface CD4 down-modulation in HIV-1-expressing OM-10.1 cells involved intracellular complexing.

Molecular association between HIV-1 gp160/120 and CD4. In TNF-α-treated OM-10.1 cells, a direct correlation was observed between the intracellular level of HIV-1 gp160/120 and the loss of surface CD4 expression. By immunoblot analysis (data not shown), HIV-1 proteins, including gp160/120, were weakly detected in unstimulated $CD4^+$ OM-10.1 cells. However, the induced expression of HIV-1 gp160/120 was evident in OM-10.1 cells after the first 4 to 6 h of TNF-α treatment and was coincident with the observed surface CD4 down-modulation. Prolonged TNF-α treatment of OM-10.1 cells resulted in the accumulation of all HIV-1 proteins, with gp160 being in 20- to 50-fold excess of gp120, and a complete absence of surface CD4 during the 36-h induction period. Because of the observed temporal association between the production of HIV-1 gp160/120 and the down-modulation of surface CD4, we attempted to verify that intracellular complexing of these proteins was a contributing factor in the CD4 down-modulation of TNF-α treated OM-10.1 cultures.

By immunoprecipitating OM-10.1 lysates with an anti-CD4 monoclonal antibody (OKT4) and then immunoblotting for associated HIV-1 proteins (Fig. 4A), an intracellular HIV-1 gp160/120-CD4 complex was revealed at time points following TNF-α treatment (36 h) when surface CD4 was completely absent from these cells. Based on the migration of the molecular weight standards, it appeared most likely that gp160 was the major viral protein complexed with CD4. Furthermore, the HIV-1 gp160 band was a specific product of the anti-CD4 immunoprecipitation because no other HIV-1 proteins were detected by immunoblotting with pooled AIDS sera (data not shown). To verify our identification of HIV-1 gp160 in this procedure, the same lysates were immunoprecipitated with an anti-gp120 monoclonal antibody and then immunoblotted with pooled AIDS sera (Fig. 4B). This combination produced a strong single band of the same electrophoretic mobility as that resolved by immunoblotting after anti-CD4 immunoprecipitation. In this procedure, gp160 was precipitated in large excess of gp120 presumably because of its abundance in OM-10.1 cell lysates, as originally determined by standard immunoblot assay.

Reciprocal oscillations of CD4 and HIV-1 expression by OM-10.1 cells. To further confirm that the state of viral activation was controlling surface HIV-1 receptor expression in these chronically infected cells, OM-10.1 cultures were first treated with TNF-α for 36 h so that maximal CD4 down-modulation and HIV-1 activation occurred. TNF-α was then removed, and the OM-10.1 cells were maintained in normal culture medium and monitored for an additional 10 days (Fig. 5A). Three days after the removal of TNF-α, CD4 surface expression could again be detected on OM-10.1 cells as supernatant RT activity began to decline, without apparent cytopathicity. By day 6 after TNF-α removal, surface CD4 expression returned to near normal levels concurrent with a continued reversion of HIV-1 to an inactive state, as evidenced by the decline of RT activity. These OM-10.1 cultures regained the resting phenotype (>90% $CD4^+$ cells) by day 9 and were again induced by TNF-α treatment on day 10. Upon restimulation, down-modulation of surface CD4 and the rise of culture supernatant RT activity occurred with similar kinetics as in the first induction period.

We had previously observed that inhibition of protein kinase activity completely blocked TNF-α-induced HIV-1 activation in OM-10.1 cultures (2). Therefore, we investigated the involvement of intracellular protein kinase activity in maintaining HIV-1 activation and depressed CD4 expression during the 4- to 5-day period after removal of the TNF-α stimulus. OM-10.1 cells were treated for 36 h with TNF-α, washed, and then placed back into medium alone or into medium containing the protein kinase inhibitor H7 or an analog without inhibitory activity, HA1004 (20). These cells were then monitored for the reappearance of CD4 over several additional days in culture (Fig. 5B). In cells placed back into medium, a 4- to 5-day period before the return of surface CD4 to pretreatment levels was again observed. However, the addition of H7 dramatically altered the kinetics of CD4 return. Pretreatment CD4 levels were found to return with enhanced kinetics (2 days) after TNF-α removal. The pattern of CD4 return in HA1004-treated OM-10.1 cells was identical to that of the medium culture. Furthermore, CD4 remained undetectable on OM-10.1 cells when maintained in the continued presence of TNF-α during the 6-day culture.

DISCUSSION

In this report we describe a novel chronically HIV-1-infected promyelocytic cell clone, OM-10.1, capable of dramatically increasing the level of HIV-1 production in response to treatment with TNF-α or phorbol esters. This increase in viral expression is due to a direct increase in the percentage of cells in culture expressing HIV-1 proteins.

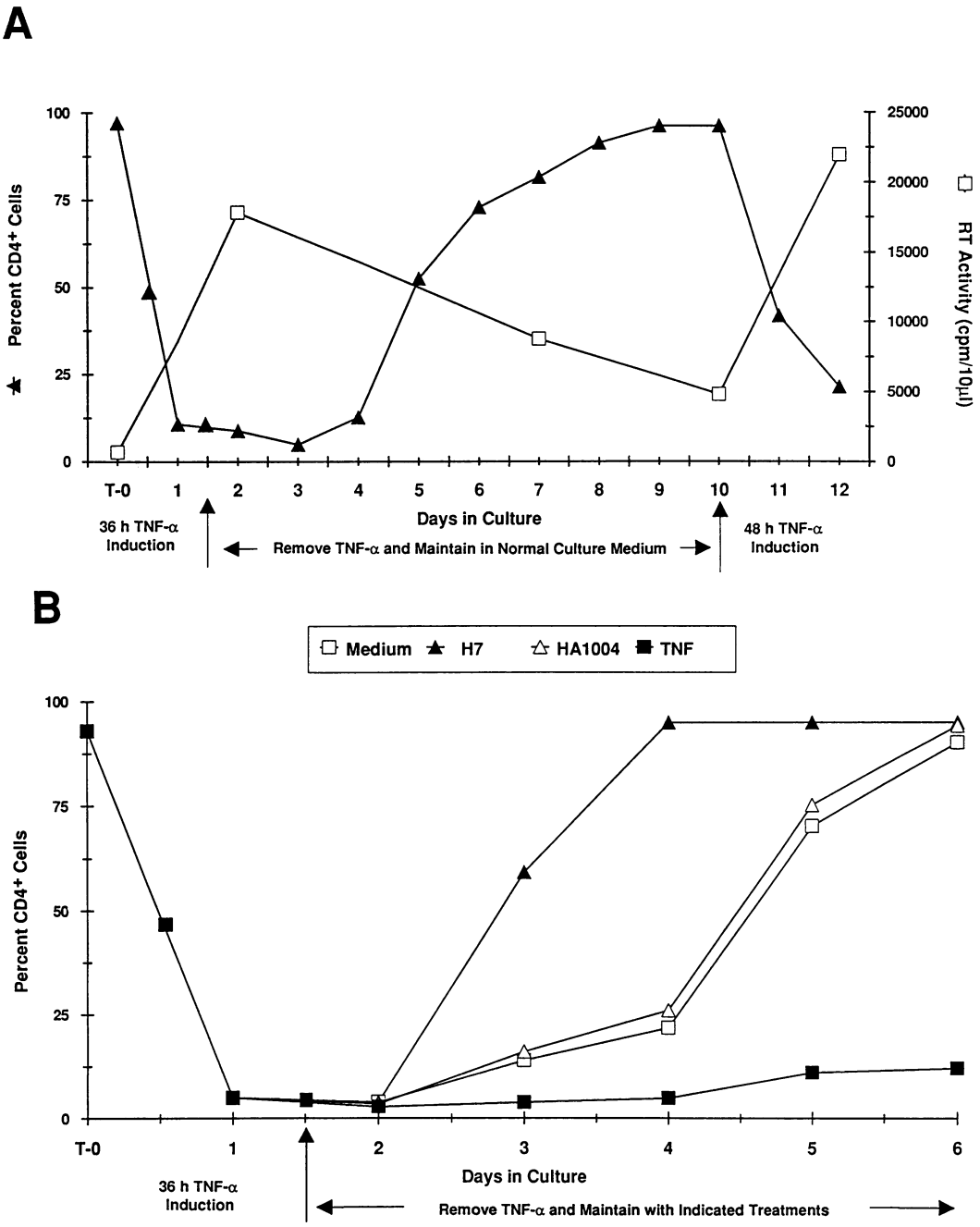

FIG. 5. Oscillations of HIV-1 production and CD4 expression by OM-10.1 cells dependent upon external stimuli. (A) OM-10.1 cells were cultured in the presence of TNF-α for 36 h; characteristically, this treatment resulted in induction of HIV-1 (as measured by RT activity of culture supernatants) and down-modulation of surface CD4. After 36 h, TNF-α was removed and the cells were maintained in normal medium. HIV-1 expression slowly declined and CD4 surface expression returned after an initial lag period of 2 to 3 days. When the same OM-10.1 culture was again exposed to TNF-α treatment (at day 10), the same sequence of events occurred. (B) OM-10.1 cells were treated for 36 h with TNF-α, after which the cells either were placed into medium alone or medium containing a protein kinase inhibitor (H7 or HA1004) or were maintained in the presence of TNF-α. The cultures were monitored for CD4 surface expression, as a measure of HIV-1 activation, over a period of 6 days.

However, unlike other cell models of chronic HIV-1 infection (4, 13, 42), OM-10.1 cells are able to maintain surface CD4 expression until viral activation occurs. CD4 down-modulation in OM-10.1 cells is regulated by the state of viral activation and results, at least in part, from intracellular HIV-1 gp160-CD4 complexing. In light of the major HIV-1 reservoir in vivo being a population of $CD4^+$ cells (35, 40), the fact that OM-10.1 cells remain $CD4^+$ until HIV-1 activation makes this line a new and important physiologic model of HIV-1 latency.

Intracellular HIV-1 gp160/120-CD4 complexing has been repeatedly suggested as a mechanism of surface CD4 down-modulation during HIV-1 expression (7, 25, 27). A recent study indicates that an intracellular association between

CD4 and HIV1 gp160 prevents transport of the complex through the endoplasmic reticulum as a consequence of the inefficient transport of gp160 itself (7). As predicted from acute infection models (16, 22, 25, 27, 39, 47), surface CD4 expression by OM-10.1 cells declines rapidly upon HIV-1 activation. We present evidence that this effect is due neither to a reduction of available CD4 mRNA, as previously shown in other cell systems (22, 39), nor to a shedding of CD4 from the cell surface. CD4 down-modulation by OM-10.1 cells occurred coordinately with HIV-1 gp160/120 expression and returned when HIV-1 reverted to a state of latency. Furthermore, intracellular CD4 remained detectable in OM-10.1 lysates during TNF-α-induced virus expression and was associated with HIV-1 gp160. Taken together, these observations with OM-10.1 cells support the hypothesis that CD4 down-modulation during HIV-1 expression results from intracellular complexing which prevents CD4 transport to the cell surface.

However, other mechanisms may be acting in conjunction with intracellular complexing to cause the CD4 modulation upon HIV-1 expression by OM-10.1 cells. The possibility exists that CD4 translation has also been altered upon HIV-1 expression (16, 47), a cytopathic feature of infection in cells of monocytic origin (16). Furthermore, expression of the HIV-1 *nef* regulatory gene product has recently been demonstrated to cause a loss of CD4 surface expression (15). It will be of continued interest to explore these alternative mechanisms and determine their relative contribution, in concert with intracellular complexing, during oscillation of the HIV-1 surface receptor in this synchronized cell model.

It has been suggested that intracellular gp160/120-CD4 complexing during HIV-1 expression participates in the cytopathic effect of this virus (22, 27), possibly by disrupting molecular flow through nuclear pores (27). In the OM-10.1 cell system, such complexing accounts for CD4 down-modulation; however, no cytopathicity is associated with viral expression by these cells. Additional reports demonstrate that the cytopathic effect of HIV-1 expression is related to the level of surface CD4 antigens (28, 38). Since HL-60 and OM-10.1 cells express levels of surface CD4 below that of most T cells, this relationship remains as a possible explanation for the lack of cytopathicity upon HIV-1 activation in OM-10.1 cells. Alternatively, OM-10.1 cells may lack additional surface structures, like cellular adhesion molecules, which are necessary for the HIV-1 cytopathic effect (21). We have determined that the absence of cytopathicity in OM-10.1 cultures is not due to an inherent defect in the virus expressed by these cells. Accordingly, supernatants from induced OM-10.1 cultures were capable of infecting A3.01 T-cell targets with similar efficiency and cytopathicity as the original LAV virus stock over three serial passages (2).

Because OM-10.1 cells remain $CD4^+$ until viral activation, the possibility exists for superinfection of nonproducing cells by virions released from the small percentage of cells constitutively expressing HIV-1. An acute superinfection of OM-10.1 cells is responsible for an accumulation of extrachromosomal HIV-1 DNA (1), as demonstrated in other HIV-1-infected cell systems (1, 32, 33, 37). Azidothymidine treatment of OM-10.1 cultures prevents the accumulation of unintegrated HIV-1 DNA (1) without affecting the activation of the integrated HIV-1 provirus by TNF-α (2, 34). We are currently examining the possibility that viral products transcribed from the extrachromosomal HIV-1 genome (41) may influence the integrated latent provirus in $CD4^+$ chronically infected cells.

Another important finding demonstrated here was that $CD4^+$, latently HIV-1-infected cells, at least of the myelomonocytic lineage, can undergo reciprocal oscillations of virus production and loss of CD4 expression without cytolysis. Once the external stimulus is removed, virus expression slowly ceases and CD4 antigen returns to the cell surface. This finding provides new insight into the possible activities of $CD4^+$ cells as a reservoir of HIV-1 (35, 40). Such cells in vivo may periodically begin to express HIV-1 as they encounter the right combination of extracellular stimuli. When that stimulus is removed, the cell may return to its $CD4^+$ phenotype and remain as a potential target for HIV-1 superinfection or reactivation. However, during this event the cell has contributed to a process that may permit maintenance and spread of HIV-1 to other susceptible targets.

Using the OM-10.1 cell line, we establish that protein kinase activity is essential for maintaining the state of HIV-1 activation. Protein kinase second messenger pathways also function in the activation of HIV-1 from latency, both in this (2) and in other cell systems (26). Because of the multiple pathways which require protein kinase activity, it is difficult to determine at which level protein kinases may be acting to regulate HIV-1 expression. HIV-1 activation in chronically infected cells can be mediated through an increased binding of the nuclear transcription factor NF-κB (9, 19, 31). Furthermore, the inhibitor of NF-κB must be phosphorylated to permit NF-κB release and migration to the nucleus (18). Therefore, inhibition of NF-κB release may restrict viral activation and return HIV-1 to a state of latency. However, additional protein kinase activities may directly phosphorylate the viral proteins themselves (5, 26) or permit the activity of viral regulatory proteins (24).

The OM-10.1 cell line presents a promising new reagent for use in addressing many aspects of HIV-1 activation from latency. Because of the pluripotential nature of promyelocytes (6, 8), the OM-10.1 cell line can also be used to further address the relationships between differentiation and HIV-1 expression. The availability of additional cell models of chronic HIV-1 infection will expand our ability to observe novel virus-host interactions and permit a more precise understanding of the events leading to the development of AIDS.

REFERENCES

1. **Besansky, N. J., S. T. Butera, S. Sinha, and T. M. Folks.** 1991. Unintegrated human immunodeficiency virus type 1 DNA in chronically infected cell lines is not correlated with surface CD4 expression. J. Virol. **65**:2695–2698.
2. **Butera, S. T.** Unpublished data.
3. **Butera, S. T., V. L. Perez, N. J. Besansky, W. C. Chan, B.-Y. Wu, G. J. Nabel, and T. M. Folks.** 1991. Extrachromosomal human immunodeficiency virus type-1 DNA can initiate a spreading infection of HL-60 cells. J. Cell. Biochem. **45**:366–373.
4. **Clouse, K. A., D. Powell, I. Washington, G. Poli, K. Strebel, W. Farrar, P. Barstad, J. Kovacs, A. S. Fauci, and T. M. Folks.** 1989. Monokine regulation of human immunodeficiency virus-1 expression in a chronically infected human T cell clone. J. Immunol. **142**:431–438.
5. **Cochrane, A. W., E. Golub, D. Volsky, S. Ruben, and C. A. Rosen.** 1989. Functional significance of phosphorylation to the human immunodeficiency virus *rev* protein. J. Virol. **63**:4438–4440.
6. **Collins, S. J.** 1987. The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression. Blood **70**:1233–1244.
7. **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.
8. **De Benedetti, F., L. A. Falk, L. R. Ellingsworth, F. W. Ruscetti, and C. R. Faltynek.** 1990. Synergy between transforming growth factor-β and tumor necrosis factor-α in the induction of monocytic differentiation of human leukemic cell lines. Blood **75**:626–632.
9. **Duh, E. J., W. Maury, T. M. Folks, A. S. Fauci, and A. B. Rabson.** 1989. Tumor necrosis factor-alpha activates human immunodeficiency virus-1 through induction of nuclear factor binding to the NF-κB sites in the long terminal repeat. Proc. Natl. Acad. Sci. USA **86**:5974–5978.
10. **Fauci, A. S.** 1988. The human immunodeficiency virus: infectivity and mechanisms of pathogenesis. Science **239**:617–622.
11. **Folks, T. M., K. Clouse, J. Justement, A. Rabson, E. Duh, J. H. Kehrl, and A. S. Fauci.** 1989. Tumor necrosis factor-alpha induces expression of human immunodeficiency virus in a chronically infected T-cell clone. Proc. Natl. Acad. Sci. USA **86**:2365–2368.
12. **Folks, T. M., J. Justement, A. Kinter, C. A. Dinarello, and A. S. Fauci.** 1987. Cytokine-induced expression of HIV-1 in a chronically infected promonocytic cell line. Science **238**:800–802.
13. **Folks, T. M., J. Justement, A. Kinter, S. Schnittman, J. Orienting, G. Poli, and A. S. Fauci.** 1988. Characterization of a promonocyte clone chronically infected with HIV and inducible by 13-phorbol-2-myristate acetate. J. Immunol. **140**:1117–1122.
14. **Folks, T. M., D. Powell, M. Lightfoote, S. Koenig, A. S. Fauci, S. Benn, A. Rabson, D. Daugherty, H. E. Gendelman, M. D. Hoggan, S. Venkatesan, and M. A. Martin.** 1986. Biological and biochemical characterization of a cloned Leu-3$^-$ cell surviving infection with the acquired immune deficiency syndrome retrovirus. J. Exp. Med. **164**:280–290.
15. **Garcia, J. V., and A. D. Miller.** 1991. Serine phosphorylation-independent downregulation of cell-surface CD4 by *nef*. Nature (London) **350**:508–511.
16. **Geleziunas, R., S. Bour, F. Boulerice, J. Hiscott, and M. A. Wainberg.** 1991. Diminution of CD4 surface protein but not CD4 messenger RNA levels in monocytic cells infected by HIV-1. AIDS **5**:29–33.
17. **Gendelman, H. E., W. Phelps, L. Feigenbaum, J. M. Ostrove, A. Adachi, P. M. Howley, G. Khoury, H. S. Ginsberg, and M. A. Martin.** 1986. Trans-activation of the human immunodeficiency virus long terminal repeat sequence by DNA viruses. Proc. Natl. Acad. Sci. USA **83**:9759–9763.
18. **Ghosh, S., and D. Baltimore.** 1990. Activation in vitro of NF-κB by phosphorylation of its inhibitor IκB. Nature (London) **344**: 678–682.
19. **Griffin, G. E., K. Leung, T. M. Folks, S. Kunkel, and G. J. Nabel.** 1989. Activation of HIV gene expression during monocytic differentiation by induction of NF-κB. Nature (London) **339**:70–73.
20. **Hidaka, H., M. Inagiki, S. Kawamote, and Y. Sasaki.** 1984. Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry **23**:5036–5041.
21. **Hildreth, J. E. K., and R. J. Orentas.** 1989. Involvement of a leukocyte adhesion receptor (LFA-1) in HIV-induced syncytium formation. Science **244**:1075–1078.
22. **Hoxie, J. A., J. D. Alpers, J. L. Rackowski, K. Huebner, B. S. Haggarty, A. J. Cedarbaum, and J. C. Reed.** 1986. Alterations in T4 (CD4) protein and mRNA synthesis in cells infected with HIV. Science **234**:1123–1127.
23. **Hoxie, J. A., B. S. Haggarty, J. L. Rackowski, N. Pillsbury, and J. A. Levy.** 1985. Persistent noncytopathic infection of normal human lymphocytes with AIDS-associated retrovirus. Science **229**:1400–1402.
24. **Jakobovits, A., A. Rosenthal, and D. J. Capon.** 1990. *Trans*-activation of HIV-1 LTR-directed gene expression by *tat* requires protein kinase C. EMBO J. **9**:1165–1170.
25. **Kawamura, I., Y. Koga, N. Oh-Hori, K. Onodera, G. Kimura, and K. Nomoto.** 1989. Depletion of surface CD4 molecule by the envelope protein of human immunodeficiency virus expressed in a human CD4$^+$ monocytoid cell line. J. Virol. **63**:3748–3754.
26. **Kinter, A. L., G. Poli, W. Maury, T. M. Folks, and A. S. Fauci.** 1990. Direct and cytokine-mediated activation of protein kinase C induces human immunodeficiency virus expression in chronically infected promonocytic cells. J. Virol. **64**:4306–4312.
27. **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.
28. **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.
29. **Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss, and R. Axel.** 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47**:333–348.
30. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Osborn, L., S. Kunkel, and G. J. Nabel.** 1989. Tumor necrosis factor α and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor κB. Proc. Natl. Acad. Sci. USA **86**:2336–2340.
32. **Pauza, C. D., J. E. Galindo, and D. D. Richman.** 1990. Reinfection results in accumulation of unintegrated viral DNA in cytopathic and persistent human immunodeficiency virus type 1 infection of CEM cells. J. Exp. Med. **172**:1035–1042.
33. **Pauza, C. D., and M. K. Singh.** 1990. Extrachromosomal HIV-1 DNA in persistently infected U937 cells. AIDS Res. Hum. Retroviruses **6**:1027–1030.
34. **Poli, G., J. M. Orienting, A. Kinter, T. M. Folks, and A. S. Fauci.** 1989. Interferon-α but not AZT suppresses HIV expression in chronically infected cell lines. Science **244**:575–577.
35. **Psallidopoulos, M. C., S. M. Schnittman, L. Thompson III, M. Baseler, A. S. Fauci, H. C. Lane, and N. P. Salzman.** 1989. Integrated proviral human immunodeficiency virus type 1 is present in CD4$^+$ peripheral blood lymphocytes in healthy seropositive individuals. J. Virol. **63**:4626–4631.
36. **Ranki, A., S. L. Valle, M. Krohn, J. Antonen, J. P. Allain, M. Leuther, G. Franchini, and K. Krohn.** 1987. Long latency preceded overt seroconversion in sexually transmitted human-immunodeficiency-virus infection. Lancet **ii**:589–593.
37. **Robins, H. L., and D. M. Zinkus.** 1990. Accumulation of human immunodeficiency virus type 1 DNA in T cells: result of multiple infection events. J. Virol. **64**:4836–4841.
38. **Rossi, A. D., G. Franchini, A. Aldovini, A. D. Mistro, L. Chieco-Bianchi, R. C. Gallo, and F. Wong-Staal.** 1986. Differential response to the cytopathic effects of human T-cell lymphotropic virus type III (HTLV-III) superinfection in T4$^+$ (helper) and T8$^+$ (suppressor) T-cell clones transformed by HTLV-I. Proc. Natl. Acad. Sci. USA **83**:4297–4301.
39. **Salmon, P., R. Olivier, Y. Riviere, E. Brisson, J.-C. Gluckman, M.-P. Kieny, L. Montagnier, and D. Klatzmann.** 1988. Loss of CD4 membrane expression and CD4 mRNA during acute human immunodeficiency virus replication. J. Exp. Med. **168**: 1953–969.
40. **Schnittman, S. M., M. C. Psallidopoulos, H. C. Lane, L. Thompson, M. Baseler, F. Massari, C. H. Fox, N. P. Salzman, and A. S. Fauci.** 1989. The reservoir for HIV-1 in human peripheral blood is a T cell that maintains expression of CD4. Science **245**:305–308.
41. **Stevenson, M., S. Haggerty, C. A. Lamonica, C. M. Meier, S.-K. Welch, and A. J. Wasiak.** 1990. Integration in not necessary for expression of human immunodeficiency virus type 1 protein products. J. Virol. **64**:2421–2425.
42. **Stevenson, M., X. Zhang, and D. J. Volsky.** 1987. Downregulation of cell surface molecules during noncytopathic infection of T cells with human immunodeficiency virus. J. Virol. **61**:3741–3748.
43. **Strohman, R. C., P. S. Moss, J. Micou-Eastwood, D. Spector, A. Przbyla, and B. Paterson.** 1977. Messenger RNA for myosin polypeptides: isolation from single myogenic cell cultures. Cell **10**:265–273.
44. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic

transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.

45. **Tsang, V. C., J. M. Peralta, and A. R. Simons.** 1983. Enzyme-linked immuno-electrotransfer blot techniques for studying the specificities of antigens and antibodies separated by gel electrophoresis. Methods Enzymol. **92**:377–391.
46. **Willey, R. L., D. H. Smith, L. A. Lasky, T. S. Theodore, P. L. Earl, B. Moss, D. J. Capon, and M. A. Martin.** 1988. In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. J. Virol. **62**:139–147.
47. **Yuille, M. A. R., M. Hugunin, P. John, L. Peer, L. V. Sacks, B. J. Poiesz, R. H. Tomar, and A. E. Silverstone.** 1988. HIV-1 infection abolishes CD4 biosynthesis but not CD4 mRNA. J. Acquired Immune Defic. Syndr. **1**:131–137.