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Gradual Shutdown of Virus Production Resulting in Latency Is the Norm during the Chronic

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Most $CD4^+$ lymphocytes in lymph nodes of both asymptomatic HIV-1-infected individuals and AIDS patients are nonproductively or latently infected. It is not clear how these cells come about because infection of resting lymphocytes results in abortive infection and infection of activated lymphocytes results in productive infection. The frequency and mechanisms underlying nonproductive or latent HIV infections of normal $CD4^+$ lymphocytes largely remain unexplored, and because HIV latency has principally been studied in latently infected cell clones of established cell lines, it is not even clear how often this type of infection occurs in cell lines. We demonstrate herein that chronic HIV replication in populations of normal phytohemagglutinin-stimulated peripheral blood $CD4^+$-enriched lymphocytes, as well as an established T-cell line (CEM), gradually shuts down in the vast majority of cells. The nonproducing cells in these cultures still harbored HIV provirus, and HIV could be reactivated in CEM cells by treatment with phorbol ester, showing that this was latent infection. Thus, HIV's life cycle should probably be considered as consisting of two phases: an acute exponential rise in production of virus progeny which levels at some peak, followed by a gradual decline of progeny production during the chronic phase leading to viral latency. Temporal analyses of the steady-state levels of viral mRNAs in populations of chronically infected CEM cells as virus production declined revealed the two mechanisms of HIV latency which have previously been described in the OM-10.1 and U1 or ACH-2 latently infected cell clones (i.e., apparent overall shutdown of HIV transcription and "blocked early-stage latency" involving enhanced splicing of viral pre-mRNAs). However, which mechanism was employed, as well as the rate of shutdown, depended on the virus strain.

INTRODUCTION

Chronic infection by prototypic lentiviruses (Visnavirus, EIAV) appears to result in a significant extent of latent infection *in vivo* (Haase, 1986; Peluso *et al.,* 1985). Similarly, HIV-1 infection *in vivo* appears to result in a significant number of infected nonproducing cells (for review, see Aiuti *et al.,* 1993; Bednarik *et al.,* 1992; Butera *et al.,* 1992; Fauci, 1988; and Pantaleo *et al.,* 1993a). The evidence for this comes from studies examining the frequencies of cells harboring HIV proviral DNA versus cells expressing viral RNA in peripheral lymphoid tissues (spleen and lymph node) or peripheral blood of infected individuals. In general, the percentage of cells expressing HIV RNA were very low during most stages of the disease, but a considerably greater number of cells possessed proviral DNA (Embretson *et al.,* 1993a,b; Harper *et al.,* 1986; Psallidopoulas *et al.,* 1989; Schnittman *et al.,* 1989). Recent *in situ* PCR studies have shown that up to 20% (in asymptomatic infected patients) or 80% (in AIDS patients) of the lymphocytes and monocytes in lymph nodes harbored silent HIV proviruses (Bagasra *et al.,* 1993; Embretson *et al.,* 1993a,b). It was also shown that about 3 to 6% of lymphocytes and macrophages in these tissues expressed low copy numbers (30–60) of viral RNA and only $6–8 \times 10^{-4}$ cells expressed high copy numbers (250–500) (Embretson *et al.,* 1993b). Cumulatively, these data strongly demonstrate that a significant number of infected lymphocytes in patients at any given time are nonproductively or latently infected.

It might be reasonable to assume, then, that nonproductive or latent infection is not a rare fate of lymphocytes infected with HIV *in vivo.* It is not clear, however, what are the proportions of cells which become truly latently infected (i.e., virus able to be reactivated) versus nonproductively infected (i.e., not able to be reactivated) versus lytically infected. Productive lytic infection appears to be the norm for peripheral blood lymphocytes (PBLs) infected *in vitro* following phytohemagglutinin (PHA) stimulation. However, lymphocytes *in vivo* may behave differently than PBLs PHA-stimulated and grown *in vitro.* It is not clear whether latently or nonproductively infected lymphocytes *in vivo* result from productively infected lymphocytes that survived and eventually shut down virus production or whether they result from acute infection that never had a productive phase. *In vitro* infection of

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resting PBLs appears to predominantly result in an abortive infection in which integration of provirus does not occur (Stevenson *et al.,* 1990; Zack *et al.,* 1990). Estimates of the half-life of the unintegrated viral DNA intermediates have varied, but it appears to be between 4 and 72 hr (Zack *et al.,* 1990). Other studies reported that HIV infection of resting PBLs resulted in stalled virus replication which could resume upon stimulation with mitogen up to 10 days later (Spina *et al.,* 1995). Caution is warranted, however, with such studies because "resting" PBLs always have a very small percentage of cells that are partially or fully activated and these may be harboring replicating virus which can spread through the rest of the cells after they are stimulated with PHA. It is also possible that, unlike nonstimulated PBLs *in vitro,* some "resting" $CD4^+$ lymphocytes *in vivo* may marginally support HIV replication, which could lead to latent infections. In support of this idea is the recent demonstration that unstimulated $CD4^+$ lymphocytes taken from skin and cultured on dermal dendritic cells can be productively infected with HIV (Pope *et al.,* 1994).

Because nonproductive or latent HIV infections appear to be frequent *in vivo,* they may be important in pathogenesis. These states are used by several viruses to establish persistence, allowing the infected cells to escape recognition by the anti-viral immune response.

Studies of HIV latency have largely examined cell clones derived from established cell lines that are already latently infected (U1, ACH-2, OM-10.1, and J1.1). This is because an early study by Folks and co-workers demonstrated that loss of HIV production in populations of chronically infected A3.01 T-cells was due to overgrowth by uninfected cells (Folks *et al.,* 1986). This would be expected because 5% of the cells in this line (A3.01) are CD4-negative and uninfectable, while the $CD4^+$ cells are susceptible to HIV-induced CPE (Folks *et al.,* 1986). We are not aware of studies that have quantitated the frequency of cells in an acutely infected cell population that became latently infected or nonproductive overtime, and it is not clear if the occurrence of nonproducing infected cells is relatively rare or if this is a common fate of most infected cells if they survive acute virus replication. We are also not aware of any data indicating that nonproductive or latent infection occurs to any extent in stimulated normal $CD4^+$ lymphocytes infected *in vitro.* Therefore, it is not clear how the significant percentage of latently infected lymphocytes *in vivo* arise. Finally, it is not clear that the latently infected transformed cell clones (e.g., U1 and ACH-2) are representative of normal lymphocytes that become latently or nonproductively infected *in vivo.*

We initiated this study to determine the frequency of nonproductive or latent infection in populations of both a T-cell line highly susceptible to HIV infection and normal PHA-stimulated $CD4^+$ PBLs acutely and chronically infected *in vitro* with HIV and to characterize the nature of nonproductive infection, if it occurred.

MATERIALS AND METHODS

Cell culture and HIV infection

CEM cells, a $CD4^+$ T-lymphoid line, were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS), 5% transferrin-supplemented calf serum, and antibiotics. Two molecularly cloned HIV strains — HIV_{213} and HIV_{MCK} (Cloyd *et al.,* 1990) — and five other strains — HIV_{RF}, HIV_{R6}, HIV_{MN}, $HIV_{AC\text{-}1}$, and HIV_{IIIB} (Race *et al.,* 1991; Sarngadharan *et al.,* 1984) — were used in this study. All of these belong to clade B. Virus stocks were made in H9 cells and were titrated on 1×10^4 C8166 T-cells in microtiter plates, which were scored for the endpoint (the highest dilution) that productively infected by the end of 2 weeks of culture. All infections to study HIV chronic infection were performed at multiplicity's of infection (m.o.i.) of about 1. Twenty-four hours after infection, the cells were refed with fresh culture medium and the cells were subcultured every 3 to 4 days thereafter.

Purification and *in vitro* culture of $CD4^+$ normal lymphocytes from peripheral blood

Peripheral blood lymphocytes (PBLs) were isolated from 40 to 50 ml of venous blood using lymphocyte separation medium (Organon Teknika) and centrifugation. The $CD8^+$ T-lymphocytes were depleted by cell panning with OKT8, and the purity of nonadherent cells was monitored by immunostaining with OKT8, OKT4, and OKT3 monoclonal antibodies and flow cytometry. The PBLs were then stimulated with 4 μg/ml of PHA (Sigma) and cultured in RPMI 1640 supplemented with 15% heat-inactivated FBS for 3 days. After that, the cells were cultured in medium containing 20 U/ml of recombinant human IL-2 (Fisher Scientific). HIV infections of CD4-enriched PBLs were performed 5 days after initiation of the cultures using m.o.i.s approximating 1. The next day, culture supernatants were changed with fresh media containing IL-2 and cultured routinely for up to 3 weeks.

Single-cell cloning

CEM cells were acutely infected with HIV_{213} or HIV_{MCK} at an m.o.i. of 0.5 and cultured until the majority of the population recovered from any virus-induced cytopathic effects (CPE). By fixed-cell immunofluorescence for HIV p24 antigen, approximately 100% of the cells expressed p24. Single cell clones were then obtained by limited dilution plating in Terisaki plates. Wells with single cells were scored after plating and the cells were cultured and expanded for several weeks. When the clones had expanded to a number that could be transferred to 96-

well plates, an aliquot was removed, fixed in cold acetone, and stained for HIV p24. These cultures were further expanded and tested weekly by fixed-cell immunofluorescence for HIV p24.

Monitoring of HIV replication in the infected cells

Three methods were used: *in situ* indirect HIV p24 immunofluorescence (IFA) of acetone-fixed cells spotted onto multiwell microscope slides, indirect immunofluorescence of cells fixed in suspension with acetone and stained for HIV p24 followed by analysis in an EPICS Profile flow cytometer, and p24 antigen-capture EIA of culture supernatants. For IFA, 40 μl of cell suspension was spotted onto wells of multiwell slides, allowed to settle, fixed with cold acetone for 5 min, and allowed to air dry. After incubation with a monoclonal antibody (M26), (kindly provided by Dr. R. C. Gallo) against viral core protein p24 or pooled HIV-positive patient sera (diluted 1:100) at room temperature for 45 min, the cells were incubated with goat anti-mouse or anti-human IgG antibodies (Sigma) conjugated with FITC. The slides were observed under a Zeiss epifluorescence microscope and the percentage of fluorescing cells was determined by counting and scoring for fluorescence of all cells in 5–10 fields (~100–200 cells).

For flow cytometry, the cell pellets were suspended in 50 μl of phosphate-buffered saline (PBS) and fixed with 750 μl of acetone at 4° overnight. After washing with PBS–2% bovine serum, the cells were incubated with 150 μl of M26 antibody or patient pooled sera at room temperature for 2 hr. The same FITC-conjugated antibodies were used as secondary antibodies. After washing and suspension in PBS, the cells were analyzed in an EPICS Profile flow cytometer.

The amount of viral p24 released into culture supernatants was tested by antigen-capture EIA (Coulter Inc.) according to the manufacturer's instructions.

Southern blot quantitation of proviral genomes in infected CEM cells

For isolation of cellular DNA, 5×10^6 HIV-infected and uninfected CEM cells were washed with PBS and suspended in 50 μl PBS. The cells were lysed in 400 μl of lysis buffer containing 20 m*M* Tris–HCl (pH 7.4), 20 m*M* EDTA, and 0.5% SDS and digested with 20 μg/ml Proteinase K at 50° overnight. After extraction with phenol:chloroform:isoamyl alcohol (25:24:1) once and chloroform:isoamyl alcohol (24:1) twice, the DNA was precipitated with 1/20 vol of 5 *M* potassium acetate (pH 7.5) and 2 vol of ethanol at −70° for 45 min. The DNA was pelleted by centrifugation (12,000 *g*) at 4°, washed with 70% ethanol once, and resuspended in 300 μl 1× TE (10 m*M* Tris–HCl, pH 8.0, and 1 m*M* EDTA, pH 8.0). Contaminating cellular RNA was digested with 200 μg/ml DNase-free RNase A at 37° for 2 hr, followed by extraction with phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol one time each and precipitation as above. The DNA was quantitated in a spectrophotometer (Hitachi) by absorbence at 260 and 280 nm. Ten micrograms of DNA per lane was electrophoresed in 0.6% agarose gels and the gels were sequentially treated with 0.25 *N* HCl for 15 min, 0.5 *N* NaOH/1 *M* NaCl for 20 min, and 0.5 *M* Tris–HCl (pH 7.4)/1 *M* NaCl for 30 min. The DNA was then transferred by capillary blotting onto Zeta-probe membranes (Bio-Rad) in 10× SSC for 24 hr. The membranes were rinsed in 2× SSC and the blotted DNA was immobilized by baking at 80° for 1 hr under vacuum. Prehybridization was carried out at 56° overnight with 6× SSPE, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA. A 3.3-kb HIV DNA probe spanning *gag* and *pol* regions (*Pst*I–*Eco*RI) was radiolabeled with [α-^{32}P]dCTP by random priming and used for hybridization. The hybridization was carried out at 56° for 36 hr with the same solution as that used for prehybridization. The membrane was sequentially washed once in 6× SSPE/0.5% SDS at room temperature for 15 min and twice in 1× SSPE/0.5% SDS twice in 0.5× SSPE/0.5% SDS, each at 56° for 20 min, followed by exposure to X-ray film.

Semiquantitative PCR assessing proviral genomes in infected PBLs

Total cellular DNA was harvested from HIV-infected PBLs with the same method as that used in CEM cells above. Since the amount of DNA at each time point was limited, we optimized a PCR protocol to quantitate the levels of proviral DNA in the infected PBLs. Proviral DNA was amplified by a pair of HIV primers spanning the 3′ end of LTR and the 5′ end of *gag* (1.3 kb). The sequences of sense and antisense primers are 5′ CCTTGATCTGTGGATCTACCACACAC 3′ and 5′ GGGTGGCTCCTTCTGATAATGCTGAA 3′, respectively. One microgram of cellular DNA was added into a 50-μl reaction containing 5 μl of 10× *Taq* DNA polymerase buffer (Promega), 0.2 m*M* of each dNTPs, 2 m*M* $MgCl_2$, 0.25 pmol of each primer, and 2 U of *Taq* DNA polymerase (Promega) and amplified at 94° for 1 min, 58° for 1 min, and 72° for 2 min for 25 cycles, followed by extension at 72° for 7 min. Twenty microliters of PCR product was then electrophoresed in a 1% agarose gel and blotted onto a Zeta-probe membrane. A *Kpn*I–*Sph*I (1.6-kb) fragment was used as HIV probe and radiolabeled with [α-^{32}P]dCTP by random priming. Prehybridization and hybridization were carried out as above. The same amount of DNA was amplified by a pair of control IFNγ gene primers: 5′ GCAGGTCATTCAGATGTAGCTGTGAT 3′ for sense and 5′ AGCAAGACCCTCGGCAATGAAACCC 3′ for anti-sense using a similar PCR protocol, to confirm the actual loading amount of cellular DNA in the sample. The PCR products

were analyzed by 1.2% gel electrophoresis and EtBr staining.

Northern blot quantitation of HIV transcripts

After infection, 1×10^7 cells were harvested at different time points and washed with Hanks' buffered saline solution (HBSS). Total cellular RNA was isolated using RNAzol B (Biotex). The RNA was subjected to 1.2% formaldehyde–agarose gel electrophoresis and then capillary transferred onto Zeta-probe membranes in 10× SSC for 24 hr. Hybridization and the subsequent washing conditions were according to the manufacturer's recommendation. The hybridization stringency was adjusted to provide clear results for each membrane. A full-length HIV genome DNA was used as the probe and radiolabeled with [α-^{32}P]dCTP by random priming. After autoradiography, the blotted membranes were stripped in 0.1× SSC/0.5% SDS at 95° for 20 min twice and rehybridized with a human β-actin (Biotech) cDNA probe as an internal control.

Semiquantitative RT–PCR for multispliced HIV transcripts

The anti-sense primer (5′ CTTAAGCAGTGGGTTCCC 3′) used for reverse transcription was complementary to the HIV poly(A) site. Five micrograms of total cellular RNA was subjected to reverse transcription in a 50-μl reaction mixture containing 1 m*M* dNTPs, 1× RT buffer (Promega), 40 U RNasin, 50 ng of primer, and 40 U of AMV reverse transcriptase. The reaction was carried out at 42° for 45 min and stored at −20° before PCR amplification. The strategy in amplifying and analysis of different HIV multiply spliced transcripts, which include mRNAs around 2 kb in size encoding various virus regulatory proteins, was modified from those of Arrigo *et al.* (1989) and Purcell *et al.* (1993). The sense primer (5′ TGCTGAAGCGCCCGCACGGC 3′) is located between the HIV transcriptional initiation site and the major 5′ splice donor site. The antisense primer (5′ TGTCGGGTCCCCTCGGGGTTGG 3′) is downstream of the splice acceptor sites for the multiply spliced mRNAs. After PCR amplification using this primer pair, the majority of the different multiply spliced HIV mRNA species could be separated according to their sizes by 8% polyacrylamide gel electrophoresis (PAGE). The sense primer was end-labeled with [γ-^{33}P]ATP (DuPont NEN). The PCR reactions in 50-μl volumes contained 1× *Taq* polymerase buffer (Mg^{2+}-free, Promega), 4 m*M* $MgCl_2$, 0.5 m*M* dNTPs, 50 ng of anti-sense primer, 2 U *Taq* polymerase, 50 ng of labeled sense primer, and 2.5 μl of reverse transcription mixture. The reaction was amplified for 20 cycles, each at 94° for 45 sec, 60° for 1 min, and 72° for 2 min. Twenty microliters of PCR products was analyzed by 8% PAGE and autoradiography.

Induction of HIV production in latently infected CEM cell populations

CEM cells chronically infected with HIV_{213} and HIV_{MCK} (for 2 years and for 6 months, respectively) and nonproducing by *in situ* immunostaining were used to determine if virus production could be induced by phorbol ester treatment. Cells were washed twice in RPM1 1640 without serum, centrifuged at 1200 rpm, 4° for 5 min, and resuspended at 0.25×10^6 cells/ml in normal culture medium with 400 ng/ml of phorbol 12-myristate 13-acetate (PMA, Sigma). After 2 days, the culture was split once, with an equal volume of fresh medium added, and cultured for another 3 days. Culture supernatants at 2 and 5 days after addition of PMA were collected, and the amount of p24 antigen in the supernatant was determined by antigen-capture EIA (Coulter, Inc.). In addition, aliquots of the cells were spotted onto multiwell microscope slides, allowed to settle, and gently fixed with cold acetone for 10 min. After air drying, the cells were stained for HIV p24 as described above.

RESULTS

Populations of T-cells chronically infected with different HIV strains gradually cease virus production

To determine the frequency of cells in a population of T-cells which become productively infected, abortively infected, or latently infected, CEM cells were acutely infected with different HIV-1 strains at m.o.i.s of about 1. CEM cells were chosen because these cells appear not to contain a CD4-negative subpopulation and are highly susceptible to infection by many strains of HIV-1 (unpublished observations). Virus production reached a peak within a few days after virus was detected in the culture supernatant, at which time virus-induced cytopathic effect (CPE) became maximal if the virus strain could injure these cells (Fig. 1). With each virus, approximately 100% of the cells appeared to produce HIV p24, and there was no evidence of a nonproducing cell population (Fig. 1A). However, following peak virus production and CPE (Figs. 1A, 1B, and 1C), the surviving cell population gradually decreased virus p24 expression over the ensuing weeks. HIV-1 strains IIIB and HIV_{213} demonstrated slower rates of virus diminution or, alternatively, longer duration of chronic virus production than HIV_{MCK} and HIV_{R6}. HIV_{MCK} differed in that it was lowly cytopathic, but it ceased virus production at the faster rate (Figs. 1A and 1C). Under the fluorescent microscope, the intensity of p24 immunostaining was very strong at the point of peak virus replication and during the following week, but after that, the surviving cells in the population, which appeared normal and grew normally, stained for p24 with decreasing intensity.

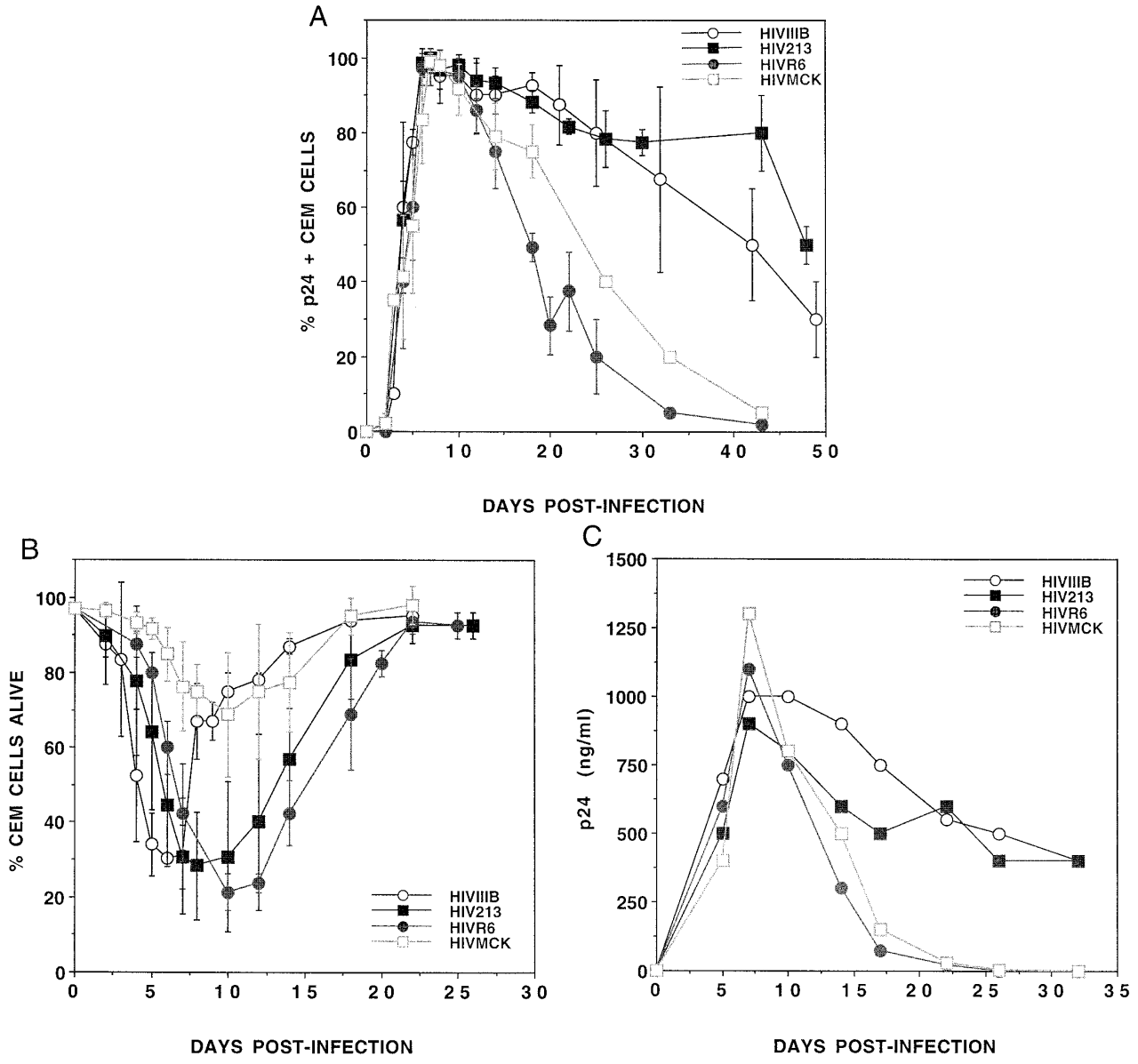

FIG. 1. (A) Percentages of CEM cells expressing HIV p24 during chronic infections with different HIV-1 isolates at m.o.i.s of about 1. At indicated time points, cells were spotted onto multiwell slides and allowed to settle for 2 hr at room temperature. After fixation with cold acetone for 5–10 min and air drying, the cells were incubated with monoclonal antibody against p24 (M26), followed by washing in PBS for 5 min and incubation with FITC-conjugated goat anti-mouse antibody. The percentage of cells expressing p24 was scored on a fluorescence microscope. (B) The percentages of viable cells at each time point were determined by trypan blue exclusion and cell counting under a hemocytometer. Data are from three separate infections with each virus performed in duplicate each time. (C) Quantitation of virus released into culture supernatants using p24 antigen capture EIA.

Over time, the percentage of cells that scored positive for HIV p24 continued to decrease. Since p24 protein is the capsid protein essential for production of progeny virions, its diminution indicated shutdown of virus production. Since we routinely monitor HIV production by *in situ* immunostaining for HIV p24, we have always observed gradual diminution of p24 immunofluorescence in all cell lines (T-cell and monocyte) infected chronically (unpublished observations), similarly to what we demonstrate here for CEM cells. However, the rate of diminution varied greatly in different lines, with some lines taking many months to go negative.

Decrease of HIV production in chronically infected cells is not due to overgrowth of uninfected cells, but rather to decline in virus production per infected cell

Gradual cessation of HIV production in chronically infected cells could be due to any one of a number of events: (1) death or slowing in the growth of infected

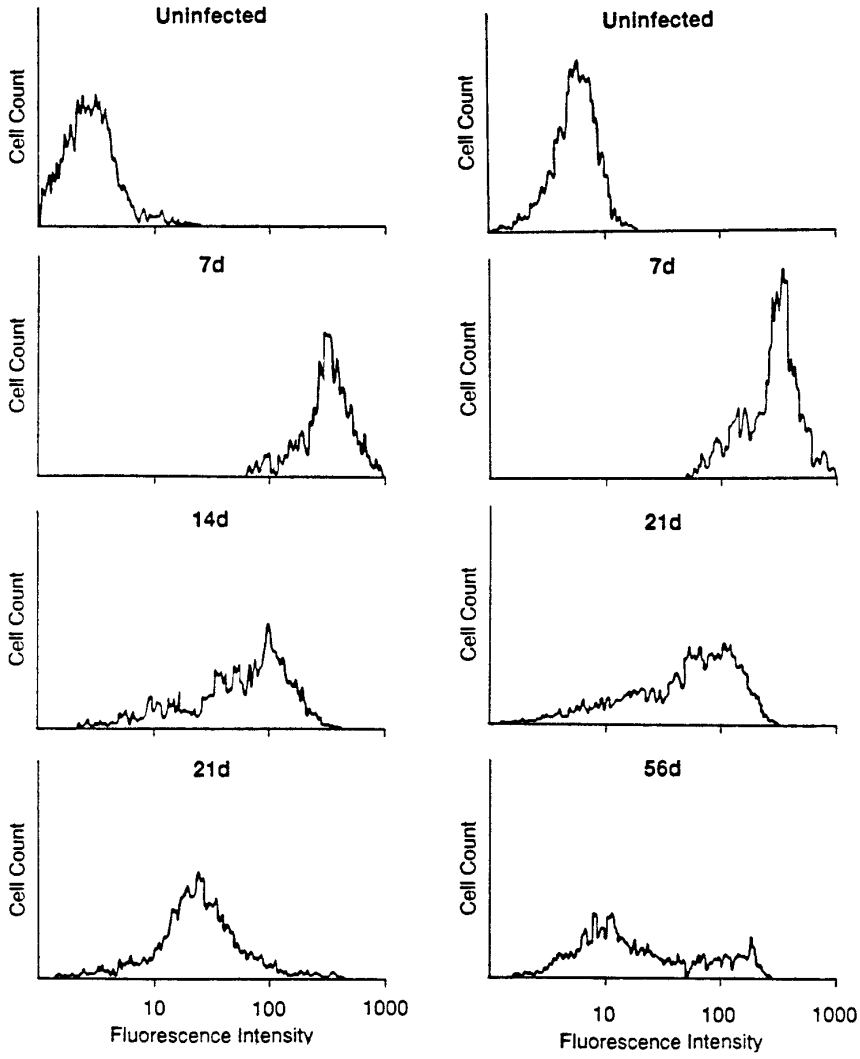

FIG. 2. Expression of HIV p24 in infected CEM populations decreased over time during chronic infection. CEM cells were infected with HIV_{213} (right panels) and HIV_{MCK} (left panels). At the indicated days (d) postinfection, cells were collected and washed in PBS, fixed in cold acetone, and washed again before incubation with M26 antibody at room temperature for $1\frac{1}{2}$ hr. After washing once in PBS–2% FBS, the cells were incubated with FITC-conjugated goat anti-mouse antibody for 30 min, followed by one more washing in PBS. The cells were finally resuspended in 0.2–0.5 ml 2% paraformaldehyde–PBS and analyzed by flow cytometry. The histograms were generated by Profile version 2.1 program.

cells with resulting overgrowth of uninfected cells; (2) elimination of proviral genome from infected cells; or (3) down-regulation of virus production. To determine which was occurring, we first analyzed the fluorescence intensities of immunostained p24-positive cells in infected CEM cultures by flow cytometry at various time points postinfection. This would reveal whether overgrowth of uninfected cells occurred in contrast to gradual reduction of virus production from each infected cell. The results are shown in Fig. 2. At 7 days postinfection, just after peak virus production, approximately 100% of the cells were p24 positive and the average intensity of p24 staining was high, again showing all the cells became productively infected. One week later, the culture still contained 100% p24-positive cells, but the fluorescence intensities shifted toward the left along the *X*-axis, which revealed that the average amount of p24 in each cell had diminished. There was no evidence of an expansion of a p24-negative population. During the subsequent weeks, the average fluorescence intensity of the cells declined even further until more and more cells stained negative. Therefore, HIV p24 production in the majority of chronically infected cells decreased over time in each cell.

To further demonstrate that the loss of HIV expression over time was due to slow shutdown of virus production, we examined cell clones derived from similarly infected populations of CEM cells. After the peak of acute virus replication, single cells were cloned by limited dilution.

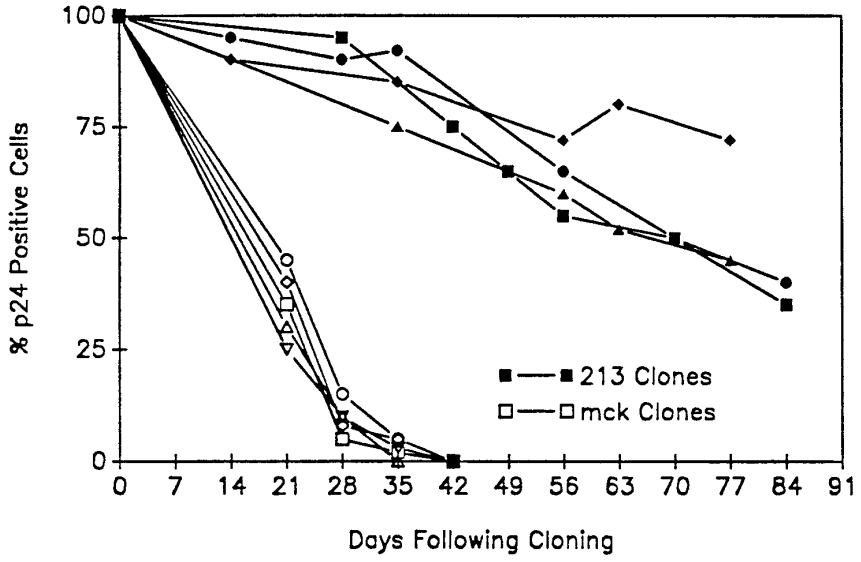

FIG. 3. Expression of HIV p24 antigen in cell clones derived from CEM populations after infection with HIV_{213} and HIV_{MCK}. Five clones from an HIV_{mck}-infected cell population and four from an HIV_{213}-infected population were established by limited dilution. The percentages of HIV p24-expressing cells within the expanded cell clones were scored over time by the method described in Fig. 1.

The expanded clones were tested for their expression of p24 antigen by immunostaining. Five clones infected with HIV_{213} and four with HIV_{MCK} were followed (Fig. 3), and all of these clones exhibited decreasing p24 protein expression over time. The differential rate of decrease for clones infected with HIV_{213} versus HIV_{MCK} were similar to the rates for uncloned infected cell populations (see Fig. 1). These observations further demonstrate that gradual loss of HIV expression in chronically infected cells was not due to dying out of infected cells nor overgrowth of uninfected cells, but was due to gradual cessation of HIV production in the vast majority of the productively infected cells.

Levels of integrated HIV proviral DNA were constant throughout chronic virus shutdown

To examine the possibility that elimination of proviral genome from infected cells was occurring, we compared

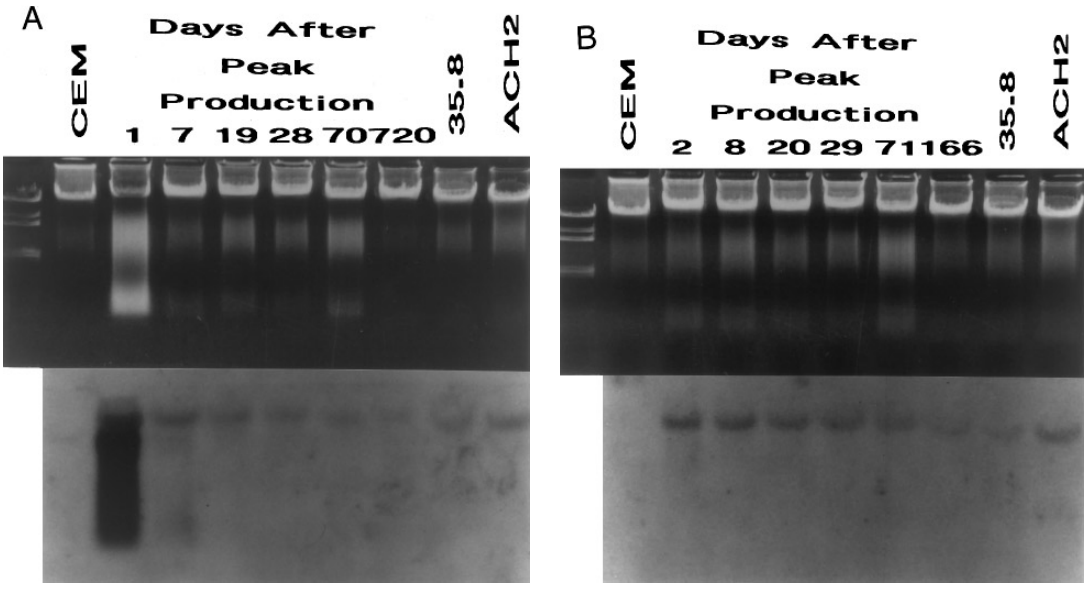

FIG. 4. Southern blot hybridization of integrated proviral DNA in CEM cells during chronic infection with HIV_{213} (A) and HIV_{MCK} (B). Cellular DNA was prepared at the indicated time points from the infected cell populations. 10 μg of uncut DNA per lane was electrophoresed on a 0.6% agarose gel. EtBr staining of the gels before transferring to membranes is shown above the results of hybridization with an HIV-specific probe followed by autoradiography. DNA samples from uninfected CEM cells were used as negative control, and DNA from clone 35.8 cells and ACH_2 cells were used as positive controls.

the levels of integrated proviral DNA at various time points following infection. One cell clone (35.8) which was derived from HIV_{213}-infected CEM cells and constitutively produces low levels of HIV was used as a positive control. We also used the ACH-2 cell line as a positive control, which has been shown to possess one to two copies of HIV proviral DNA per cell (Clouse *et al.,* 1989). Southern blot analysis of uncut chromosomal DNA from chronically infected CEM cultures taken at various time points as the cells ceased virus production revealed persistence of integrated HIV proviral DNA at about one copy per cell in cultures infected with either HIV_{213} or HIV_{MCK} (Fig. 4). This approach was taken because single restriction enzyme digestion would only show a smear for integrated proviruses, and double digestion could not distinguish integrated from unintegrated proviral DNAs. These data confirmed that the vast majority of CEM cells chronically infected *in vitro* with HIV-1 gradually shut down virus production without loss of the integrated provirus.

Virus production in PHA-stimulated normal $CD4^+$ lymphocytes also declined following acute replication

We next wanted to determine if a detectable degree of nonproductive infection occurs in acutely infected normal lymphocytes and if virus shutdown occurs during chronic infection as it does in cell lines. Using panned PBLs enriched for $CD4^+$ cells ($\sim$90–95% $CD4^+$), we monitored virus production in these cells stimulated with PHA and infected at m.o.i.s of approximately 1 with six different HIV strains. HIV_{213} and HIV_{RF} killed all the lymphocytes (data not shown), which terminated those experiments, but the other four HIV isolates did not harm the cells. Figure 5 summarizes these results and shows the percentage of HIV-expressing lymphocytes over time, the number that died, and whether CD8 cells overgrew the culture. Approximately 90–95% of the cells expressed HIV antigens by 5–7 days postinfection (Fig. 5). Following this, virus expression rapidly declined and by 3 weeks postinfection only 5–10% of the lymphocytes were expressing detectable levels of HIV antigens. These cells were still $CD4^+$ and the percentage of $CD8^+$ cells did not increase but slightly declined over the 3 weeks, showing that $CD8^+$ lymphocytes did not overgrow these cultures. Thus, chronic infection of normal $CD4^+$ lymphocytes appeared to also result in gradual shutdown of virus production, but information on the presence of proviral DNA was needed.

Since it was difficult to obtain enough infected PBLs to perform Southern blot hybridization such as we did for CEM cells, semiquantitative PCR amplification of HIV proviral genome in DNA from these PBLs was performed. This showed that the nonexpressing PBLs also harbored an amount of proviral DNA similar to that in the same number of clone 35.8 and ACH_2 cells (Fig. 6). Thus, the nonproducing normal $CD4^+$ PBLs generated during chronic infection were infected, and uninfected cells did not overgrow the cultures.

HIV production could be induced from chronically infected nonproducing CEM cell populations

We next determined if virus production in nonproducing chronically infected CEM populations could be reactivated. This would demonstrate whether this gradual shutdown of virus productions in chronically infected cells leads to latent infection or not. HIV production can be induced in the latently infected U1 and ACH-2 cell clones by factors like PMA, TNF, sodium butyrate, and certain physical agents such as UV light. Nonproductive populations of CEM cells which had been infected by HIV_{213} or HIV_{MCK} for 2 years and for 6 months, respectively, were treated with PMA and the amounts of viral p24 released into culture supernatants at 2 and 5 days were quantitated. Reactivation of virus was most obvious with 0.2, 0.4, and 0.8 μg of PMA per milliliter, but higher doses injured the cells and resulted in lower amounts of the virus produced (data not shown). Shown in Fig. 7, after 2 days of treatment with 0.4 μg per milliliter of PMA, a greater than 10-fold increase in p24 production occurred, and after 5 days, virus production was increased by about 20-fold. The low base levels of virus produced from the HIV_{MCK} latently infected cells before treatment probably resulted from a very small percentage ($<$1%) of the cells still producing virus. Figure 8 shows that the majority of cells in the PMA-treated cultures began producing HIV p24. These data demonstrated that the nonproducing CEM cells resulting from long-term chronic infection actually contained latent provirus that could be reactivated by induction with PMA. Thus, shutdown during the chronic phase of HIV replication led to latent infection.

Studies of the molecular mechanisms underlying shutdown of virus production in chronically infected cell populations

Previous studies of the established latently infected cell clones (Butera *et al.,* 1994; Chen *et al.,* 1994; Michael *et al.,* 1991; Pomerantz *et al.,* 1990, 1992) have shown that several different mechanisms are operating to lead to loss of virus production. The U1 clonal line appears to have a problem with Tat function. ACH-2 appears to have a problem at *tar* because exogenously added Tat did not reactivate the virus (Butera *et al.,* 1994). With both, transcription is not stopped but the mRNAs made are completely spliced. The OM-10.1 and J1.1 cell clones appear to have total shutoff of transcription (Butera *et al.,* 1994). We then performed Northern blot analysis of

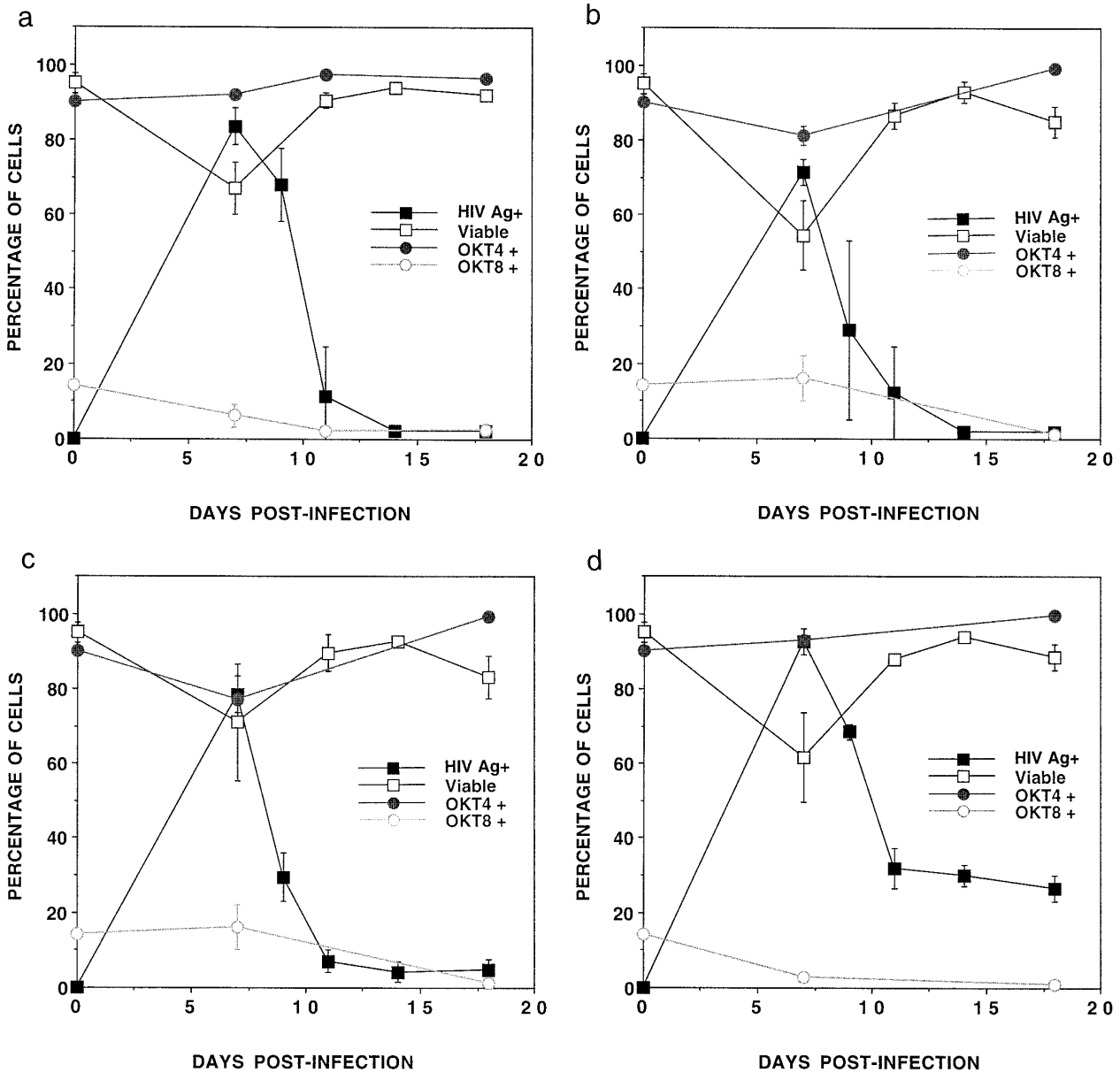

FIG. 5. Percentages of $CD4^+$ PBLs expressing HIV antigen following infections with HIV_{MCK} (a), HIV_{MN} (b), HIV_{AC-1} (c), and HIV_{R6} (d). PBLs were separated from peripheral blood of normal donors by centrifugation on LSM, and the $CD4^+$ cells were purified from the PBLs by negative selection panning with OKT8 MoAb. The $CD4^+$ cells were stimulated by PHA (4 μg/ml) and maintained in RPMI 1640 medium containing 15% FBS, 20 U/ml IL-2, and antibiotics. Infections with HIV stocks were performed at an m.o.i. of about 1, and, at the indicated time points, the percentages of cells expressing CD4, CD8, and HIV antigens in the populations were determined by staining the live cells with OKT4, OKT8, and anti-HIV antibody (appropriately diluted sera from HIV-infected patients) followed by flow cytometry. Viability of the cells was determined by trypan blue exclusion.

HIV mRNAs in chronically infected CEM populations which were shutting down virus production.

HIV Pre-mRNAs are regulated differently during shutdown of HIV_{213} versus HIV_{MCK}. Figure 9 shows Northern blot hybridization of RNA extracted from HIV_{MCK}-infected CEM cells at various time points after peak virus production. Three different HIV transcripts could be detected: the full-length (10-kb), the medium-sized or singly spliced transcripts (about 4 kb), and small-sized or multiply spliced transcripts (about 2 kb). At peak production, the quantities of medium-sized and full-length transcripts were at their highest levels. After that, quantities of full-length transcripts decreased rapidly in HIV_{MCK}-infected CEM, while the levels of medium-sized transcripts decreased more slowly. However, the levels of small-sized transcripts did not appreciably change. The levels of human β-actin mRNA served as internal controls for comparisons of loading amounts of cellular RNA. This result further showed that the majority of cells in these latently infected cell populations maintained provirus. It also

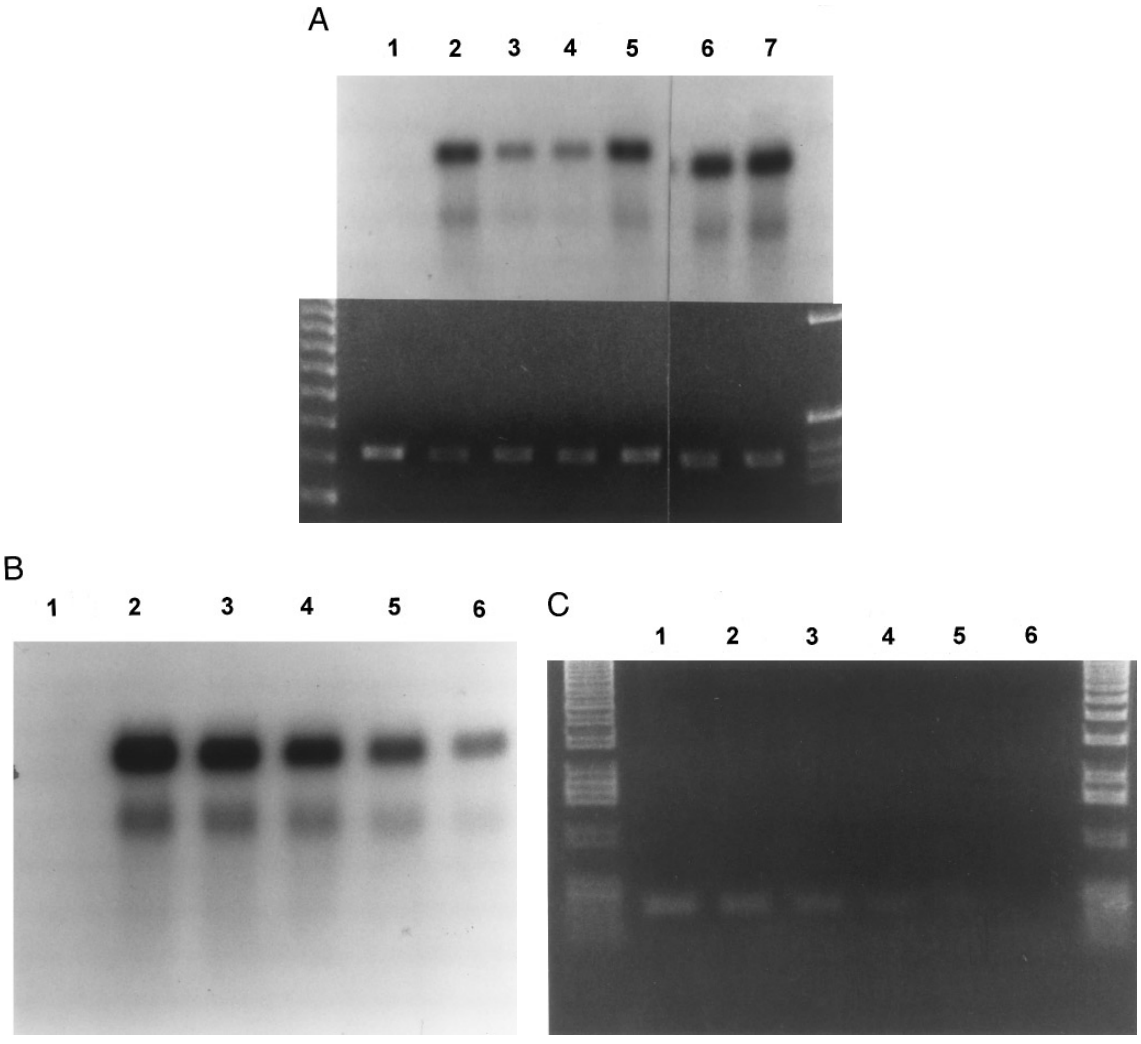

FIG. 6. Levels of proviral DNA in $CD4^+$ PBLs chronically infected by HIV_{MCK}. After infection, DNA was isolated from the cell population at various time points. One PCR assay was designed to quantitatively amplify an HIV proviral DNA sequence, and another to quantitatively amplify an IFNγ sequence was used to internally control the actual amount of DNA in each sample. (A) (Top) Hybridization with HIV probe of the PCR products after amplifying by HIV primers, agarose gel electrophoresis, and transfer to membrane; (bottom) EtBr staining of the products of PCR using IFNγ primers to show that approximately equal amounts of DNA were used in each sample. Lane 1, DNA from uninfected normal $CD4^+$ PBLs; lanes 2–5, DNA from HIV_{MCK}-infected $CD4^+$ PBLs harvested at 7, 14, 21, and 28 days postinfection; lane 6, DNA from clone 35.8 cells; lane 7, DNA from clone ACH_2 cells. (B) Hybridization of PCR products with HIV probe. DNA from clone 35.8 cells serially diluted with DNA from uninfected $CD4^+$ PBLs were amplified by HIV primers. Lanes 2–6 contained 1.0, 0.75, 0.5, 0.25, and 0.1 μg DNA from cell clone 35.8 in a total of 1.0 μg DNA; lane 1 contained 1.0 μg DNA from uninfected $CD4^+$ PBLs. (C) EtBr staining of PCR product amplified by IFNγ primers to demonstrate the sensitivity in quantitating the amount of DNA in samples. Lanes 1–6 contained 1.0, 0.75, 0.5, 0.25, 0.1, and 0 μg of DNA from uninfected CEM cells.

demonstrated that HIV transcription did not completely shut down in CEM cells chronically infected with HIV_{MCK}, but changes occurred in the steady-state levels of unspliced and singly spliced pre-mRNAs.

Since the small-sized HIV transcripts contain more than 2 dozen different HIV mRNAs coding for important viral regulatory proteins such as Tat, Rev, and Nef (Schwartz *et al.,* 1990), semiquantitative RT–PCR analysis of the major multiply spliced HIV mRNA species was performed. This would reveal the levels of the individual multiply spliced viral mRNA and whether changes were occurring in their ratios. The results (Fig. 10A) showed that the relative amounts of major species were largely maintained during chronic infection leading into latency, but the levels of *tat* mRNA (345 bp) slightly increased while *rev* mRNA slightly decreased.

The quantitative aspect of this RT–PCR assay is shown in Fig. 10B with serial dilutions of mRNA isolated at the peak of virus production. PCR amplification of the same RNA sample without RT reaction did not show any products (data not shown).

In contrast, the progression of CEM cells chronically infected with HIV_{213} into latency was associated with a concomitant decrease in the levels of all three viral tran-

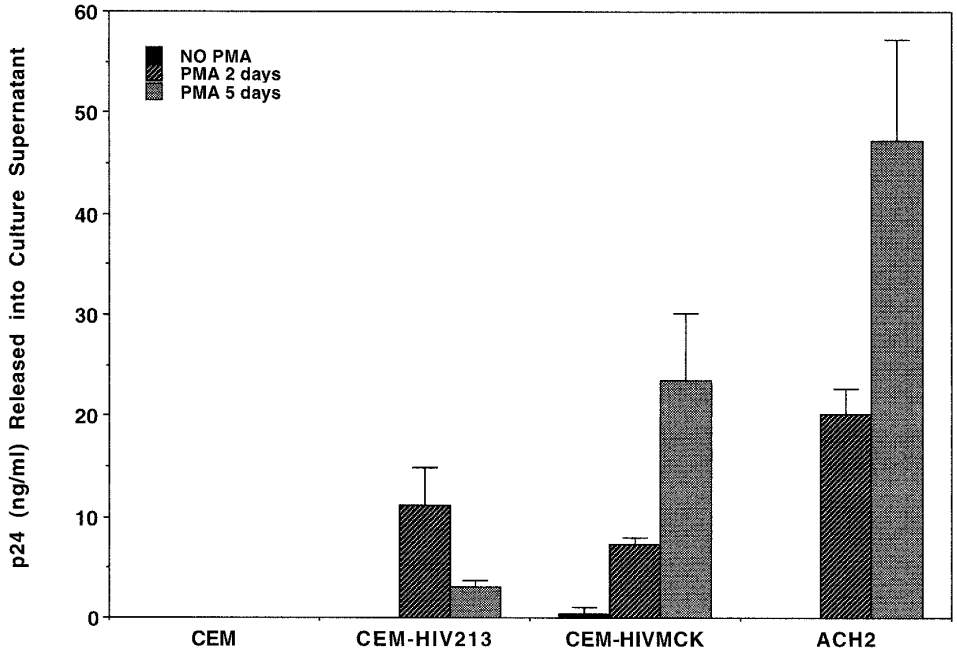

FIG. 7. Relative levels of HIV p24 antigen released into culture medium before and after treatment of latently infected CEM cells by PMA (0.4 μg/ml). CEM cells had been infected by HIV_{213} and HIV_{MCK} for 2 years and 6 months, respectively. The cells were maintained at 0.25×10^6 cells/ml and treated with PMA for 2 and 5 days. After collecting the cell-free culture supernatants, the amounts of HIV p24 in the supernatants were determined by antigen capture EIA.

scripts (Fig. 11), and the RT–PCR results (Fig. 12) confirmed that the major multiply spliced HIV mRNA species disappeared. These results showed that an apparent overall shutdown of viral gene transcription appeared to occur in CEM cells chronically infected with this HIV strain. Therefore, it appeared that the mechanisms of reduced production of virus during chronic infection can vary with different HIV isolates.

The HIV_{213} mechanism of shutdown appears common among several other HIV-1 isolates. It was a coincidence that the two HIV isolates (HIV_{MCK} and HIV_{213}) which we chose to study shut down virus production by different mechanisms. It was, then, of interest to determine which mechanism other HIV isolates used. We infected CEM cells with four randomly chosen HIV isolates, three of which are commonly used by other investigators, and examined the steady-state levels of viral gene transcripts during chronic infection. Figures 13A–13D show that HIV_{RF} (A), HIV_{MN} (B), and HIV_{R6} (C) followed the HIV_{213}-type of apparent shutdown of transcription, and HIV_{IIIB} (D) was somewhat intermediate to HIV_{213} and HIV_{MCK} in that progression into latency was associated with decreases in full-length and medium-sized transcripts, but the multiply spliced transcripts decreased more slowly. HIV_{IIIB}, however, is a mixture of at least two HIV strains, and it may not be an appropriate virus to test. Nevertheless, these results confirm our finding that the particular mechanism of virus shutdown can be determined by the virus strain, at least in CEM cells.

DISCUSSION

In vivo, up to 80% of the $CD4^+$ lymphocytes in lymph nodes appear to be nonproductively or latently infected (Embretson *et al.,* 1993b), but it is not known how these viral states develop. Infection of resting lymphocytes appears to predominantly result in abortive infection (Zack *et al.,* 1990; Spina *et al.,* 1995). Infection of mitogen-stimulated lymphocytes results in productive infection with infected cells usually dying. Chronic infection, in general, has not been studied much in either PBLs or highly susceptible T-cell lines because of the viral-mediated CPE that occurs at the peak of virus replication following acute infection and the high probability of noninfected cells overgrowing the chronically infected culture.

We therefore examined chronic HIV infection of a T-cell line highly susceptible to HIV infection and normal $CD4^+$ lymphocytes stimulated with PHA to determine if detectable frequencies of nonproductive or latent infection occurred. In earlier nonpublished studies, we found that purified $CD4^+$ PBLs lacking $CD8^+$ cells were not killed by certain HIV strains and thus chronic infections could be studied in these cells. Our data showed that most, if not all, of the CEM cells became infected, and chronic infection was established whereby the virus in the majority of the cells went into a latent state. Our CEM model for HIV latent infection was validated by our demonstration that chronic infection of PHA-stimulated normal $CD4^+$ lymphocytes also results in gradual shut-

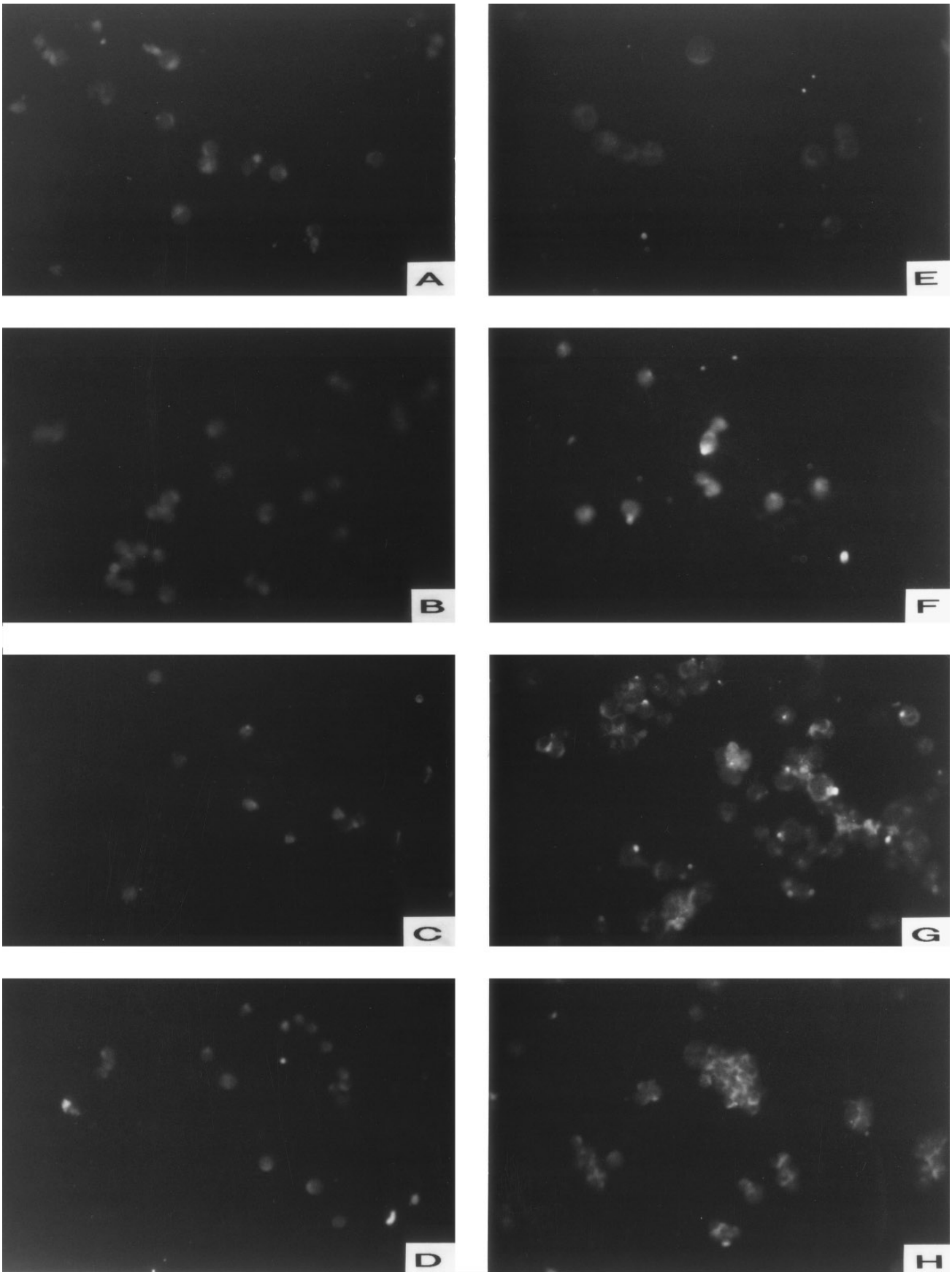

FIG. 8. HIV p24 immunostaining of PMA-treated latently infected CEM cell populations. (A) Untreated uninfected CEM (control); (E) PMA-treated uninfected CEM (control); (B) untreated CEM latently infected with HIV_{213}; (F) PMA-treated CEM latently infected with HIV_{213}; (C) untreated CEM latently infected with HIV_{MCK}; (G) PMA-treated CEM latently infected with HIV_{MCK}; (D) untreated ACH-2; (H) PMA-treated ACH-2.

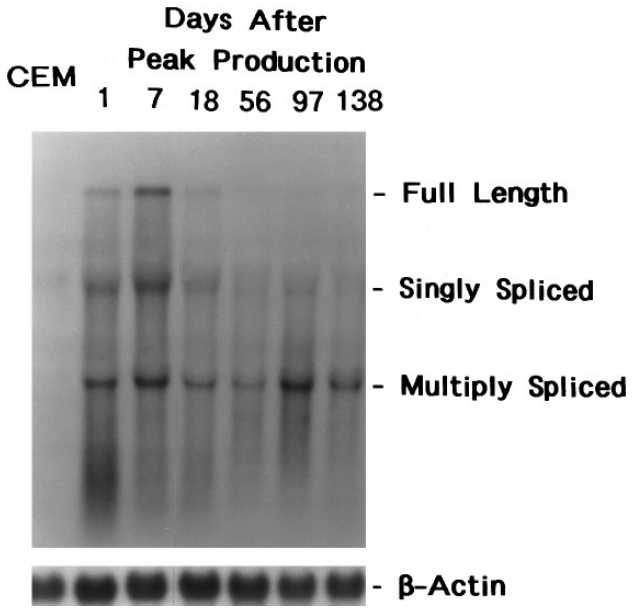

FIG. 9. Northern blot comparisons of the levels of HIV mRNAs in CEM cells during chronic infection with HIV_{MCK}. CEM cells were infected with virus at an m.o.i. of 1 and the total cellular RNA were isolated using RNAzol B (Bio 101) at the indicated time points. 30 μg total RNA was used in each lane for electrophoresis on 1.2% formaldehyde–agarose gel. The RNA was transferred onto Zeta-probe. After autoradiograph the HIV probe was stripped off and the membrane was rehybridized with a human β-actin probe (bottom).

down of virus production, although the rate was faster than what we saw in CEM cells. Similarly, the decline of virus production was not due to exclusion of infected cells nor overgrowth of uninfected lymphocytes, because provirus was present in the nonproducing lymphocytes at approximately 1 copy per cell. Thus, we can now study what happens to HIV expression in chronically infected normal lymphocytes *in vitro,* which is needed to validate the studies of latency mechanisms in the established cell clones or in cell line populations. These data differs from those of Hoxie and co-workers (1985), which showed that PBLs surviving acute HIV infection were chronic producers. Although they state that their data were from a representative experiment, it was not clear how reproducible this finding was. We have always observed, in dozens of noncytopathic infections, shutdown of HIV production from the vast majority of chronically infected normal $CD4^+$ PBLs *in vitro.* Therefore, our data indicate that gradual shutdown of virus production leading to latency is the norm for most productively infected $CD4^+$ T-cells.

These data are partially similar to those from a study by Mikovits and co-workers (1990) using THP-1 promonocytic cells. They found that chronic infection of THP-1 populations could lead to useful information concerning the fate of virus expression over time, and they found that depending on the viral strain and m.o.i., the popula-

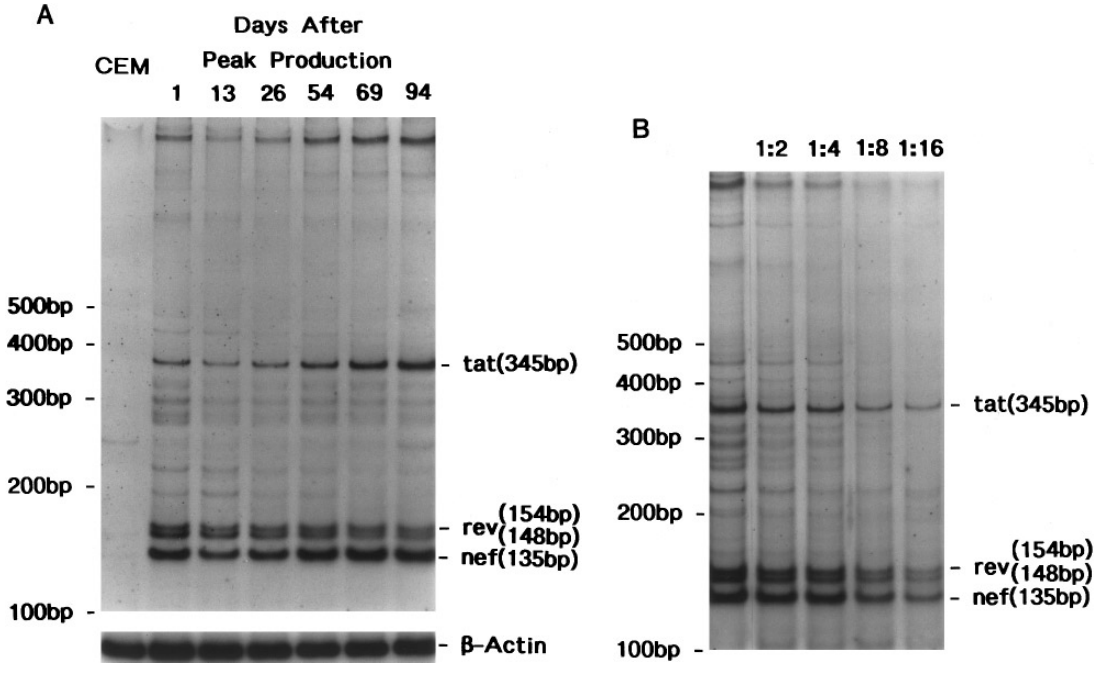

FIG. 10. Determining the levels of HIV *tat, rev,* and *nef* mRNAs in HIV_{MCK} chronically infected CEM cells by semiquantitative RT–PCR. The total RNA was isolated from the cells at the indicated time points, and 5 μg total RNA was used in each RT reaction to generate the first minus DNA strand. The products were then amplified by quantitative PCR to distinguish the major mRNA species for *tat, rev,* and *nef* according to their sizes synthesized by an HIV primer pair in the PCR. The 5′ primer was radiolabeled, and the RT–PCR products were analyzed after separation by 8% PAGE and autoradiography (A, see Materials and Methods for details). The same set of samples were also amplified by a quantitative PCR with a specific primer pair for β-actin (bottom of A). Serial dilutions of RNA isolated at the peak of HIV replication was subjected to the same RT–PCR to demonstrate the quantitation aspect of the RT–PCR (B).

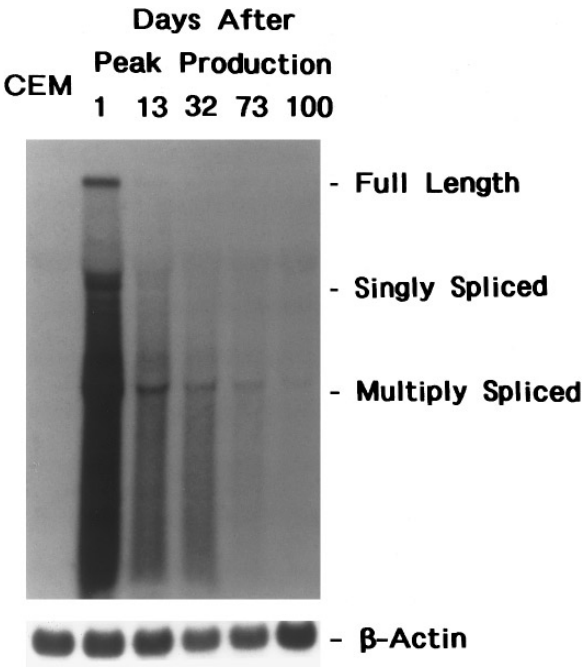

FIG. 11. Northern blot comparisons of the levels of HIV mRNA species in CEM cells during chronic infection with HIV_{213}. See Fig. 9 for details, but the stringency of hybridization was increased. Using hybridization stringency similar to that of Fig. 9 resulted in very dark smears of all lanes. Therefore, to obtain adequate visualization of the major RNA species, the hybridization stringency was increased.

tion could be latently infected, have restricted low level expression, or be continuous producers. Another report by Mustafa and Robinson (1993) studied the influence of auxiliary viral genes in determining the duration of chronic HIV expression in H9 T-cells and also showed that mass cultures transfected with the viruses displayed reproducible patterns in the rate of virus shutdown, or duration of chronic production, over a 20-day period. Thus, it appears that cell populations can be useful in studying chronic HIV infection and the processes involved in chronic shutdown or latency, but a thorough study of the cell population needs to confirm that uninfected cells are not overgrowing infected ones or that provirus is not lost from the majority of the cells.

Because of the latter possibilities, most studies of HIV latency have been performed in select cell clones which are already latently infected (Butera *et al.,* 1994; Chen *et al.,* 1994; Michael *et al.,* 1991; Pomerantz *et al.,* 1990, 1992). Although these clones have been very useful, there are inherent problems with using clones. First, do the clones represent the majority of cells in a population? These clones have demonstrated at least three mechanisms of virus shutdown. Which of these are the predominant mechanisms in the majority of chronically infected cells? Finally, does a transformed cell line chronically infected with HIV represent what occurs in normal lymphocytes?

Nevertheless, the mechanisms underlying HIV nonproductive infection have been studied to a great extent in these clones. The mechanisms leading to establishment of latent infections were conjectured from the observed changes following reactivation of the virus with cytokines or chemicals. A limitation of these studies is that the virus is already shutdown in these clones, and the process of shutdown can not be studied, only its reactivation. In addition, different HIV strains or cell types cannot be used. As we demonstrated above, different HIV isolates can employ different mechanisms and have different rates in progressing into chronic nonproductivity. The mechanisms of virus shutdown we observed in our chronically infected cell populations do appear to be similar to those found in the U1, ACH-2, or J1.1 clones. HIV_{MCK}, which went into latency at a fairly rapid rate, did not totally shut down HIV transcription, and steady-state levels of multiply spliced mRNAs remained near normal throughout the chronic infection period, even when the cells were nonproductive. However, the steady-state levels of full-length and medium-sized mRNAs did decrease during latency induction, with the full-length transcripts decreasing faster than the singly spliced transcripts. These data indicated that although transcription may be reduced somewhat during HIV_{MCK}'s decent into latency, the major mechanisms appears to be changes in the pattern of pre-mRNA splicing, with all of the mRNAs becoming completely spliced. This is similar to the mechanism of latency in the ACH-2 and U1 clones (Michael *et al.,* 1991; Pomerantz *et al.,* 1990). Likewise, similar

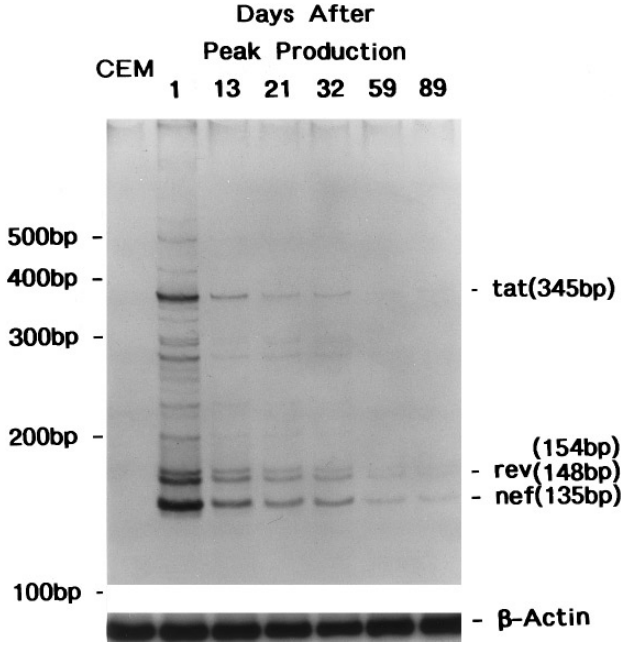

FIG. 12. Semiquantitative RT–PCR analyses of the levels of HIV *tat, rev,* and *nef* mRNAs in CEM cells chronically infected with HIV_{213}. See Fig. 10 for details.

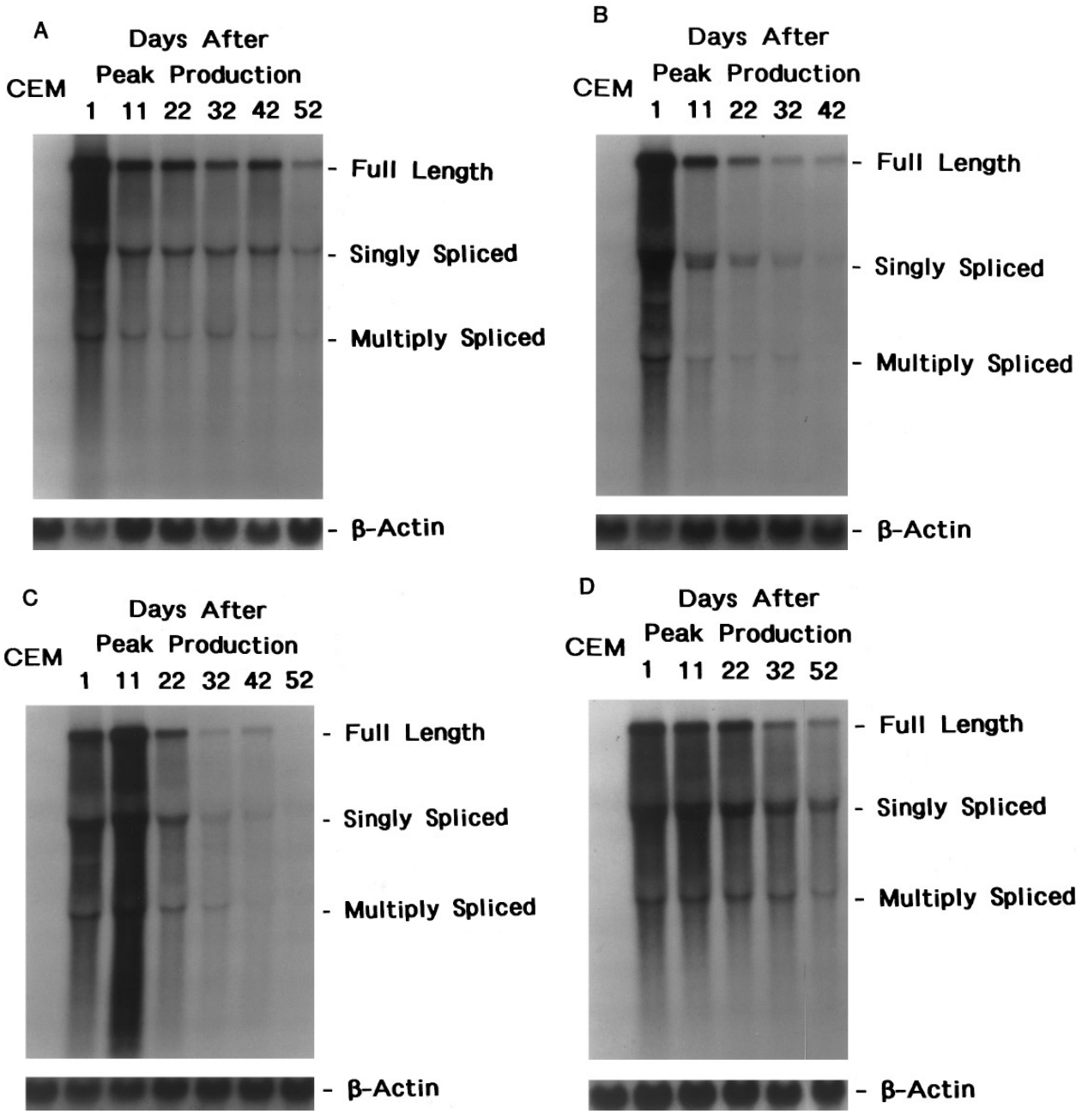

FIG. 13. Northern blot comparisons of the levels of HIV mRNA species in CEM cells during chronic infections with HIV_{RF} (A), HIV_{MN} (B), HIV_{R6} (C), and HIV_{IIIB} (D). See Fig. 9 for details.

changes in SIV mRNA splicing has been observed during chronic infection in that system (Pantaleo *et al.,* 1993; Vigliati *et al.,* 1992). Recent studies dissecting the molecular basis of this "blocked early-stage latency" observed in ACH-2 and U1 cells have shown that the mechanisms of virus shutdown appear to be different in the two cells (Michael *et al.,* 1991; Pomerantz *et al.,* 1990; Seshamma *et al.,* 1992). U1 shutdown appears to be due to suboptimal levels of functional Tat protein, because addition of Tat increased virus production. However, addition of Tat did not increase expression of latent HIV in the ACH-2 cells (Duan *et al.,* 1994). Addition of Rev expressed from a vector only marginally stimulated virus production in both U1 and ACH-2 (Duan *et al.,* 1994). Examining the cellular environment by superinfection with reporter viruses, Chen and Kim and their co-workers (Chen *et al.,* 1994; Kim *et al.,* 1993) found that U1 actually decreased reporter expression, while the ACH-2 cells drastically increased it. They concluded that U1 has a defect in the cell environment which can be described as a *trans*-effect, while HIV latency in ACH-2 cells appears to be specific for the integrated provirus and is probably a *cis*-

effect due to the site of integration (Chen *et al.,* 1994). Inherent is this system, then, is the likelihood that reduced transcription leads to complete splicing of the mRNA that is made. Another study has indicated that the site of integration may play important roles in determining whether an infected cell remains a constitutive producer or becomes a nonproducer (Winslow *et al.,* 1993), but it seems unlikely that the site of integration is playing much role in the CEM populations we studied. An idea put forth by Mustafa and Robinson (1993) stemming from their data indicating a correlation between shutdown of HIV and its cytopathicity concerns the possibility that the rate of shutdown of HIV expression could be due to opposing effects of virus-induced cytostatic effects and cellular responses to them that limit virus gene expression. We likewise suspect that HIV shutdown involves induction of cellular factors, but we do not observe that the rate of shutdown correlates with the degree of cytopathicity. HIV_{213}, which proceeded into latency at a very slow rate, was more cytopathic than HIV_{MCK}, which went into latency faster. HIV_{213} appeared to possess a different mechanism of latency, involving an apparent shutdown of transcription. This is similar to the mechanisms in OM-10.1 and J1.1 clones (Butera *et al.,* 1994). From the small sample of HIV strains tested, this mechanism of nonproductivity may be one most HIV strains use. However, examinations of PBLs from HIV-infected patients have implicated both the "blocked early-stage latency" and the HIV_{213}-type mechanisms (Arens *et al.,* 1993; Michael *et al.,* 1995; Seshamma *et al.,* 1992). RT–PCR results have shown no or reduced levels of HIV full-length transcripts in PBLs of asymptomatic patients, but as clinical symptoms progress, the levels of HIV full-length transcripts increase. Some of this may be reactivation of latent HIV, but that has not been formally proved. Further studies of the mechanisms underlying latent HIV infection in normal lymphocytes are warranted.

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