JOURNAL OF VIROLOGY, Mar. 1992, p. 1321–1328
0022-538X/92/031321-08$02.00/0

Vol. 66, No. 3

Alpha Interferon Inhibits Early Stages of the Human Immunodeficiency Virus Type 1 Replication Cycle

YASAMAN SHIRAZI[1] AND PAULA M. PITHA[1,2]*

Oncology Center[1] and Department of Molecular Biology and Genetics,[2] The Johns Hopkins University School of Medicine, Baltimore, Maryland 21231

Received 10 October 1991/Accepted 22 November 1991

In this study, we have analyzed the effect of human alpha interferon (IFN-α) on a single replication cycle of human immunodeficiency virus type 1 (HIV-1) infection in the lymphocytic cell line CEM-174, which is highly sensitive to the antiviral effects of IFN. Pretreatment of cells with 50 to 500 U of recombinant human IFN-α per ml resulted in a marked reduction in viral RNA and protein synthesis. The effect of IFN-α was dose dependent and was amplified in multiple infection cycles. IFN-induced inhibition of viral protein synthesis could be detected only when cells were treated with IFN-α prior to infection or when IFN-α was added up to 10 h postinfection, but not if IFN-α was added at the later stages of HIV-1 replication cycle or after the HIV-1 infection was already established. Analysis of the integrated HIV-1 provirus showed a marked decrease in the levels of proviral DNA in IFN-treated cells. Thus, in contrast to the previous studies on established HIV-1 infection in T cells, in which the IFN block appeared to be at the posttranslational level, during de novo infection, IFN-α interferes with an early step of HIV-1 replication cycle that occurs prior to the integration of the proviral DNA. These results indicate that the early IFN block of HIV-1 replication, which has been previously observed only in primary marcophages, can also be detected in the IFN-sensitive T cells, indicating that the early IFN block is not limited to macrophages.

Host response to viral infection involves immune-mediated events, including cellular and humoral immune responses, release of various inflammatory mediators, and induction of cytokines with antiviral properties such as those described for interferons (IFNs). IFNs are believed to play a major role in the early response against virus infection by inhibiting the replication of a wide variety of viruses, including a number of animal retroviruses and lentiviruses. The antiviral effects of IFNs are generally associated with the induction of at least two enzymatic activities: (i) the double-stranded RNA-dependent 68-kDa protein kinase that catalyzes phosphorylation of the α subunit of the protein synthesis initiation factor eIF-2, and (ii) 2′,5′-oligoadenylate synthetase (2′,5′-OAS), which in the presence of double-stranded RNA activates the latent endonuclease, RNase L, responsible for degradation of viral and cellular RNAs (12). Inhibition of viral protein synthesis observed in IFN-treated cells appears to be a consequence of induction of these two enzymatic systems. By contrast, the IFN-mediated inhibition of murine leukemia virus (MuLV) replication is probably not dependent on the induction of these two enzymes and occurs posttranslationally at the levels of assembly and maturation of virus particles (7). In IFN-treated cells, MuLV particles were poorly released from the plasma membranes, were noninfectious, and were missing the major envelope glycoprotein, gp71 (3). The molecular mechanism by which IFN alters retrovirus assembly is not well understood, but it has been suggested that the alteration in assembly may be a consequence of IFN-induced changes in the fluidity of plasma membranes (20).

IFNs have been shown to inhibit human immunodeficiency virus type 1 (HIV-1) replication both in primary cells such as peripheral blood lymphocytes and macrophages and in monocyte and T-cell lines (6, 8, 10, 15, 16, 19, 22, 36, 37). The molecular nature of this inhibition, however, appears to be cell type dependent. Thus, in de novo-infected primary macrophages, IFN was shown to interfere with an early step of the HIV-1 replication cycle by preventing formation of the HIV-1 provirus (16), although addition of IFN to HIV-1-infected primary macrophages has been shown to substantially reduce the relative levels of HIV-1 RNAs without affecting provirus formation (8). By contrast, in the majority of established T-cell lines and peripheral blood lymphocytes, IFN significantly suppressed the levels of released HIV-1 virions in the culture supernatants but did not affect viral protein synthesis (6, 22, 37). Thus, the results from a majority of these studies suggest that, similar to the effect of IFN on MuLV infection, the effect of IFN on de novo infection in T cells is primarily at the posttranslational level, although an early block in the HIV-1 replication cycle has been observed in T cells producing IFN-α (2).

We have been interested in the molecular mechanism(s) by which IFN-α mediates the restriction of HIV-1 replication in acutely infected T cells. While others have studied the effect of IFN-α on HIV-1 replication in either chronically infected T-cell lines or acutely infected cells during multiple infection cycles, we have analyzed the effect of human recombinant IFN-α (IFN-α_2) on HIV-1 replication in a single infection cycle. Using an established cell line, CEM-174, which is both highly sensitive to IFN-α and permissive to HIV-1 replication, we found that IFN-α can effectively inhibit the first replication cycle of HIV-1 by decreasing the relative levels of viral RNA and proteins in the cells. We also found that the levels of integrated HIV-1 provirus were markedly lower in IFN-treated cells than in the untreated controls, suggesting that the IFN-α-mediated interference is at the level of either proviral DNA formation or proviral DNA integration.

* Corresponding author.

MATERIALS AND METHODS

Cell culture. CEM-174 and A3.01 cells were maintained in RPMI 1640 (Life Technologies, Inc., Gaithersburg, Md.) containing 2 mM L-glutamine, 10% heat-inactivated fetal calf serum (Life Technologies), 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, and 50 μg of gentamicin per ml at a density of 3×10^5 cells per ml.

Virus stock and infection protocol. The HIV-1 virus stock was generated in A3.01 cells by electroporation of 10^7 cells with DNA (10 μg) of the infectious molecular clone of HIV-1 (pNL4-3) (1). The virus was harvested on day 7 after electroporation, and its titer was determined by infecting A3.01 cells with serial dilutions of the virus stock and determining the endpoint dilution of infectivity by the reverse transcriptase (RT) activity. The infections were performed in the presence of 8 μg of Polybrene (Sigma Chemical Co., St. Louis, Mo.) per ml. In single-cycle infection studies, CEM-174 cells (3×10^5/ml), pretreated with human recombinant IFN-α (Hoffman LaRoche, Nutley, N.J.) for 24 h, were infected with a 10^{-1} dilution of the HIV-1 stock (1.9×10^5 cpm/ml) either in the presence or in the absence of IFN-α. After an adsorption period of 2 to 5 h, virus was removed, and cells were washed three times with Hanks medium containing 0.5 mM EDTA and cultured in fresh RPMI with or without IFN-α for various times. In some experiments, IFN-α was added at various times after HIV-1 infection. Treatment with 50 to 200 U of IFN-α per ml did not inhibit the growth of CEM-174 cells, as assessed by cell count.

Metabolic labeling and pulse-chase experiments. CEM-174 cells, pretreated with IFN-α, were infected with a high dose of HIV-1 (1.9×10^5 cpm/ml) as described above. Approximately 36 h after infection, cells were washed with methionine-free RPMI 1640 containing 5% fetal calf serum, pulsed with 250 μCi of [^{35}S]methionine (1 mCi/0.088 ml; ICN, Costa Mesa, Calif.) for 30 min, resuspended in RPMI 1640 medium containing 10% fetal calf serum, and chased for various periods of time. Cell lysates as well as lysates from the culture supernatants were prepared according to Samelson et al. (28). The HIV-1 proteins were detected by immunoprecipitation using human serum containing anti-HIV-1 antibodies as described previously (34). Briefly, HIV-1 proteins were precipitated with the antibody adsorbed to protein A-agarose beads (Life Technologies). The precipitates were then washed, resuspended in sodium dodecyl sulfate (SDS) sample buffer (17), dissociated from agarose by boiling, and subjected to electrophoresis on an 8% SDS-polyacrylamide gel under reducing conditions (28). Molecular weight markers were included in all experiments. The gel was treated with an enhancer (En^{3}Hance; New England Nuclear, Boston, Mass.), dried, and autoradiographed at −70°C, using an intensifying screen.

Western immunoblot analysis of HIV-1 proteins. Uninfected and infected CEM-174 cells were washed two times with phosphate-buffered saline, and the cell pellets were lysed in a triple-detergent lysis buffer containing 50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 2 mM phenylmethylsulfonyl fluoride. Approximately 50 to 100 μg of cellular proteins was precipitated by acetone, recovered by centrifugation, dissolved in SDS sample buffer, and subjected to electrophoresis on a 10% SDS-polyacrylamide gel under reducing conditions. Proteins were transferred to a nitrocellulose filter (Millipore, Bedford, Mass.) by electroblotting, and filters were incubated with human sera containing anti-HIV-1 antibodies. Antigen-antibody complexes were then detected with ^{125}I-labeled protein A (New England Nuclear) as described previously (33).

Probes. To detect the HIV-1 transcripts, plasmid pJM105 (2) was linearized with *Bam*HI; an [α-^{32}P]GTP-labeled RNA probe was prepared by using SP6 polymerase (Promega, Madison, Wis.) as described previously (13) and used in Northern (RNA) hybridization. The HIV-1 proviral DNA was detected with DNA probes prepared from the pHXBc2 HIV plasmid (2) digested with *Bam*HI and *Eco*RI. Two HIV-1 DNA fragments of approximately 1,000 and 3,000 bp were isolated, labeled with [α-^{32}P]dCTP (Amersham, Chicago, Ill.) by random priming using an oligolabeling reaction kit (Pharmacia, Piscataway, N.J.), and used in Southern hybridization.

Preparation and analysis of total RNA. Total cellular RNA was isolated by the guanidium isothiocyanate method (5). Ten micrograms of total RNA was electrophoresed through a 0.8% agarose gel containing 7.2% formaldehyde, transferred onto nitrocellulose, and hybridized with an RNA probe labeled with [α-^{32}P]GTP (New England Nuclear). Blots were then washed under stringent conditions as described previously (18) and autoradiographed at −70°C.

Preparation and analysis of DNA. Genomic DNA was prepared as described previously (27). Genomic DNA was digested with *Eco*RI and *Bam*HI which recognize unique restriction sites within the HIV-1 long terminal repeat at nucleotides 5740 and 8470, respectively. Digested DNA was then electrophoresed on a 0.8% agarose gel and transferred to a nylon membrane (Zetaprobe; Bio-Rad, Rockville Centre, N.Y.) in 0.4 N NaOH. The hybridization and prehybridization conditions were as described previously (27).

RT assay. The magnesium-dependent RT activity in the culture supernatants of cells infected with HIV-1 was determined by a modification of the method of Willey et al. (35). Briefly, duplicate samples (25 μl) were mixed with 50 μl of RT reaction mixture containing a template primer of poly(A) and oligo(dT) (1.57 μg/ml; Pharmacia) in 50 mM Tris (pH 7.8), 7.5 mM KCl, 2 mM dithiothreitol (Sigma), 5 mM $MgCl_2$, 0.05% Nonidet P-40 (Sigma), and 5 μCi of [α-^{32}P]dTTP (3,000 Ci/mmol; ICN) and incubated for 2 h at 37°C. Fifty microliters of the reaction mixture was then applied to DE81 paper (Whatman), using a dot blot apparatus. The filter was then washed two times with 2× SSC (0.3 M sodium chloride and 0.63 M sodium citrate) and once with 95% ethanol, dried, and autoradiographed at −70°C. After exposure, radioactivity on the filters was determined by counting in a liquid scintillation counter.

RESULTS

Effect of IFN-α on HIV-1 replication in a multiple-cycle infection. We have shown that CEM-174 cells are highly sensitive to the antiviral effect of human IFN-α, since pretreatment of cells with 10 to 100 U of IFN-α per ml was able to decrease replication of vesicular stomatitis virus by 10- to 100-fold (data not shown). To determine whether IFN-α also inhibits HIV-1 replication in these cells, CEM-174 cells were pretreated with 20 to 500 U of IFN-α per ml and kept in the continuous presence of IFN-α for 7 days after HIV-1 infection, at which time the levels of virus (measured by RT activity) in the medium were determined. The results indicated a dose-dependent reduction in the levels of HIV-1 virions in the IFN-treated culture supernatants compared with the untreated control cells (Fig. 1A). In the absence of IFN-α, the levels of virus released from cells infected with

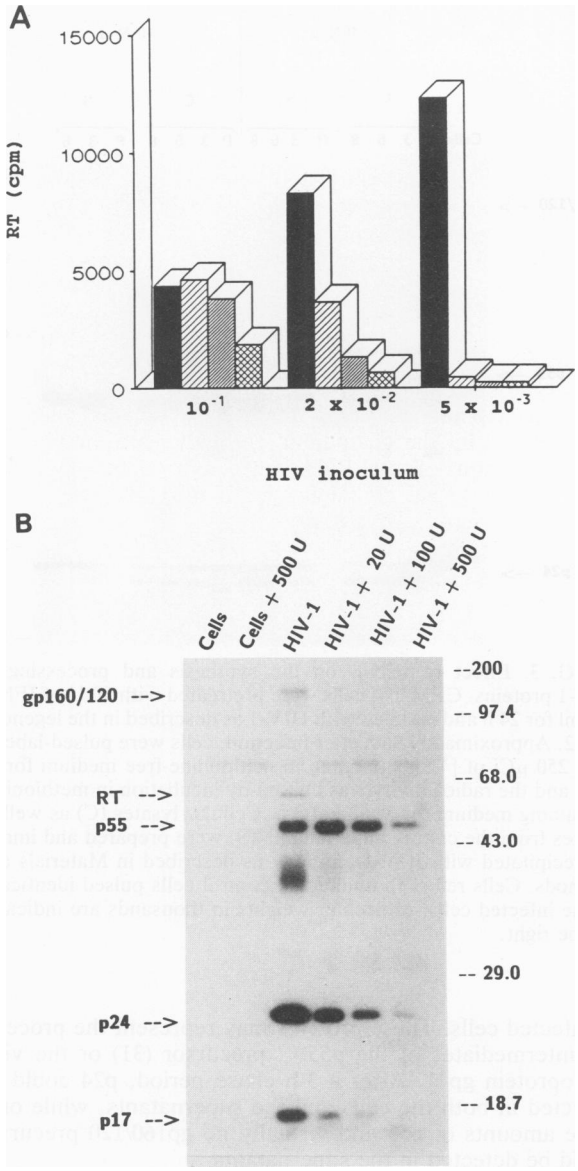

FIG. 1. Effect of IFN-α on HIV-1 infection in vitro. (A) CEM-174 cells were untreated (■) or pretreated with 20 (▨), 100 (▨), and 500 (▩) U of IFN-α per ml for 24 h, and duplicate samples were infected with various doses of HIV-1 in a 24-well tissue culture plate (3×10^5 cells per ml) (Costar). A 10^{-1} dilution of the virus stock is equivalent to 1.9×10^5 cpm/ml, 2×10^{-2} equals 1.9×10^4 cpm/ml, and 5×10^{-3} equals 4.3×10^3 cpm/ml, as measured by RT assay. Cells were refed every 3 days with fresh medium with or without IFN-α, and culture supernatants were removed on day 7 postinfection and assayed in duplicate for RT activity. Results are expressed as counts per minute per 50 μl of the reaction mixture as described in Materials and Methods. (B) Cell lysates from cells infected with HIV-1 at a dose of 2×10^{-2} (1.9×10^4 cpm/ml) were prepared and analyzed by Western blot analysis as described in Materials and Methods. Molecular weights in thousands are indicated on the right.

1.9×10^5 cpm of HIV-1 per ml were much lower than those released from cells infected with 1.5×10^4 or 4.3×10^3 cpm/ml, primarily due to cell killing by the high dose of virus. In addition, the effect of IFN treatment was indirectly related to virus inoculum, and the inhibition was significantly greater with infection of low doses of HIV-1. Removal of IFN-α from the culture medium 5 days after infection did not result in the recovery of RT activity in the medium (data not shown). To further investigate whether the reduction in the RT activity in the medium was associated with changes in the steady-state levels of viral proteins in the cells, the levels of HIV-1 proteins in cells infected with 1.9×10^4 cpm of HIV-1 per ml were determined by Western blot analysis. As shown in Fig. 1B, treatment with IFN-α led to a dose-dependent reduction of all HIV-1 proteins in the cells. IFN-α treatment did not significantly affect cell growth (data not shown).

Effect of IFN-α on HIV-1 replication in a single infection cycle. The effect of IFN-α on HIV-1 replication in multiple infection cycles may be highly amplified mainly by preventing reinfection and virus spread. Therefore, we examined the effect of IFN-α on HIV-1 replication in a single infection cycle. CEM-174 cells were pretreated with IFN-α (500 U/ml) for 24 h and infected with a high dose of HIV-1 (1.9×10^5 cpm/ml) for 5 h, at which time the residual virus was removed, and fresh IFN-α was added after virus adsorption. The levels of HIV-1 proteins and HIV-1 RNA transcripts were then analyzed at 24 and 48 h postinfection. Infection with this dose of HIV-1 led to the appearance of cell-associated HIV-1 at 36 h postinfection (see Fig. 3) and virus particles in the medium by 48 h postinfection (data not shown), indicating that the single cycle of infection was about 48 h. Figure 2A shows a Northern blot analysis of the total RNA in infected IFN-treated and untreated cells. The 9.2-kb RNAs, representing the nonspliced RNAs, and trace amounts of 4.2-kb transcripts, corresponding to the envelope mRNAs, could be detected as early as 5 h postinfection in both IFN-treated and untreated cells. We assume, however, that these transcripts represent the input virion HIV-1 RNA rather than de novo transcripts. No 2.0-kb transcripts representing the doubly spliced, nonstructural *tat*, *rev*, and *nef* mRNAs could be detected at this time. An increase in the relative levels of 9.2-kb RNAs as well as in the levels of 4.2- and 2.0-kb RNAs could be seen in infected cells at 24 and 48 h postinfection. Surprisingly, the relative increase in the spliced HIV-1 RNAs at 24 h was higher than that of the 9.2-kb RNAs, an observation somewhat similar to that reported earlier (14). By contrast, neither an increase in 9.2-kb HIV-1 RNAs nor an increase in 4.2- and 2.0-kb RNAs could be detected in IFN-treated cells at 24 h, and the relative levels of all HIV-1 transcripts at 48 h were much lower in IFN-treated cells than in the untreated control cells. These data indicate that the expression of HIV-1 provirus is significantly inhibited in IFN-treated cells. The levels of HIV-1 proteins in HIV-1-infected and IFN-treated and untreated cells are shown in Fig. 2B. The $p55^{gag}$ precursor was present in infected cells as early as 24 h postinfection; however, at this time, very little if any of this protein was processed, and no p24 and p17 proteins could be detected. At 48 h postinfection, high levels of p55 as well as of p24 and p17 could be readily detected in the infected cells. We assume that the 75- and 32-kDa proteins detected represent cross-reactivity of the human serum with cellular proteins, since these proteins were also detected in the uninfected control cells (Fig. 2B). In IFN-treated cells, the steady-state levels of all viral proteins were significantly lower than those in the untreated controls, a phenomenon also observed in peripheral blood lymphocytes infected with HIV-1 in a single infection cycle (data not shown).

Effect of IFN-α on the synthesis and processing of HIV-1

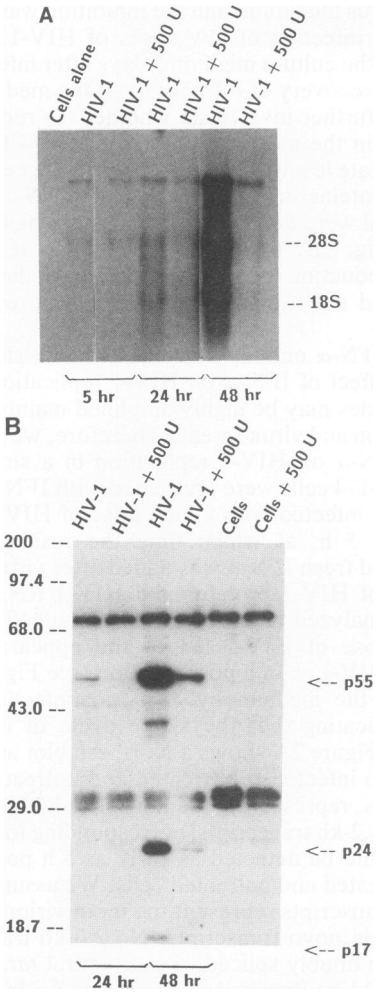

FIG. 2. Evidence that IFN-α inhibits HIV-1 RNA and protein synthesis in the first replication cycle. CEM-174 cells were pretreated with 500 U of IFN-α per ml for 24 h and infected with a high dose of HIV-1 (1.9×10^5 cpm/ml) at 37°C. Virus was removed after 5 h; cells were washed and incubated in medium with or without 500 U of IFN-α per ml for 24 and 48 h. Total RNA for Northern blot analysis (A) and cell lysates for analysis of HIV-1 proteins by Western blot (B) were prepared as described in Materials and Methods. In panel B, molecular weights in thousands are indicated at the left.

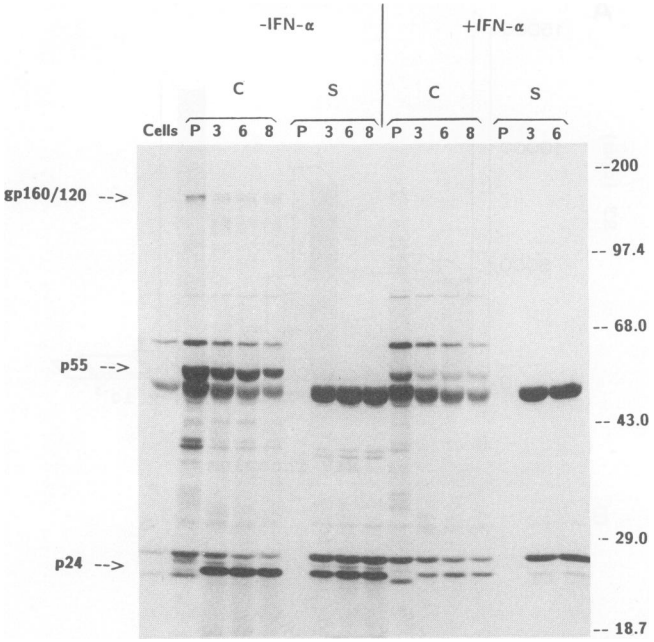

FIG. 3. Effect of IFN-α on the synthesis and processing of HIV-1 proteins. CEM-174 cells were pretreated with 50 U of IFN-α per ml for 24 h and infected with HIV-1 as described in the legend to Fig. 2. Approximately 36 h after infection, cells were pulsed-labeled with 250 μCi of [^{35}S]methionine in methionine-free medium for 30 min, and the radioactivity was chased by incubation in methionine-containing medium for 3, 6, and 8 h. Cellular lysates (C) as well as lysates from the culture supernatants (S) were prepared and immunoprecipitated with HIV-1 antibody as described in Materials and Methods. Cells refers to uninfected control cells pulsed identically to the infected cells. Molecular weights in thousands are indicated on the right.

proteins. The decrease in the levels of HIV-1 proteins in IFN-treated cells could be the consequence not only of decreased levels of HIV-1 mRNAs in these cells but also of an inhibition in the synthesis of viral proteins or their processing. To examine these possibilities, CEM-174 cells were treated with low levels of IFN-α (50 U/ml) that were not able to completely block viral protein synthesis and were infected with HIV-1 for 36 h. At this time, IFN-treated and untreated cells were pulse-labeled with [^{35}S]methionine and chased with methionine-containing medium for various lengths of time; [^{35}S]labeled viral proteins were then detected by immunoprecipitation. The pulse-chase analysis of viral proteins in the cellular lysates as well as in the supernatants of infected cells is shown in Fig. 3. During the 30-min labeling period, we could detect the synthesis of both gp160/120 and p55^{gag} precursors. In addition, two proteins of about 42 and 41 kDa were detected in infected but not in uninfected cells. These proteins may represent the processing intermediates of the p55^{gag} precursor (31) or the viral glycoprotein gp41. After a 3-h chase period, p24 could be detected in both the cells and the supernatants, while only trace amounts of p55 and virtually no gp160/120 precursor could be detected in the supernatants.

Viral protein synthesis was decreased by about threefold following IFN-α treatment with a low dose of IFN-α (50 U/ml; Fig. 3), and the inhibition was more significant with 100 and 500 U/ml (data not shown). The levels of p55^{gag} precursor in pulse-labeled IFN-treated (50 U/ml) cells were about three times lower than in the untreated infected cells; consequently, about three times lower p24 levels were detected in the 3-h chase period in IFN-treated cells. Surprisingly, in IFN-treated cells, the amounts of p24 released into the supernatants were much lower than in the infected control cells. Thus, under conditions in which the effect of IFN-α on viral protein synthesis in the cells was low (3-fold inhibition), the effect on virus assembly was more significant (about 10-fold inhibition). These data indicate that at least two mechanisms contribute to the IFN-mediated inhibition of HIV-1 replication: (i) a decrease in viral protein synthesis, reflecting the low levels of HIV-1 mRNAs (Fig. 2A), and (ii) an inhibition of p24 release from the infected cells into the supernatants, indicating inhibition of virus particle assembly or release.

Effect of IFN-α on established HIV-1 infection. To determine whether IFN can also block virus replication in CEM-

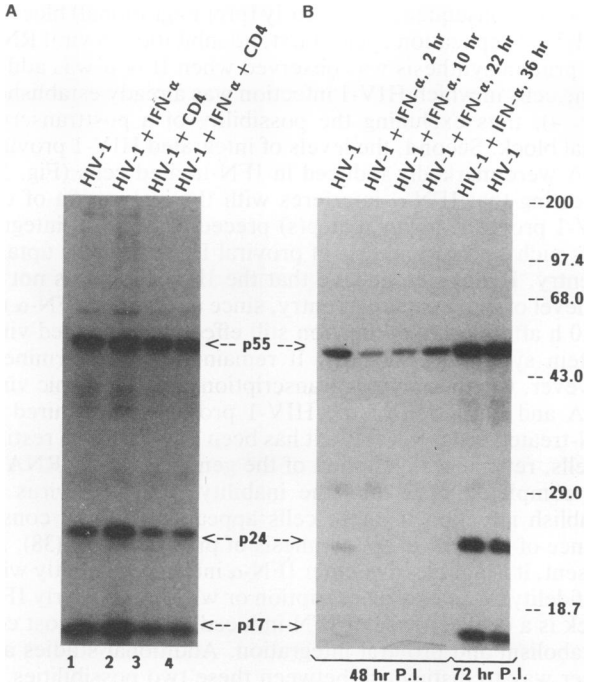

FIG. 4. Effect of IFN-α on an established HIV-1 infection. (A) CEM-174 cells were infected with HIV-1 as described in the legend to Fig. 2. Approximately 36 h after infection, cells were treated with (lanes 2 and 4) 500 U of IFN-α per ml in the presence (lanes 3 and 4) or in the absence (lanes 1 and 2) of recombinant soluble CD4 (50 μg/ml). Twenty-four hours later, cell lysates were prepared and HIV-1 proteins were detected by Western blot analysis as described in Materials and Methods. (B) CEM-174 cells were infected with HIV-1 as in panel A, and IFN-α (500 U/ml) was added at 2, 10, 22, and 36 h after infection. Cell lysates were prepared at 48 or 72 h postinfection (P.I.), and HIV-1 proteins were detected by Western blot analysis. Molecular weights in thousands are indicated on the right.

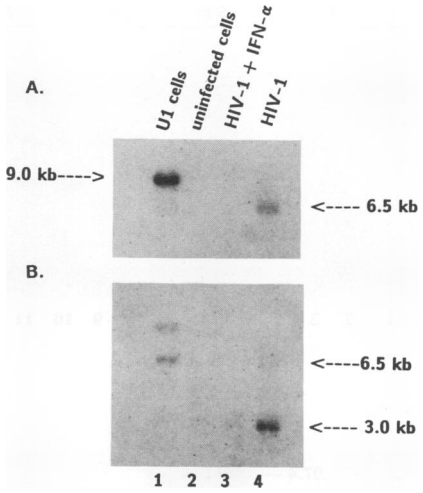

FIG. 5. Effect of IFN-α on integration HIV-1 provirus. CEM-174 cells were pretreated with 500 U of IFN-α per ml and infected with HIV-1 as described in the legend to Fig. 2. Approximately 48 h after infection, cells were harvested and genomic DNA was isolated, restricted with *Bam*HI and *Eco*RI, and analyzed by Southern hybridization using ^{32}P-labeled DNA fragments of the pHXBc2 HIV-1 DNA clone as described in Materials and Methods. Lanes: 1, DNA from the chronically infected macrophage cell line U1, which contains two copies of HIV-1 provirus per cell; 2, control, uninfected CEM-174 cells; 3, infected CEM-174 cells treated with 500 U of IFN-α per ml; 4, infected CEM-174 cells. The product of *Bam*HI-*Eco*RI digestion in lane 4 was detected as an approximately 6.5-kb fragment when the 1,000-bp probe was used (A); a 3.0-kb fragment was detected when the 3,000-bp fragment was used (B). In panel B, the faint 6.5-kb band in lane 4 represents the residual radioactivity from panel A.

174 cells in which HIV-1 infection has been already established, cells were infected with a high dose of HIV-1 (1.9×10^5 cpm/ml), and at 36 h post-infection, 500 U of IFN-α per ml was added, either in the presence or in the absence of soluble recombinant CD4 (50 μg/ml) (Biogen, Cambridge, Mass.), to block reinfection and virus spread. Cell lysates were prepared 24 h after addition of IFN-α, and the presence of HIV-1 proteins in cells was assessed by Western blot analysis. As shown in Fig. 4A, addition of IFN-α to the HIV-1-infected cells 36 h after infection did not inhibit viral protein synthesis (compare lanes 1 and 2). In addition, the presence of soluble CD4 did not significantly alter the levels of viral proteins in HIV-1-infected cells (compare lanes 1 and 3), indicating that at this stage of infection, reinfection did not play a major role. These results suggest that the effect of IFN-α on de novo infection occurs at the early stages of the HIV-1 replication cycle.

To determine precisely which of the early steps in the HIV-1 replication cycle are blocked by IFN-α, cells were infected with HIV-1 for 2 h and washed extensively to remove the residual virus, and IFN-α (500 U/ml) was added either immediately after washing (2 h) or at 10, 22, and 36 h after infection. The levels of HIV-1 proteins in cell lysates were analyzed at either 48 or 72 h postinfection. As shown in Fig. 4B, addition of IFN-α as late as 10 h postinfection resulted in a significant inhibition of $p55^{gag}$ precursor synthesis measured at 48 h. By contrast, addition of IFN-α at 22 and 36 h postinfection had no significant effects on the levels of viral proteins measured at 48 and 72 h, respectively. These results further suggest that the IFN block is at the very early stages of the HIV-1 replication cycle.

Effect of IFN-α on provirus integration. To investigate whether IFN-α interferes with the integration of the proviral DNA, IFN-treated and untreated CEM-174 cells were infected with a high dose of HIV-1 (1.9×10^5 cpm/ml), and the presence of HIV-1 provirus in the genomic DNA was analyzed by Southern hybridization at 48 h postinfection. The pNL4-3 HIV-1 provirus contains internal *Eco*RI and *Bam*HI sites at nucleotides 5740 and 8470, respectively. As shown in Fig. 5, digestion of the genomic DNA from HIV-1-infected cells (lane 4) by *Eco*RI and *Bam*HI resulted in the detection of two fragments of 6.5 and 3.0 kb. The 3.0-kb fragment represents the internal pNL4-3 fragment, while the 6.5-kb fragment corresponds to a digestion within the pNL4-3 provirus and the 3′ flanking sequences of the human DNA. In Fig. 5A, a 1-kb DNA fragment of plasmid pHXBc2 was used as a probe; in Fig. 5B, the same blot was reprobed with the 3-kb fragment of plasmid pHXBc2 (see Materials and Methods). The results indicate that treatment with IFN-α resulted in a marked reduction in the levels of the integrated proviral DNA (Fig. 5A, lane 3). The genomic DNA from the chronically infected macrophage cell line U1, which contains two copies of the proviral DNA (24), was also digested with *Eco*RI and *Bam*HI and used as a positive control (lane 1).

Induction of 2′,5′-OAS by IFN-α. Our results show that the

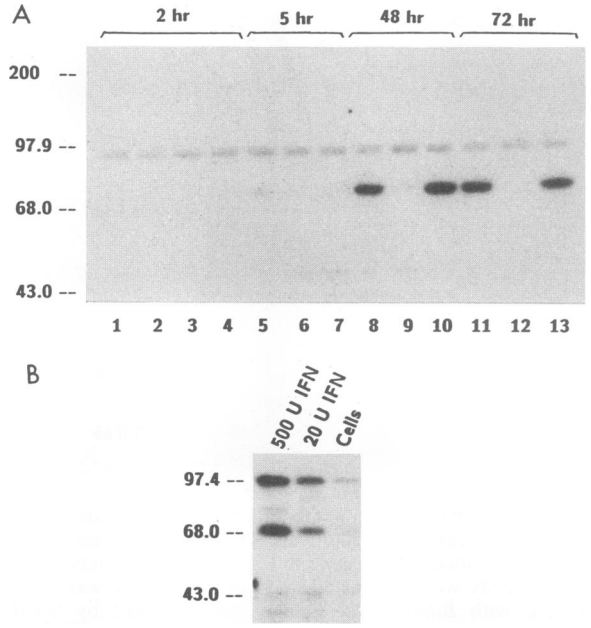

FIG. 6. Induction of 2',5'-OAS by IFN-α in CEM-174 cells. (A) CEM-174 cells were infected with HIV-1 for 2 h; cells were washed as described in Materials and Methods, and immediately after washing, 500 U of IFN-α per ml was added. Samples were collected at 2, 5, 48, and 72 h after infection, and the presence of the p68/69 species of 2',5'-OAS in cell lysates was detected by Western blot analysis using an anti-p68/69 antibody. Lanes: 1, untreated cells; 2, 5, 8, and 11, cells treated with 500 U IFN-α per ml; 3, 6, 9, and 12, cells infected with HIV-1; 4, 7, 10, and 13, cells infected with HIV-1 and treated with 500 U of IFN-α per ml. (B) CEM-174 cells were treated with 20 or 500 U of IFN-α per ml for 24 h, and 2',5'-OAS proteins were detected by Western blot analysis using a polyclonal antibody cross-reacting with all species of 2',5'-OAS. Molecular weights in thousands are indicated on the left.

replication of HIV-1 provirus is highly sensitive to the IFN-mediated inhibition in CEM-174 cells. To determine whether this high sensitivity to IFN correlates with the induction of IFN-inducible enzymes, we analyzed the levels of 2',5'-OAS enzymes in CEM-174 cells treated with 20 and 500 U of IFN-α per ml for various periods of time. Using Western blot analysis with polyclonal antibodies that recognize both the large and small species of 2',5'-OAS enzymes (4), we found that treatment of CEM-174 cells with 20 U of IFN-α per ml resulted in the induction of three species of 2',5'-OAS proteins, p40 to p46 (seen after prolonged exposure), p68/69, and p100 (Fig. 6B). Induction kinetics of the p68/69 2',5'-OAS by IFN-α was also examined by using a monoclonal antibody to p68/69 2',5'-OAS (11) (Fig. 6A). Induction of p68/69 2',5'-OAS could be detected in the cells as early as 5 h after IFN-α treatment and reached a steady state by 72 h. In contrast to a previous report (29), HIV-1 infection alone did not induce expression of 2',5'-OAS genes (Fig. 6A, lanes 3, 6, 9, and 12).

DISCUSSION

In this report, we have demonstrated that in the CEM-174 cell line, IFN-α can interfere with HIV-1 infection in the first replication cycle and that the relative levels of HIV-1 RNA transcripts and viral proteins were significantly reduced in IFN-treated cells. Two observations suggest that this inhibition is a consequence of an early (preintegrational) block in the HIV-1 replication cycle. First, no inhibition in viral RNA and protein synthesis was observed when IFN-α was added to the cells in which HIV-1 infection was already established (Fig. 4), thus excluding the possibility of a posttranscriptional block. Second, the levels of integrated HIV-1 proviral DNA were markedly reduced in IFN-treated cells (Fig. 5), indicating that IFN-α interferes with the integration of the HIV-1 provirus and/or a step(s) preceding proviral integration, such as the synthesis of proviral DNA or virus uptake or entry. Hence, we believe that the IFN-α block is not at the level of virus uptake or entry, since addition of IFN-α up to 10 h after de novo infection still effectively reduced viral protein synthesis (Fig. 4B). It remains to be determined, however, whether reverse transcription of the genomic viral RNA and formation of the HIV-1 provirus is impaired in IFN-treated cells. Recently, it has been shown that in resting T cells, reverse transcription of the genomic HIV-1 RNA is not completed, and thus the inability of HIV-1 virus to establish infection in these cells appears to be the consequence of a defect in the synthesis of proviral DNA (38). At present, it is not clear whether IFN-α interferes directly with the fidelity of reverse transcription or whether the early IFN block is a consequence of IFN-induced changes in host cell metabolism and proviral integration. Additional studies are under way to distinguish between these two possibilities.

Studies by others have suggested that the mechanisms by which IFN inhibits HIV-1 replication in T cells and macrophages may not be identical. Thus, while in infected T cells or primary lymphoblast cultures, IFN was generally found to inhibit virus assembly and release, in persistently infected primary macrophages, IFN appears to interrupt an early step of the HIV-1 replication cycle. In these cells, IFN-α reduced the relative levels of viral RNA and proteins without affecting the levels of integrated proviral DNA (8). In addition, in acutely infected primary macrophages, IFN also decreased the levels of integrated provirus (15, 16). However, this early effect of IFN was limited to the primary macrophages and has not been observed in monocyte cell lines such as U937 cells. The data presented in this study indicate that the early IFN block of HIV-1 replication is not limited to cells of the monocyte/macrophage origin but can also be observed in T cells that are sensitive to IFN. Since most of the T-cell lines used for HIV-1 propagation in vitro, as well as U937 cells, are rather insensitive to the antiviral effect of IFN (unpublished results), we suggest that the differential effects of IFN on the early and late stages of HIV-1 replication cycle are determined not by the cell type but rather by their sensitivity to the antiviral effects of IFN. Furthermore, the inability to detect an early IFN effect in T cells may also be due to the fact that all of the previous studies measured virus replication in a multiple infection cycle. We have recently shown that IFN-α enhances degradation and processing of the 9.2-kb genomic HIV-1 RNA and increases the levels of spliced HIV-1 mRNAs in cells containing latent HIV-1 provirus (23). Thus, in addition to its ability to inhibit an early step of the HIV-1 replication cycle, IFN-α appears to affect HIV-1 replication also at the posttranscriptional level.

While treatment of CEM-174 cells with IFN-α resulted in a general decrease in the synthesis of all HIV-1 proteins (Fig. 2B), we have not observed any significant effect on the processing of viral polypeptides (Fig. 3). However, an inhibition of viral p24 release into the culture supernatants was noted even under conditions in which the effect on viral protein synthesis was only marginal (Fig. 3). Previously, an

inhibition of virus particle release was observed in IFN-α-treated T cells chronically infected with HIV-1 (22, 26, 32), and this inhibition was observed in the absence of IFN effect on viral protein synthesis. Recent studies have shown that the C-terminal region of the HIV-1 capsid precursor contains sequences that are critical for viral assembly and release from the cell surface (9). Particularly, mutations in the p6 region resulted in a reduction in virus p24 antigen release, a phenomenon that was mimicked by IFN-α treatment. Additional work will be required to determine the molecular mechanism(s) by which IFN-α affects assembly of HIV-1 virions and their release from the cells, during both de novo and chronic infections.

The role of IFN-induced enzymes such as 2′,5′-OAS and double-stranded RNA-dependent protein kinase in the regulation of HIV-1 infection has been implicated (29, 30). Our results indicate that the induction of 2′,5′-OAS in CEM-174 cells (Fig. 6) was not sufficient to inhibit HIV-1 replication in cells in which HIV-1 infection was already established (Fig. 4). It is possible, however, that the observed inhibition of provirus integration in IFN-treated cells is a consequence of the degradation of the input virion-associated genomic RNA mediated by the 2′,5′-OAS-activated RNase L. Interestingly, in A3.01 T-cell lines, IFN-α (500 U/ml) neither induced 2′,5′-OAS production nor inhibited HIV-1 replication in a single infection cycle (data not shown). Thus, the inability of IFN-α to inhibit an early step of HIV-1 replication in A3.01 cells correlates with its inability to induce 2′,5′-OAS enzymes. Further work will be needed to clarify the specific roles and targets of the 2′,5′-OAS system in the inhibition of HIV-1 replication by IFN-α.

In summary, several major differences have emerged between the effects of IFN on MuLV and HIV-1 replication. In contrast to the murine retrovirus system, in which the major IFN effect is at the posttranslational level in both de novo and established infections (3, 21, 25), the IFN-mediated block of HIV-1 replication occurs at multiple levels and it is at least partially determined by the sensitivity of the cells to the antiviral effect of IFN. These observations suggest that IFN may have a broad spectrum of action in vivo and that its effect may be the result of both the anticellular and antiviral activities.

ACKNOWLEDGMENTS

We thank J. Chebath and A. Hovanessian for gifts of the anti-2′,5′-OAS antibodies and M. Martin for the pNL4-3 HIV-1 clone. We are especially grateful to Ron Willey for technical advice on immunoprecipitation of HIV-1 proteins. We also express our appreciation to J. Pozsgay for critical reading of the manuscript, B. Schneider for excellent secretarial assistance, and J. Simkins for preparation of plasmids.

This work was supported by Public Health Service grant AI-26123, American Foundation for AIDS Research/Michael Chernow Trusts research grant 000947-7-RGR to P.M.P., and award FG-882-A-1 from the National Multiple Sclerosis Society to Y.S.

REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Robson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59**:284–291.
2. **Bednarik, D. P., J. D. Mosca, N. B. K. Raj, and P. M. Pitha.** 1989. Inhibition of human immunodeficiency virus (HIV) replication by HIV-transactivated α_2-interferon. Proc. Natl. Acad. Sci. USA **86**:4958–4962.
3. **Bilello, J. A., N. A. Wivel, and P. M. Pitha.** 1982. Effect of interferon on the replication of MCF virus in murine cells: synthesis, processing, assembly, and release of viral proteins. J. Virol. **43**:213–222.
4. **Chebath, J., P. Benech, A. Hovanessian, J. Galabru, and M. Revel.** 1987. Four different forms of interferon-induced 2′,5′-oligo(A) synthetase identified by immunoblotting in human cells. J. Biol. Chem. **262**:3852–3857.
5. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
6. **Dolei, A., A. Fattorossi, R. D'Amelio, F. Aiuti, and F. Dianzani.** 1986. Direct and cell-mediated effects of interferon-α and -γ on cells chronically infected with HTLV-III. J. Interferon Res. **6**:543–549.
7. **Friedman, R. M., and P. M. Pitha.** 1984. The effect of interferon on membrane-associated viruses, p. 319–341. *In* R. M. Friedman (ed.), Interferons, vol. 3. Elsevier Science Publishers, B.V., Amsterdam.
8. **Gendelman, H. E., L. M. Baca, J. Turpin, D. C. Kalter, A. Hansen, J. M. Orenstein, C. W. Dieffenbach, R. M. Friedman, and M. S. Meltzer.** 1990. Regulation of HIV replication in infected monocytes by IFN-α. Mechanisms for viral restriction. J. Immunol. **145**:2669–2676.
9. **Gottlinger, H. G., T. Dorfman, J. G. Sodroski, and W. A. Haseltine.** 1991. Effect of mutations affecting the p6 *gag* protein on human immunodeficiency virus particle release. Proc. Natl. Acad. Sci. USA **88**:3195–3199.
10. **Hartshorn, K. L., D. Neumeyer, M. W. Vogt, R. T. Schooley, and M. S. Hirsch.** 1987. Activity of interferons alpha, beta, and gamma against human immunodeficiency virus replication in vitro. AIDS Res. Hum. Retroviruses **3**:125–133.
11. **Hovanessian, A. G., A. G. Laurent, J. Chebath, J. Galabru, N. Robert, and J. Svab.** 1987. Identification of 69-kd and 100-kd forms of 2-5A synthetase in interferon-treated human cells by specific monoclonal antibodies. EMBO J. **6**:1273–1280.
12. **Johnston, M. I., and P. F. Torrence.** 1984. The role of interferon induced proteins, dsRNA and 2′,5′-oligoadenylate in the interferon-mediated inhibition of viral translation, p. 189–298. *In* R. M. Friedman (ed.), Interferons, vol. 3. Elsevier Science Publishers, B.V., Amsterdam.
13. **Kelley, K. A., and P. M. Pitha.** 1985. Differential effect of poly rI.rC and Newcastle disease virus on the expression of interferon and cellular genes in mouse cells. Virology **147**:382–393.
14. **Kim, S., R. Byrn, J. Groopman, and D. Baltimore.** 1989. Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential gene expression. J. Virol. **63**:3708–3713.
15. **Kornbluth, R. S., P. S. Oh, J. R. Munis, P. H. Cleveland, and D. D. Richman.** 1989. Interferons and bacterial lipopolysaccharide protect macrophages from productive infection by human immunodeficiency virus in vitro. J. Exp. Med. **169**:1137–1151.
16. **Kornbluth, R. S., P. S. Oh, J. R. Munis, P. H. Cleveland, and D. D. Richman.** 1990. The role of interferons in the control of HIV replication in macrophages. Clin. Immunol. Immunopathol. **54**:200–219.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Lieberman, A. P., P. M. Pitha, H. S. Shin, and M. L. Shin.** 1989. Production of tumor necrosis factor and other cytokines by astrocytes stimulated with lipopolysaccharide or a neurotropic virus. Proc. Natl. Acad. Sci. USA **86**:6348–6352.
19. **Michaelis, B., and J. A. Levy.** 1989. HIV replication can be blocked by recombinant human interferon beta. AIDS **3**:27–31.
20. **Pitha, P. M.** 1980. The effects of interferon in mouse cells infected with MuLV. Ann. N.Y. Acad. Sci. **350**:301–313.
21. **Pitha, P. M., W. P. Rowe, and M. N. Oxman.** 1976. Effect of interferon on exogenous, endogenous and chronic murine leukemia virus infection. Virology **70**:324–338.
22. **Poli, G., J. M. Orenstein, 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.

23. **Popik, P., and P. M. Pitha.** Submitted for publication.
24. **Rabson, A. (Center for Advanced Biotechnology and Medicine).** 1991. Personal communication.
25. **Riggin, C. H., and P. M. Pitha.** 1982. Effect of interferon on the exogenous friend murine leukemia virus infection. Virology **118**:202–213.
26. **Rodriguez-Ortega, M., and P. M. Pitha.** Unpublished data.
27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., p. 9.14–9.55. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
28. **Samelson, L. E., J. B. Harford, and R. D. Klausner.** 1985. Identification of the components of the murine T cell antigen receptor complex. Cell **43**:223–231.
29. **Schroder, H. C., R. Wenger, Y. Kuchino, W. E. G. Muller.** 1989. Modulation of nuclear matrix-associated 2′,5′-oligoadenylate metabolism and ribonuclease L activity in H9 cells by human immunodeficiency virus. J. Biol. Chem. **264**:5669–5673.
30. **SenGupta, D. N., and R. H. Silverman.** 1989. Activation of interferon-regulated, dsRNA-dependent enzymes by HIV-1 leader RNA. Nucleic Acids Res. **17**:969–978.
31. **Smith, A. J., M.-I. Cho, M.-L. Hammarskjold, and D. Rekosh.** 1990. Human immunodeficiency virus type I $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ expressed from a simian virus 40 late replacement vector are efficiently processed and assembled into viruslike particles. J. Virol. **64**:2743–2750.
32. **Smith, M. S., R. J. Taresher, and J. Pagano.** 1991. Inhibition of human immunodeficiency virus type 1 morphogenesis in T cells by alpha interferon. Antimicrob. Agents Chemother. **35**:62–67.
33. **Strebel, K., T. Klimkat, and M. A. Martin.** 1988. A novel gene of HIV, *vpu*, and its 16-kilodalton product. Science **241**:1221–1223.
34. **Willey, R. L., J. S. Bonifacino, B. J. Potts, M. A. Martin, and R. D. Klausner.** 1988. Biosynthesis, cleavage, and degradation of the human immunodeficiency virus 1 envelope glycoprotein, gp160. Proc. Natl. Acad. Sci. USA **85**:9580–9584.
35. **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.
36. **Yamada, O., N. Hattori, T. Kurimura, M. Kita, and T. Kishida.** 1988. Inhibition of growth of HIV by human natural interferon in vitro. AIDS Res. Hum. Retroviruses **4**:287–294.
37. **Yamamoto, J. K., F. Barre-Sinoussi, V. Bolton, N. C. Pedersen, and M. B. Gardner.** 1986. Human alpha- and beta-interferon but not gamma- suppress the *in vitro* replication of LAV, HTLV-III, and ARV-2. J. Interferon Res. **6**:143–152.
38. **Zack, J. A., S. J. Arrigo, S. R. Weitsman, A. S. Go., A. Haislip, and I. S. Y. Chen.** 1990. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell **61**:213–222.