Hydroxyurea and Interleukin-6 Synergistically Reactivate HIV-1 Replication in a Latently Infected Promonocytic Cell Line via SP1/SP3 Transcription Factors*

Raphael M. Oguariri, Terrence W. Brann, and Tomozumi Imamichi†

From the Laboratory of Human Retrovirology, Clinical Services Program, Science Applications International Corporation-Frederick Inc., NCI-Frederick, National Institutes of Health, Frederick, Maryland 21702

The existence of viral latency limits the success of highly active antiretroviral therapy. With the therapeutic intention of reactivating latent virus to induce a cure, in this study we assessed the impact of cell synchronizers on HIV gene activation in latently infected U1 cells and investigated the molecular mechanisms responsible for such effect. Latently infected U1 cells were treated with 10 drugs including hydroxyurea (HU) and HIV-1 replication monitored using a p24 antigen capture assay. We found that HU was able to induce HIV-1 replication by 5-fold. HU has been used in the clinical treatment of HIV-1-infected patients in combination with didanosine; therefore, we investigated the impact of HU on HIV-1 activation was enhanced by 90-fold, whereas TNF-α-mediated activation was inhibited by >30%. A reporter gene assay showed that HU and IL-6 synergized to activate HIV promoter activity via the Sp1 binding site. Electrophoretic mobility shift and supershift assays revealed increased binding of the Sp1 and Sp3 transcription factors to this region. Western blot analysis showed that HU and IL-6 co-stimulation resulted in increased levels of Sp1 and Sp3 proteins. In contrast, treatment with HU plus TNF-α down-regulated the expression of NF-κB. These findings suggest that Sp1/Sp3 is involved in controlling the HU/IL-6-induced reactivation of HIV-1 in latently infected cells.

Hydroxyurea (HU)2 or hydroxycarbamide is a ribonucleotide reductase inhibitor and has been extensively used in medical practice including treatment of chronic leukemia and sickle cell anemia (1). HU has been reported to possess antiretroviral activity due to its ability to deplete the intracellular dNTP pool, thereby directly inhibiting viral DNA synthesis (2–5). It is thought that by reducing endogenous levels of deoxynucleoside triphosphates, HU may potentiate the effects of certain reverse transcriptase inhibitors (6). However, HU alone is not potent enough to reduce initial viral load in treatment-naïve patients (7); however, in combination with didanosine (ddI) HU has been shown to induce substantial and durable reductions in human immunodeficiency virus (HIV) RNA levels (5, 8–12). As a result, the HU plus ddI combination has been proposed as maintenance therapy in patients on prolonged successful highly active antiretroviral therapy (HAART) (12).

HIV-1 encodes a transactivator protein, Tat (13), that stimulates transcription elongation through interaction with a transactivation-responsive element located at the 5’ end of the nascent transcript (14). High level expression of HIV requires transactivation by Tat (15). Sp1 is one member of a multigene family (16), has been shown to contribute significantly to the expression of the HIV-1 LTR (17), and plays a pivotal role in Tat activation of LTR-driven transcription (18). Post-translational modification of Sp1 through glycosylation and phosphorylation (19), rather than absolute increase in protein levels, has also been implicated to be important to HIV-1 gene expression (20).

The persistence of latent HIV-infected cellular reservoirs despite prolonged treatment with HAART represents the major hurdle to virus eradication. These latently infected cells are a permanent source for virus reactivation and lead to a rebound of the virus load after interruption of HAART (21, 22). The cellular reservoirs for HIV-1 infection could be macrophages (23) or CD4+ T lymphocytes not fully activated, which carry the integrated provirus in a non-replicative state until the activation process is complete (24). The reservoir that appears to be the major barrier to eradication is composed of latently infected cells that carry an integrated provirus that is transcriptionally silent (25, 26). The extremely long half-life of these cells combined with a tight control of HIV-1 expression has been reported (24) to make the reservoir ideally suited to maintain hidden copies of the virus, which are in turn able to trigger a novel systemic infection upon discontinuation of therapy. At the cellular level, preintegration and postintegration latency have been described in HIV-1 (27). The U1 promonocytic cell line is one of the most thoroughly characterized models of postintegration latency. The U1 cell line was derived from a

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population of U937 cells and harbors two copies of integrated proviruses (28, 29). In the absence of stimulation, U1 cells maintain a pattern of low viral mRNA expression. Several studies have shown that the relative state of latency in U1 cells is as a result of defective Tat, the HIV transactivating protein (30, 31) One rational strategy of purging the latent HIV reservoir is to understand the molecular mechanisms regulating viral latency and reactivation (32).

To activate latently infected HIV in vitro, proinflammatory cytokines such as granulocyte macrophage colony-stimulating factor, interleukin (IL) 3, IL-6, and tumor necrosis factor (TNF)-α have been used (28, 29). These agents have been shown to activate HIV-1 expression in U1 cells by affecting distinct steps of the virus life cycle, including NF-κB-dependent transcription in the case of TNF-α and phosphol 12-myristate 13-acetate (33, 34) or a posttranscriptional event(s) in cells stimulated with IL-6 (35).

Our laboratory previously reported that a low concentration of actinomycin D, a known transcription inhibitor and a cell cycle synchronizer, induces HIV-1 activation in latently infected cells and HTLV-1-transformed cells (36, 37). To further understand the mechanism of HIV gene activation, we investigated the impact of another synchronizer, HU, on HIV-1 gene activation in latently infected U1 cells. We demonstrate here for the first time that HU activates HIV-1 replication in latently infected U1 cells and synergizes with IL-6 to enhance HIV-1 gene reactivation and that binding of the Sp1 and Sp3 transcription factors to the LTR promoter is critical for this synergism.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—The promonocytic cell line, U1 (28), was obtained from the AIDS Research and Reference Reagent Program, NIAID, National Institutes of Health (Rockville, MD). U1 cells were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan UT), 10 mm l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Quality Biologics Inc., Gaithersburg, MD). HU was purchased from Calbiochem. Recombinant IL-6 and recombinant TNF-α were purchased from R&D systems (Minneapolis, MN).

**Plasmid Constructions**—The 5′ LTR region of HIV was subcloned upstream of luciferase reporter gene as follows. MluLTR5 (5′-ACGCGTTCAGGCTATCATCCGCTATTTT) and HindLTR3 (5′-AAGCGTTCAGGCTATCATCCGCTATTTT) primers were used for amplification of full-length of the 5′ LTR from a cloned proviral pNL4.3 (obtained from M. Martin through the AIDS Research and Reference Reagent Program) (38). The amplified product was purified and cloned into the TOPO TA cloning pCR2.1 vector (Invitrogen). The resultant clone, pCRLTR, was then digested with MluI and HindIII, and a 0.6-kbp fragment containing the LTR region was subcloned into the corresponding sites of similarly digested pGL3-Basic vector (Promega, Madison, WI). The resultant clone is named p461. To construct a clone (designated as p470) lacking the sequence upstream of the NF-κB binding site from the full-length of 5′ LTR, pCRLTR was digested with MluI and then an inverted PCR was performed using Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) with a primer set of MluR (5′-TAGCGTAGCCGAATTCAGCCGTAATCCGACAC-3′) and NF-κB (5′-GGAACTTTCCGCTGGGAGCTT-3′). PCR product was purified, treated with T4 kinase (Promega), and then self-ligated using PanVera Takara ligation kit II (Madison, WI). The intended clone was digested with MluI and HindIII, and the 180-bp fragment was subcloned into the pGL3-Basic vector. Using the same procedure, clones lacking the upstream sequence from Sp1 site (p5006) or TATA box (p5007) were constructed from pCRLTR using primer sets of the MluR and Sp1F (5′-GGCGGCGCTGGGAGTGCCGAG3′) or MluR and TATAF (5′-CATAAGCTGCGTGTCTCTGTTG-3′). To construct a Tat expression plasmid, the full-length of Tat gene including intron region in pNL4.3 was PCR amplified using the primers FTAT/F (5′-ATGGAGCCAGTGATCTCAGT-3′(sense)) and FTAT/R (5′-ATATTCCTTGGGCCGTGTCCGG-3′(antisense)). A 2585-bp PCR product obtained was cloned into pcDNA4/HisMax TOPO vector (Invitrogen). To delete the intron region in the PCR product (216-2539 nucleotides), an inverted PCR was performed using Pfu Turbo DNA polymerase (Stratagene) with the primer set Tat/R (5′-TGCTTTGATAGAGAAGCTTGATG) and Tat/F (5′-ACCACCTCCCAATCCCGAG-3′). The PCR product was purified and treated with T4 kinase (Promega) and then self-ligated using the PanVera Takara ligation kit II (Madison, WI). The resultant clone named p494 was verified by Western blot to confirm the expression of Tat protein. All the constructs were confirmed by nucleotide sequencing.

**HIV Replication Assays**—Latently infected U1 cells were seeded at 2.0 × 10⁵/ml. All conditions were performed in 96-well microplates for 4 days at 37 °C. On day 4, cell-free supernatants were collected, and virus replication was measured using a p24 antigen capture assay (Beckman-Coulter, Miami, FL). Each culture was performed in quadruplicate. Results are the means of three independent experiments. For cytokine assays, U1 cells were seeded at 2.0 × 10⁵/ml and stimulated with HU (200 μM), IL-6 (15 ng/ml), TNF-α (5 ng/ml), or a combination of HU and IL-6 or HU and TNF-α. Replication assays were carried out as above. HIV-1 replication kinetics was performed as previously described (36). Briefly, U1 cells were cultured at 2.0 × 10⁵/ml in the presence or absence of stimulation. Culture supernatants were collected every day for 4 days, and HIV-1 replication was monitored using the p24 antigen capture assay.

**Cell Cycle Analysis**—U1 cells were treated with 200 μM HU at 37 °C for 24, 48, 72, or 96 h and washed with phosphate-buffered saline. Cell cycle analysis was performed as previously described (36). Briefly, cells were fixed by the addition of 70% ethanol. The pellet was washed with phosphate-buffered saline and treated with DNase-free RNase (Roche Applied Science) at 37 °C for 15 min. Propidium iodide was added to the cell suspension, which was then incubated on ice for at least 30 min. The stained cells were analyzed for red fluorescence (FL3) on a Coulter XL flow cytometer (Beckman-Coulter) with double discrimination achieved with an amorphous gate based on linear and peak FL3 signal, and the distribution of cells in the G₁, S, and G₂/M phases of the cell cycle was calculated from the resulting DNA histogram with Multicycle AV software, based...
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on a zero order polynomial S-phase model (Phoenix Flow Systems, San Diego, CA).

**Enzyme-linked Immunosorbent Assay**—Levels of IL-6 and TNF-α production in culture supernatants were quantified using enzyme-linked immunosorbent assay kits for human IL-6 and human TNF-α (R&D systems), respectively, according to the manufacturer’s instructions.

**Northern Blot Analysis**—Total cellular RNA was extracted at 48 or 72 h from treated and untreated cells using the RNeasy kit (Qiagen). A total of 30 μg of RNA was loaded per lane and separated on a 1.2% agarose, formaldehyde gel and transferred to a Nytran SuperCharge nylon membrane using the TurboBlotter System (Schleicher & Schuell). After transfer, RNA was cross-linked onto the membrane by UV irradiation. Gene-specific DNA fragments were confirmed by DNA sequencing and specific DNA fragments were confirmed by DNA sequencing and specific DNA fragments were confirmed by DNA sequencing and specific DNA fragments were confirmed by DNA sequencing.

**Western Immunoblot Assays**—U1 cells were transiently transfected with Tat plasmid (p494) and either p461, p5006, or p5007 reporter constructs and cultured in the presence or absence of HU plus IL-6 for 48 h. Cells were washed, lysed, and assayed for luciferase activity using the Luciferase Assay System (Promega). Luciferase activity was normalized by total cellular protein measured with the BCA protein assay kit (Pierce).

**Electrophoretic Mobility Shift Assays (EMSAs)**—Nuclear extracts were prepared from U1 cells treated or untreated with HU (200 μM) IL-6 (15 ng/ml), TNF-α (5 ng/ml), or a combination of HU plus IL-6 or HU plus TNF-α according to the manufacturer’s instructions (Active Motive, Carlsbad, CA), and protein concentrations were determined by the BCA assay. Sense and corresponding antisense synthetic oligonucleotides were annealed to form double-stranded oligonucleotides. Sp1 consensus and HIV-specific Sp1 oligonucleotides were end-labeled with [γ-32P]ATP (Amersham Biosciences) using T4 polynucleotide kinase (Promega). A total of 10 μg of nuclear extract was first incubated at room temperature for 10 min in binding buffer (Promega) in the absence of the probe. A 50-fold excess of cold probe or AP2 oligonucleotides was added to the reaction mixture as specific and nonspecific competitor DNAs, respectively. 20,000 cpm of probe was then added to the mixture and incubated at room temperature for 20 min. For the supershift assay, polyclonal antibodies against Sp1, Sp3, STAT3, and C/EBP (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the reaction mixture and incubated for 30 min on ice before the addition of radiolabeled probe. Samples were subjected to electrophoresis on native 6% polyacrylamide gels (Invitrogen). The gel was dried and exposed to x-ray film for 20 min at ~80 °C.

**Reverse Transcriptase (RT)-PCR**—U1 cells were seeded at 2.0 x 10^5/ml, treated or untreated with 200 μM HU and 15...
ng/ml IL-6, and cultured at 37 °C. Total cellular RNAs were isolated at 24 and 48 h using RNeasy kit (Qiagen). After DNase treatment, the RNA was reverse-transcribed using the SuperScript First-Strand Synthesis system for RT-PCR according to the manufacturer’s instructions (Invitrogen). The cDNA was amplified by PCR using the Expand High Fidelity PCR System (Roche Applied Science). Sp1 gene-specific primers used were sense, 5'-TGT TGG CAA GAC GGG CAA TG-3', and antisense, 5'-GGA AAA AGA CTT CCG AGG GTA GC-3', and antisense, 5'-GCA AGG TGG TCA GTC ATA AAG C-3'. β-Actin gene-specific primers were used as the internal control. PCR conditions were 94 °C for 2 min, 30 cycles (30 s at 94 °C, 30 s at 55 °C, and 45 s at 72 °C), then 7 min at 72 °C. PCR-generated fragments were verified on 1.2% agarose gel. The PCR products were confirmed by DNA sequence.

Real-time Quantitative PCR—As above, U1 cells were mock-treated or treated with HU and IL-6, and total RNAs were extracted after 24 and 48 h. RNAs were DNase-treated and reverse-transcribed using the TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). Real-time quantitative PCR was performed using iCycler iQ real-time PCR detection system (Bio-Rad) employing a TaqMan Universal PCR Master Mix (Applied Biosystems). The oligonucleotide primers used for the detection of Sp1, Sp3, or glyceraldehyde-3-phosphate dehydrogenase were obtained from TaqMan gene amplification assays (Applied Biosystems). In each instance the amount of real-time quantitative PCR product for the genes of interest (Sp1 and Sp3) was normalized to the amount of the reference gene glyceraldehyde-3-phosphate dehydrogenase in the same sample.

RESULTS

**HU Activates HIV-1 Replication in the Chronically Infected Promonocytic Cell Line, U1**—U1 cells, an HIV-1 latently infected cell line, harbors two integrated provirus DNA copies (28, 29). In the absence of stimulation with certain stimulants, culturing of U1 cells leads to little virus expression. To investigate the molecular process that leads to HIV-1 reactivation, U1 cells were treated with 10 known drugs (actinomycin D, N-acetyl-Leu-Leu-norleucinal, butyrolactone, trichostatin A, paclitaxel, dexamethasone, acyclovir, tunicamycin, rapamycin, or HU) for 7 days. HIV-1 replication was monitored by measuring p24 antigen in the cell culture supernatant. Of the drugs tested, only actinomycin D
and HU induced HIV-1 activation by 5–7-fold (Fig. 1A). Because HU has been used in clinical treatment of HIV-infected patients in combination with ddl, a nucleotide reverse transcriptase inhibitor, we further investigated in detail the molecular mechanisms of HU-induced HIV-1 reactivation in U1 cells. U1 cells were mock-treated or treated with increasing concentrations of HU (0, 12.5, 25, 50, 100, 200, 400, and 800 μM) and assayed for p24 antigen in culture supernatant after 4 days of culture. Results presented in Fig. 1B showed that HU induced HIV reactivation in dose-dependent manner. At 200 μM HU, enhanced the reactivation by 7-fold compared with control. Concentration of HU greater than 800 μM did not induce the reactivation (data not shown). This was due to cytotoxicity. Because the highest increase in replication was observed at 200 μM HU, this concentration was, therefore, used in all stimulations with HU in the present study. It is known that HU is able to induce cell cycle arrest. To determine whether HU induced an arrest in U1 cells, cell cycle analysis was performed. U1 cells were treated or untreated with HU (200 μM), cultured for 24, 48, 72, or 96 h, and subjected to the analysis. Our data showed an overall increase in the S and G2/M phases of the cell cycle and no arrest at the G1 phase (Fig. 2).

HU Enhances IL-6-mediated HIV-1 Activation, Suppresses TNF-α—Several proinflammatory cytokines, for example IL-6 and TNF-α, induce HIV-1 activation in U1 cells (28, 33–35). To investigate the impact of HU on HIV-1 activation in the presence of these cytokines, U1 cells were stimulated or unstimulated with HU, IL-6, or HU plus IL-6 and in parallel with TNF-α or HU plus TNF-α for 4 days. HIV-1 replication was detected by measuring p24 antigen in the culture supernatant. As shown in Fig. 3A, HU or IL-6 alone enhanced p24 to 13 or 18 ng/ml (~13–18-fold untreated cells), respectively, whereas a combination of HU plus IL-6 at the same concentrations resulted in an increase in p24 to 90 ng/ml (~90-fold compared with the untreated control). As shown in Fig. 3B, TNF-α alone induced HIV replication in latently infected U1 cells by ~500-fold compared with the unstimulated control. Surprisingly, in the presence of HU, the TNF-α-mediated HIV induction was inhibited by >30%. This indicates that HU synergizes with IL-6 in reactivating and enhancing HIV-1 replication, whereas it suppresses TNF-α-mediated HIV-1 activation. Fig. 4 shows the kinetic study of these co-stimulations (HU plus IL-6 or HU plus TNF-α). Whereas the synergistic effect of HU on IL-6 peaks at 48 h after stimulation (Fig. 4A), its inhibitory effect on TNF-α-mediated activation starts immediately after stimulation (Fig. 4B).

HU and IL-6 Enhance HIV-1 Transcription—Having established that HU synergizes with IL-6 to up-regulate viral replication and partially inhibits TNF-α-mediated viral replication, we investigated by Northern blot analysis the effect of these co-stimulations on HIV-1 viral RNA transcription. We observed that HU or IL-6 alone did not induce significant amounts of HIV-1 RNA (~2-fold increase each compared with control) (Fig. 5A), whereas TNF-α alone induced substantial accumulation of HIV-1 RNA to 52-fold over the constitutive level (Fig. 5B). In contrast, co-stimulation of U1 cells with HU plus IL-6, but not with HU plus TNF-α, resulted in synergistic accumulation of HIV-1 RNA (Figs. 5, A and B). No difference was observed between cell culture for 48 and 72 h post-stimulation (Figs. 5, C and D). Quantification of the 9.2-kb HIV-1 genomic mRNA transcript (Fig. 5A) showed an 18-fold
enhancement of transcription in cells co-stimulated with HU plus IL-6 compared with the untreated control. The observed increase in HIV mRNA, however, may be due to a combination of transcription and post-transcriptional effects resulting in increased mRNA stability.

**HU and IL-6 Synergistically Activate HIV-1 Promoter Activity via the Sp1 Binding Site**—We have shown that co-stimulation of latently infected U1 cells with HU and IL-6 enhances HIV-1 replication by substantially inducing HIV-1 mRNA. To investigate whether this co-stimulation activates the LTR promoter activity, U1 cells were transiently transfected with a plasmid construct (p461) consisting of the luciferase reporter gene controlled by the complete 5' LTR. Cells were treated or untreated with HU (200 μM), IL-6 (15 ng/ml), or a combination of HU and IL-6 for 4 days (8). Virus replication was quantified by measuring p24 antigen in the supernatant everyday for 4 days. Results are from three experiments. Error bars represent the S.D.

**FIGURE 4.** Kinetic studies of HU, IL-6, and TNF-α effects in U1 cells. U1 cells were treated or untreated with HU (200 μM), IL-6 (15 ng/ml) or HU and IL-6 for 48 h in the presence or absence of HU (200 μM), IL-6 (15 ng/ml), or HU and IL-6 (A and C) and HU (200 μM), TNF-α (5 ng/ml), or HU and TNF-α (B and D). Virus replication was quantified by measuring p24 antigen in the supernatant everyday for 4 days. Results are from three experiments. Error bars represent the S.D.

**FIGURE 5.** HU and IL-6 enhance accumulation of HIV-1 RNA in U1 cells. U1 cells were seeded at 2.0 × 10⁴/ml and cultured for 48 and 72 h in the presence or absence of HU (200 μM), IL-6 (15 ng/ml), or HU and IL-6 (A and C) and HU (200 μM), TNF-α (5 ng/ml), or HU and TNF-α (B and D). RNA was isolated using RNeasy kit (Qiagen). 30 μg of total RNA was used in Northern blot analysis (upper). The 28 S and 18 S RNAs stained with ethidium bromide (bottom) were used as control for equal loading. The 9.2-kilobase HIV-1 genomic RNA was quantified using FujiFilm FLA 5100 phosphorimaging. kb, kilobases.

untreated with HU (200 μM), IL-6 (15 ng/ml), or a combination of HU and IL-6 for 48 h and assayed for luciferase activity. As expected, in the absence of stimulation, only basal activity was observed. Treatment with HU or IL-6 alone minimally increased the basal transcription to 2- and 9-fold, respectively. In contrast, treatment with HU plus IL-6 remarkably activated the promoter activity by ~40-fold (Fig. 6A) above the basal level, demonstrating an important synergism between HU and IL-6. To identify the cis-regulatory region responsible for the HU and IL-6-induced activation of the HIV-1 LTR transcription, plasmid constructs consisting of serial deletions of the 5' upstream sequence of p461 construct (Fig. 6A) were made. The full-length construct (p461) and the deletion mutants (p470, p5006, and p5007) were mock-treated or treated with a combination of HU and IL-6 and assayed for luciferase activity. The activities obtained from mock-treated controls were normalized to 1. As shown in Fig. 6C, gene expressions of p461, p470, and p5006 were significantly activated by HU and IL-6 treatment to 18-, 14-, and 19-fold, respectively. However, deletion of the Sp1 binding site (p5007) eliminated the observed synergistic activation of the LTR by HU and IL-6. These results demonstrate that HU and IL-6 functionally synergize to activate the HIV-1 promoter even in the absence of NF-κB (p5006) but are strictly dependent on the intact Sp1 binding site. This indicates that the Sp1 binding site is critical for the HU/IL-6-induced HIV-1 promoter activation.
HU and IL-6 Enhance Tat Activation of HIV-1 LTR-driven Transcription via the Sp1 Binding Site—Sp1 has been shown to be important for Tat activation of LTR-driven transcription (18), and in the normal latency model, synthesis of Tat is quickly triggered. In this paper we have shown that HU and IL-6 enhance HIV-1 transcription via Sp1 binding site. To investigate what happens to transcription when HU plus IL-6 trigger Tat synthesis, U1 cells were transiently co-transfected with Tat and either p461, p5006, or p5007 plasmids. Cells were stimulated or unstimulated with HU and IL-6 for 48 h and assayed for luciferase activity. As shown in Fig. 6D, cells transfected with p461, p5006, or p5007 reporter alone showed only basal luciferase activity. Co-transfection of the reporters with Tat plasmid but no HU and IL-6 stimulation minimally increased the basal transcription to 4.9-, 1.3-, and 2-fold, respectively. Interestingly, gene expressions of p461 and p5006 co-transfected with Tat were significantly enhanced by HU and IL-6 stimulation to ~32- and 23-fold, respectively. In contrast, the deletion of Sp1 binding site (p5007) diminished the observed cooperative activation of the LTR by HU and IL-6 and Tat. Taken together, these results demonstrate that HU and IL-6 functionally cooperate with Tat to activate the HIV-1 transcription and that the

FIGURE 6. Promoter activity of HIV-1 gene in U1 cells. A, U1 cells were transiently transfected with plasmid DNA containing HIV-1 LTR-luciferase reporter gene. Cells were cultured in the presence or absence HU (200 μM), IL-6 (15 ng/ml), or HU plus IL-6 for 48 h and assayed for a marker of transient gene expression (luciferase). Luciferase activity was normalized by total cellular protein obtained by BCA assay (Pierce). B, schematic representations of the deleted 5’ LTR promoter-luciferase constructs. The plasmids were constructed as described under “Experimental Procedures.” Transactivation-responsive element as shown in the schematic is important as an RNA sequence and not as a DNA sequence (14). TAR, transactivation-responsive element. C, all of the constructs were transiently transfected into U1 cells using FuGENE 6 as described under “Experimental Procedures.” Cells were untreated (white bars) or treated with HU + IL-6 (black bars) for 48 h. Luciferase activity was measured and normalized to the total cellular protein amount. The basal luciferase activities in untreated cells were shown relative to the other reporters. D, the reporters, p461, p5006, and p5007, were transiently co-transfected with Tat expression plasmid, p494, into U1 cells using FuGENE 6 as described above. Cell were untreated (gray bars) or treated with HU + IL-6 (black bars) for 48 h. Cells transfected in the absence of Tat and HU + IL-6 (white bars) served as control. Luciferase activity was measured and normalized as above.
Huang and colleagues investigated the role of Sp1 and Sp3 transcription factors in the HIV-1 LTR synergistic activation induced by hydroxyurea (HU) and IL-6. They found that intact Sp1 binding site but not NF-κB is required for the observed cooperative and synergistic effect.

**HU and IL-6 Increase Sp1/Sp3 DNA Binding Activity**—To assess the effect of HU and IL-6 co-stimulation on the Sp1/Sp3 DNA binding, EMSAs were performed using two double-stranded oligonucleotide probes containing the HIV-specific Sp1 oligo or the consensus Sp1 oligo. Both probes formed enhanced DNA-protein complexes with nuclear proteins obtained 48 h after HU and IL-6 co-stimulation of U1 cells (Figs. 7, A and B). We observed more than one DNA-protein complex when we used the consensus Sp1 oligo as the probe (Fig. 7B, lane 5). To evaluate the specificity of the binding, we performed competition EMSA by the addition of excess of unlabeled probes. As shown in Fig. 7A and B (lane 6), corresponding unlabeled Sp1 oligonucleotides completely abolished the binding of nuclear proteins. In contrast, an unlabeled unrelated (AP2) oligonucleotide (lane 7) had no effect on the binding. To prove further that the shifted bands were due to the Sp transcription factor family and to establish the nature of the interacting nuclear proteins, we performed supershift assays using consensus Sp1 oligonucleotide probe and specific antibodies directed against C/EBP, Sp1, Sp3, and STAT3. Anti-C/EBP antibody served as a negative control. As shown in Fig. 7C, preincubation with anti-Sp1 or anti-Sp3 antibodies selectively retarded the migration of three DNA-protein complexes, as reflected by decreased abundance of the major slower migrating complex (for both anti-Sp1 and anti-Sp3) and the disappearance of the faster migrating complex (anti-Sp3) (Fig. 7C, seventh and eighth lanes). In contrast, the formation of the DNA-protein complexes or the binding pattern was not affected by the addition of no antiserum (lane 5) or an unrelated antibody, anti-C/EBP (sixth lane). The addition of the anti-STAT3 antibody also did not affect the formation of the DNA-protein complexes (lane 9), suggesting that STAT3 does not bind to this promoter region in response to HU and IL-6 co-stimulation even when it is known that IL-6 stimulation involves STAT3. Taken together with the data in Fig. 6, we conclude that Sp1/Sp3 transcription factors are involved in the HIV-1 LTR synergistic activation induced by HU and IL-6.

**HU Plus IL-6 Up-regulate Protein Expression of Sp1/Sp3 Transcription Factors**—To elucidate whether the enhanced Sp1/Sp3 binding activity to the HIV-1 LTR promoter was due to an increase in expression of the Sp1/Sp3 transcription factors, we performed Western blot analysis using the same batch of nuclear extracts employed in EMSA. As shown in Fig. 8A, HU and IL-6 co-stimulation of U1 cells significantly increased Sp1/Sp3 protein levels, including both the full-length and truncated Sp3 isoforms, evidenced by the significant increase in the respective bands. In contrast, when nuclear extract from HU plus TNF-α co-stimulated U1 cells were analyzed, we observed a decrease in intensity of the NF-κB p50 band (Fig. 8B, upper panel) compared with TNF-α alone, confirming our earlier observations that HU suppresses TNF-α-mediated HIV-1 activation. As a control for equal loading (Fig. 8B, lower panel), a polyclonal antibody against the nuclear transcription factor TFII-1 was used to reprobe the membranes.
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![Western blot analysis of Sp1, Sp3, and NF-κB in nuclear extracts of U1 cells. A, 30 μg of nuclear extracts from untreated or HU, IL-6, or HU- and IL-6-treated U1 cells was separated on a 10% SDS-polyacrylamide gel and immunoblotted using monoclonal anti-Sp3 (upper) and anti-Sp1 (lower) IgG as described under “Experimental Procedures.” B, 15 μg of nuclear extracts from untreated or HU, TNF-α, or HU- and TNF-α-treated U1 cells was separated on a 10% SDS-polyacrylamide gel and immunoblotted using monoclonal anti-p50 NF-κB antibody (upper) as above. Anti-TFII-I antibody (lower) was used as an internal control for equal loading. C, U1 cells were seeded at 2.0 × 10^5/ml and cultured for 24 or 48 h in the presence or absence of HU and IL-6. Cells were washed, and RNA was isolated. The RNA was treated with DNase and subsequently used to synthesize cDNA. The cDNA served as template for subsequent PCR to amplify Sp1, Sp3, or β-actin. β-Actin served as an internal control to monitor the efficiency of amplification of each of the RNAs.](image)

DISCUSSION

We have previously reported that low concentrations of actinomycin D, a cell cycle synchronizer and transcription inhibitor, up-regulate HIV-1 replication (36). We reasoned that other cell cycle synchronizers might activate latently infected cells and enhance replication. HU has been reported to be an inhibitor of HIV-1 replication and has been clinically used in combination with ddi (39 – 41). In the present study we have demonstrated that HU actually activates HIV-1 replication in latently infected U1 cells and that co-stimulation of HU and IL-6 resulted in profound synergistic effects on HIV viral expression.

The existence of viral reservoirs (or sanctuaries) that harbor latent forms (25, 26, 42 – 45) has limited the success of HAART. It has been suggested that reactivation of the latent reservoirs could allow effective targeting and possible eradication of virus when compared with mock-treated or HU-treated cells. Unlike TNF-α, HU in combination with IL-6 significantly enhanced the accumulation of viral RNA (Fig. 5A) and viral production (Fig. 3A). It is reported that Rev, a viral protein, is associated with positive modulation of virion production through up-regulation of mRNA transport into the cytoplasm by interaction with its RNA target, the rev-responsive element (50, 51). Thus, this dichotomous effect of HU on HIV activation suggests that HU/IL-6 may enhance the Rev/rev-responsive element (RRE) posttranscriptional pathway, whereas HU/TNF-α may interfere with the Rev/RRE elements.

It has been shown that replication of retroviruses depends on cycling cells (52). HU is reported as a cell cycle synchronizer that arrests the cell cycle at G1/S phases (2 – 4). However, in U1 cells, analysis of the cell cycle after HU treatment demonstrated substantial progression into the S and G2/M phases (Fig. 2) despite the well described activity to arrest the cell cycle. This could explain the observed reactivation of HIV-1 replication in latently infected U1 cells after HU stimulation.

Within the Sp family of transcription factors, Sp1 and Sp3 are ubiquitously expressed in mammalian cells to regulate various genes (53). Both are closely related, recognize the same DNA binding sequence (GC box), and are able to enhance or repress promoter activity (54, 55). Sp1 acts as a transcriptional activator, whereas Sp3 can activate or repress transcription depending on the promoter characteristics and cellular context (56 – 59).

Using transient transfection assays, we showed that the Sp1 binding site of the HIV-1 LTR promoter has a positive effect on (46, 47). It has been shown that the immunoregulatory cytokines IL-6 and TNF-α up-regulate HIV-1 replication in acutely as well as in chronically infected cells. But whereas TNF-α activates HIV-1 expression at the transcriptional level, IL-6 mediates its effect at the posttranscriptional level (33 – 35). We investigated the impact of HU on HIV activation in the presence of these cytokines. Our data demonstrated that co-stimulation of U1 cells with HU plus TNF-α led to a reduction in the level of viral RNA induced by TNF-α along with suppressive effects on viral protein expression (Figs. 5B and 3B). TNF-α-mediated effects are known to be dependent on activation of the NF-κB, a cellular transcription factor (33, 34, 48, 49). As expected, co-stimulation with HU plus TNF-α down-regulated the nuclear NF-κB expression (Fig. 8B). Nevertheless, cells co-stimulated with HU plus TNF-α still resulted in a significant increase in HIV-1 RNA levels (Fig. 5B) or protein expression (Fig. 3B).
luciferase reporter activity, thereby indicating a positive regulatory function of the Sp1 binding site on HIV-1 reactivation. Studies have shown that the relative state of latency in U1 cells is as a result of defective Tat, the HIV transactivating protein (30, 31). We have shown in this work that co-stimulation of latently infected U1 cells with HU and IL-6 synergistically enhances HIV-1 transcription. In the normal latency model, synthesis of Tat is quickly triggered. To investigate what happens to transcription when U1 cells are stimulated in the presence of Tat, HU, and IL-6, we performed co-transfection experiments. Our data demonstrate positive cooperative interaction between Tat and HU plus IL-6 in enhancing HIV-1 LTR transcription, suggesting that HU plus IL-6 probably trigger Tat synthesis in latently infected promonocytic U1 cells. It has been previously suggested that the Sp1 binding site is critical for Tat transactivation of HIV-1 LTR (18). In this study, by using plasmid constructs that contained (p461 and p5006) or lacked (p5007) the Sp1 binding site, we clearly show here that Sp1 binding site is key to the observed HU plus IL-6 and Tat cooperative activation of HIV-1 transcription in latently infected U1 cells, thus confirming the earlier observation by Jeang et al. (18) that Sp1 is critical for Tat activation of LTR-driven transcription. Interestingly, our data show that in the absence of HU and IL-6, Tat-mediated activation of transcription through the Sp1 binding site is very minimal but greatly enhanced by >20-fold in the presence of HU and IL-6. We also showed by in vitro binding experiments that the Sp1 and Sp3 proteins specifically bind HIV-1 LTR Sp1 binding site. In addition, we demonstrated that co-stimulation with HU and IL-6 significantly increased the expression of both Sp1 and Sp3 proteins, which bound to the Sp1 binding site of the LTR promoter to reactivate HIV-1 replication. IL-6 belongs to a family of cytokines that utilize the JAK-STAT signaling pathway (60) through gp130 signal transducer (61). A current study demonstrates that IL-6 induces a direct interaction between Sp1 and STAT3 (62). Therefore, the possibility that HU- and IL-6-mediated activation of the HIV-1 promoter may involve members of the STAT family was also considered. Surprisingly, antibody to STAT3 was not associated with a supershift, decreased binding, or disappearance of any DNA-protein complex (Fig. 7C, ninth lane), suggesting a lack of involvement of the STAT3 signaling pathway. Taken together, our findings suggest that Sp1 and Sp3 are necessary for promoting the HU plus IL-6-induced reactivation of HIV-1 genes in latently infected U1 cells. In natural SIV genomes such as Sykes, there are no NF-kB binding sites at all in the LTR (63). So whereas Sp1 sites are always conserved as three sites in all lentiviral LTRs, the NF-kB site(s) is variably two copies, one copy or zero copy in the various lentiviral LTRs. Hence, it is of more importance to understand activation from latency via Sp1 sites (which are omnipresent) than via NF-kB sites (which are imperfectly conserved).

Obviously the latent population is one critical target in the current efforts to control HIV in infected individuals. Activation of latent HIV-1 expression when combined with HAART could prevent the spread of the released virus to adjacent cells (22, 46); therefore, understanding the molecular mechanisms controlling reactivation of latent cells is important in planning efficient therapeutic strategies in the context of HAART.

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