Modulation of Human Immunodeficiency Virus 1 Replication by Interferon Regulatory Factors

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Abstract

Transcription of the human immunodeficiency virus (HIV)-1 is controlled by the cooperation of virally encoded and host regulatory proteins. The Tat protein is essential for viral replication, however, expression of Tat after virus entry requires HIV-1 promoter activation. A sequence in the 5′ HIV-1 LTR, containing a binding site for transcription factors of the interferon regulatory factors (IRF) family has been suggested to be critical for HIV-1 transcription and replication. Here we show that IRF-1 activates HIV-1 LTR transcription in a dose-dependent fashion and in the absence of Tat. This has biological significance since IRF-1 is produced early upon virus entry, both in cell lines and in primary $CD4^+$ T cells, and before expression of Tat. IRF-1 also cooperates with Tat in amplifying virus gene transcription and replication. This cooperation depends upon a physical interaction that is blocked by overexpression of IRF-8, the natural repressor of IRF-1, and, in turn is released by overexpression of IRF-1. These data suggest a key role of IRF-1 in the early phase of viral replication and/or during viral reactivation from latency, when viral transactivators are absent or present at very low levels, and suggest that the interplay between IRF-1 and IRF-8 may play a key role in virus latency.

Key words: virus replication • Tat • transcription factors • gene expression • T lymphocytes

Introduction

Replication of the HIV-1 provirus is mainly controlled at the transcriptional level and depends on a complex interplay between the viral transregulatory protein Tat and cellular transcription factors with the LTR and the intragenic enhancer in the pol gene (1–4). Several major DNase-hypersensitive sites associated with the LTR promoter and target sequences for regulatory proteins have been identified (5, 6).

In the HIV-1 LTR transcriptional regulatory elements are present both upstream and downstream the transcriptional start site. DNaseI sensitivity studies identified just downstream the 5′ LTR (5) a region spanning nt +200 to +217 that is homologous to the IFN-stimulated response element (ISRE)* present in the promoter of IFN-stimulated genes (ISGs; reference 7). This sequence is a binding site for members of the IFN regulatory factor (IRF) protein family (8) and plays a critical role in HIV-1 transcription and replication leading to the definition of a new positive transcriptional regulatory element in the HIV-1 provirus (8, 9).

IRFs play a key role in gene regulation by IFNs and viral infections as well as in several immunological and growth-related cellular functions (10, 11). Nine members of this family have been identified to date based on a homologous DNA-binding domain located at the NH_2 terminus responsible for binding to the ISRE. The less conserved COOH-terminal region acts as a regulatory domain and classifies IRFs into three groups: those that activate (IRF-1, IRF-3, IRF-7, and IRF-9/ISGF-3γ), those that repress (IRF-2, IRF-8/ICSBP), and those (IRF-2, IRF-4/LSIRF/Pip) that are able both to activate or to repress gene

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*Abbreviations used in this paper: CAT, chloramphenicol acetyltransferase; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; ISRE, IFN-stimulated response element; WB, Western blot.

transcription depending on the target gene. IRFs interact with each other and with other families of transcription factors modifying both ISRE-binding activities and the formation of initiation transcription complexes. In addition IRF-1, IRF-2, and IRF-3 can interact with components of the basal transcriptional machinery as well as with the histone-acetyltransferases (12–14).

The HIV-1 transactivator protein Tat is a ~14/15-kD protein produced early after infection and before virus integration (15), which is absolutely required for productive virus replication (16, 17). Tat has been shown to modulate viral gene expression by increasing the rate of transcription initiation, elongation, and translation of TAR-containing mRNAs (3, 18–20). Several reports also suggest that Tat can dissociate from TAR to bind either elongating RNA polymerase II (21) or DNA-tethered promoter factors (22, 23). Tat has the capability of augmenting transcription of viral as well as cellular genes by both TAR-dependent and TAR-independent mechanisms (24–28) also by acting as a DNA sequence–specific transcription factor in the absence of TAR and other HIV-1 LTR sequences (29).

Both specific and basal cellular transcription factors are key in Tat-mediated transactivation of virus gene expression, including Sp1 (22), TBP (30) and TAFII 55 (31), TAP, (32, 33), the kinases TAKs (34), and NF-κB (25). In addition, Tat relieves the transcriptionally inactive chromatin-associated proviral LTR through the recruitment of Tat-associated histone acetyltransferases TAHs (35–37).

The mechanism of action of Tat is complex and not yet completely defined. Similarly, it has not yet been completely elucidated how the viral genome initiates early transcription immediately after viral entry when Tat is still absent or at a threshold concentration, or how the integrated HIV-1 genome reactivates from latency, before viral transactivators are produced.

Here we show that upon entry, HIV-1 is able to induce IRF-1 expression before the expression of Tat. IRF-1 is capable per sé of driving LTR-mediated transactivation in a dose-dependent fashion. In addition, IRF-1 increases Tat-mediated transactivation of the HIV-1 LTR via a physical interaction of its COOH-terminal domain with Tat. This positive cooperation is blocked by IRF-8, a physiological repressor of IRF-1 activity, which inhibits Tat-mediated LTR transcription and viral replication in vivo. These results identify IRF-1 as essential for efficient HIV-1 gene expression and viral replication and indicate that the recruitment of IRF-1 to the HIV-1 promoter can be a key step in the early phases of infection or during viral reactivation from latency, in response to both viral infection and cell activation signals.

Materials and Methods

Cell Cultures and Treatments. Jurkat and 293 HEK cells were grown in RPMI 1640 medium (Bio-Whittaker) or MEM, respectively, containing 10% FCS and antibiotics (growth medium). rIFN-γ (Pepro Tech EC LTD) was used at 10 ng/ml. Human PBLs from healthy donors were isolated by Ficoll-Hypaque gradient and the $CD4^+$ T cell population purified by negative selection using magnetic beads (Miltenyi Biotech) coated with mAbs directed against CD8, CD19, CD16, CD56, and CD11b by manufacturers' instructions. The recovered cells were >96% $CD3^+$ as determined by FACS® analysis. Purified cells were cultured in growth medium and activated with anti-CD3 mAb (Clone FM-18; Biosource International).

Plasmids. The HIV-LTR-CAT construct contains the chloramphenicol acetyltransferase (CAT) gene linked to the HIV-1 LTR BH10 clone (−454 to +286) (9). Δ_1 LTR corresponds to the BH10-LD1 which is deleted in the ISRE sequence (9). Δ_2 LTR and Δ_3 LTR were obtained from the HIV-LTR-CAT and Δ_1 LTR, respectively, by site-directed mutagenesis of the NF-κB site using the QuickChange site-directed mutagenesis kit (Stratagene). The sequence of the primer used to induce the specific mutation was: 5′ CGAGCTTGCTACAACTCACCGCTGCTCACCCAGGGAGG 3′.

CMV-Tat, CMV-IRF-1, CMV-IRF-2, CMV-IRF-3.5D, and CMV-IRF-7* (S477D/S479D) expression vectors have been described previously (38–41); the IRF-8 expression vector (pTarget-ICSBP) was a gift of B. Levi, Technion-Israel Institute of Technology, Haifa, Israel; IRF-4 expression vector was a gift of I. Julkunen, National Public Health Institute, Helsinki, Finland. The pIRF-1/Hygro construct was generated by cloning the fragment excised from pUC-IRF-1 (a gift of T. Taniguchi, University of Tokyo, Tokyo, Japan) by XbaI and HindIII digestion in the pcDNA3.1/Hygro plasmid (Invitrogen Corp.).

All plasmids used in the transfection experiments were purified by cesium chloride.

Stable and Transient Transfection Experiments. Stable transfectants of Jurkat cells were obtained by electroporation with a Bio-Rad gene pulser transfection apparatus using a field strength 0.875 KV/cm, a capacitance of 25 μF and a time constant ~10 μs. Cells were selected for 2 wk with 0.5 mg/ml Geneticin G-418 sulfate (GIBCO BRL).

Jurkat cells expressing both IRF-1 and IRF-8 were obtained by transfecting the IRF-8–expressing cells with the pIRF-1/Hygro. After 10 d of selection in growth medium containing 350 μg/ml of Hygromycin (Sigma-Aldrich), cells were amplified on medium containing both hygromycin and geneticin G-418 sulfate.

Bulk populations were frozen and aliquots periodically thawed (every 4–6 wk) to maintain the identity of the polyclonal cell population. Transient transfections experiments were performed using the FuGENE 6 Transfection Reagent (Roche Laboratories) according to the manufacturer's protocol. Amounts of transfected DNA were normalized by using RcCMV vector. A cotransfected RcCMV β-gal plasmid was used to normalize for transfection efficiency.

Enzymatic Assays. CAT assay was performed as described previously (42). β-gal assay was performed using the β-galactosidase Enzyme Assay system (Promega).

EMSA. EMSA with nuclear cell lysates (6 μg; reference 43) was performed as described previously (42). For supershift analysis, nuclear extracts were incubated with polyclonal anti–IRF-1 and anti–IRF-2 (a gift of Dr. J. Hiscott) antibodies in binding buffer containing the oligonucleotide probe (C_{13} $[AACTGA]_4$) for 30 min on ice.

DNA Affinity Purification Assay. A biotinylated oligonucleotide corresponding to the HIV-ISRE (AGGGACTTGAAAGCGAAAGGGAAACCAGAG) or a mutant oligonucleotide (AGGGACTTGACCGCGGGGCCACCAGAG) were synthesized (Invitrogen) and then annealed with the corresponding antisense oligonucleotides in 1× STE buffer, containing 10 mM Tris-HCl, pH 8.0, 50 mM NaCl and 2 mM EDTA. 25 pico-

moles of biotinylated DNA were mixed with 100–200 μg of nuclear extract in 200 μl of binding buffer containing 20 mM Tris-HCl, pH 7.5, 75 mM KCl, 1 mM DTT, and 5 μg/ml BSA in the presence of 10% glycerol and 20 μg of poly(dI-dC) and incubated for 25 min at room temperature. The complex was pull-down with magnetic beads (Streptavidin MagneSphere; Promega) for 30 min at 4°C and for 10 min at room temperature by mixing with rotation. The collected beads were washed and bound material eluted by boiling in sample buffer. Eluted proteins were separated onto 10% SDS-PAGE followed by immunoblotting with antibody against IRF-1.

Western Blot Analysis. Western blot (WB) was performed as described previously (42). Polyclonal antibodies against IRF-1 was a gift of R. Pine, Public Health Institute, New York, NY.

Immunoprecipitation and Immunoblot Analysis of the Interactions between IRF-1 and Tat. 293 HEK cells were contransfected with expression plasmids encoding IRF-1 or Tat. Whole cell extracts (200–300 μg) were precleared with rabbit IgG nonimmune antisera cross-linked to ultralink immobilized protein A-G (Pierce Chemical Co.), and incubated with anti–IRF-1 antibodies (C20; Santa Cruz Biotechnology, Inc.) cross-linked to ultralink immobilized protein A-G sepharose for 1 h at 4°C. Immunoprecipitates were washed five times with lysis buffer and eluted by boiling the beads for 3 min in 1× SDS sample buffer. Eluted proteins were separated by SDS-PAGE and subjected to WB.

RNA Extraction and Protection Analysis. Total RNA was isolated by the guanidinium-cesium chloride method (44). RNase protection was performed with 5 μg of total RNA as described previously (42).

To generate the ^{32}[P]-labeled 280-bp long antisense IRF-1 RNA probe, the pBS-IRF-1 plasmid (45) was linearized with EcoRI and transcribed by T7 polymerase. To generate the ^{32}[P]-labeled 280-bp long antisense IRF-8 RNA probe, the plasmid (pBS-BP) was linearized with PvuII and transcribed by T7 polymerase. The plasmid pBS-BP was generated by cloning a 1,400-bp long fragment obtained by EcoRI digestion from the plasmid pTarget-ICSBP containing the entire IRF-8 cDNA. A 18S RNA probe was used as a control for equal RNA loading.

Virus Stock Preparation and Infection. Replication-competent T cell-tropic virus was made by transfecting Jurkat cells with the pHXB2R molecular clone, as described previously (46). Viral infection assays were performed by inoculating 10^6 cells with 1,000 or 5,000 cpm of RT activity corresponding to 0.001 or 0.005 50% TCDI/cell of HIV-1/HXB2. After 2 h, virus was washed out and cells were cultured for 96 h. Virus production was then monitored at 24, 48, 72, and 96 h after infection by measuring the levels of p24 in the culture supernatants with a commercial assay kit (p24/27 core antigen assay; Innogenetics) as specified by the manufacturer.

RT-PCR Analysis. To isolate total cellular RNA, 10×10^6 cells were processed using the RNA easy-total RNA extraction kit from QIAGEN. Total RNA was treated with RNase-free DNaseI (Boeringher Mannheim). RT was performed in 50 μl reaction volume containing 1 μg of total RNA according to the manufacturer's instruction (RNA PCR kit; Perkin Elmer). To control for the presence of genomic DNA, control cDNA reaction mixture from which RT was omitted were prepared in parallel. These were uniformly negative (data not shown).

The specific primers named M667, M668, LA45, LA41, M669, and LA23 used to amplify HIV transcripts and PCR conditions were described previously (47). To detect the PCR product of the primer pair M667/M668 and M669/LA23, ^{32}[P]-labeled primer M669 and M668, respectively, were used for hybridization. Detection of the PCR product using specific primers LA41-LA45 were revealed by hybridization with ^{32}[P]-labeled oligonucleotide designed to span in between the first and the second Tat exons: TCAAAGCAACCCACCTCCCAA.

To evaluate the expression of the IRF-1 gene, an aliquot of reverse-transcribed-RNA was amplified within the linear range by 25 PCR cycles: denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s. The RT-PCR was normalized for 26S. Each sample was electrophoresed onto 1% agarose, transferred to nylon membrane and hybridized with a specific probe. The following primers and probe for IRF-1 were used: primer 5′: GTCCAGCCGAGATGCTAAGAGC; primer 3′: GGCTGCCACTCCGACTGCTCC; and probe: GGCCAAGAGGAAGTCATGTGGG.

The primers and probe for 26S amplification were: primer 5′: GCCTCCAAGATGACAAAG; primer 3′: CCAGAGAATAGCCTGTCT; and probe: GAGCGTCTTCGATGCCTATGTGCTTCCCAA.

Construction of the Two-Hybrid Clones. The IRF-1 open reading frame (ORF) was PCR-amplified using primers that introduced an EcoRI and an XhoI site at the 5′ and 3′ ends, respectively, and inserted in the pEG202LexA yeast expression vector in frame with LexA (48). The clones were then transferred in the YEplac181GLexA202 vector and used for the yeast two-hybrid interaction assay. The truncated forms IRF-1 (1–291) and IRF-1 (1–234) were constructed by removal of the 3′ coding sequences with AccI and Bsu36I, respectively, followed by the Klenow treatment. For the construction of the VP16-Tat clone, the Tat 86 amino acids open reading frame was PCR-amplified from the CMV-Tat plasmid using primers that introduced an EcoRI and an XhoI site at the 5′ and 3′ ends, respectively, and cloned in the yeast expression vector pRS314VP16 (49).

The ORFs obtained as described above were cloned into the bacterial expression vector pGEX-4T-1 (Amersham Pharmacia Biotech) at EcoRI and XhoI sites. All the constructs were resequenced after identification.

Two-Hybrid Yeast Assay. The two-hybrid yeast assay was performed as described previously (48). Yeast cells harboring a LexA-responsive LacZ reporter plasmid were cotransformed with a LexA/IRF-1 plasmid, and a VP16/Tat plasmid. Transformants were selected at 30°C on YMM plates. From each transformation three colonies were grown in selective minimal liquid media before galactose-induced expression of the fusion proteins. After incubation of 24 h at 30°C with 2% galactose, yeast cells were used in the permeabilized cell assay (50) to determine the β-galactosidase activity resulting from the LacZ reporter gene expression.

In Vitro GST Pull-Down Experiments. GST and GST fusion proteins were expressed in *Escherichia coli* BL21:DE3(pLysS) (48). For the in vitro binding experiments, ~2 μg of GST and GST–Tat or GST–IRF-1 were mixed with the ^{35}[S]-labeled rIRFs and/or Tat proteins synthesized in vitro using the coupled TNT transcription/translation system (Promega TNT system) in 500 μl of PBS containing 0.1% BSA, 0.5% NP-40, 10% glycerol, and protease inhibitors. Binding reaction was allowed at 4°C for 90 min. Beads were washed, resuspended in sample buffer, and subjected to SDS-PAGE. Gels were analyzed by electronic autoradiography in an Instant Imager (Camberra Packard).

Results

IRF-1 Activates Transcription from the HIV-1 LTR and Increases Tat-mediated Transactivation of LTR-directed Gene Expression. The effect of IRFs on HIV-1 transactivation

was evaluated in Jurkat cells transiently cotransfected with vectors expressing IRF-1, IRF-4, or the constitutively activated forms of IRF-3 (IRF-3 5D) and IRF-7 and a HIV-1 LTR-CAT reporter construct (nt −456 to nt +286). As shown in Fig. 1 A, the basal activity of the HIV-LTR was increased only by the presence of IRF-1, whereas no or little increase was detected in the presence of the other IRFs.

Therefore, the effect of IRF-1 was further analyzed. IRF-1 increased HIV-1 LTR-directed gene expression in a dose-dependent fashion (Fig. 1 B), whereas no activation was detected by deleting the entire COOH-terminal activation domain of IRF-1 (Δ IRF-1). This indicated that upon HIV-1 infection IRF-1 can activate transcription of Tat.

To investigate whether the effect of IRF-1 was mediated by the ISRE, an ISRE-deleted (Δ_1 LTR) or a NF-κB mutated (Δ_2 LTR) construct were used. As shown in Fig. 1 C, IRF-1 was still capable of transactivating the HIV-1 LTR. On the contrary, transactivation was greatly reduced when a mutant bearing deletions in both the ISRE and the NF-κb sites (Δ_3 LTR) was used. These results indicate that the ISRE is not the major site mediating the IRF-1 effect.

To determine the effect of the simultaneous presence of IRF-1 and Tat on HIV-1 LTR transactivation, Jurkat cells were cotransfected with the HIV-LTR construct and with both Tat and IRF-1 expression vectors (Fig. 1 D). The presence of IRF-1 had additive effects on the HIV-1 LTR-CAT activity induced by suboptimal expression of Tat, whereas the cooperative effect was not evident when Tat was overexpressed (data not shown). This suggests that Tat/IRF-1 effect may be key in the very early phase of infection, when Tat is absent or still at low levels.

HIV-1 Induces IRF-1 Early Upon Infection and Prior to Expression of Tat in both T Cell Lines and Primary CD4⁺ T Cells. To determine whether IRF-1 is induced by HIV-1 and whether this occurs before Tat expression, Jurkat cells were infected with the HIV-1 IIIB strain at a low multiplicity of infection and IRF-1 RNA expression analyzed by RNase protection and tat/rev RNA by semiquantitative RT-PCR analysis at different time points after infection. As shown in Fig. 2 A, discrete basal levels of IRF-1 mRNA were detected in Jurkat cells, which increased by 3- and 2.5-fold, respectively, after 5 and 7 h after infection (Fig. 2 A and B). This increase was already detectable at 3 h after infection (data not shown) and returned to basal levels within 24 h. A parallel increase in the protein levels was also detected (Fig. 4).

Notably, at the moment of the highest IRF-1 expression (5 h after infection), no doubly/spliced HIV tat/rev RNA transcripts were detected, whereas at 24 h after infection, as expected, the tat/rev mRNA was clearly detectable. Thus, HIV-1 induces IRF-1 expression early upon infection and before production of Tat.

To assess the biological relevance of these findings, experiments were repeated with primary purified CD4⁺ T lymphocytes stimulated with anti-CD3 mAb and infected with the same virus. IRF-1 and tat/rev RNA expression were then analyzed by RT-PCR at different time points after infection.

As shown in Fig. 3, very low expression of IRF-1 was present in freshly isolated cells, which increased upon stimulation with anti-CD3 antibody (approximately twofold), consistently with previous data (51). However, starting from 5 h after infection IRF-1 mRNA increased by four-

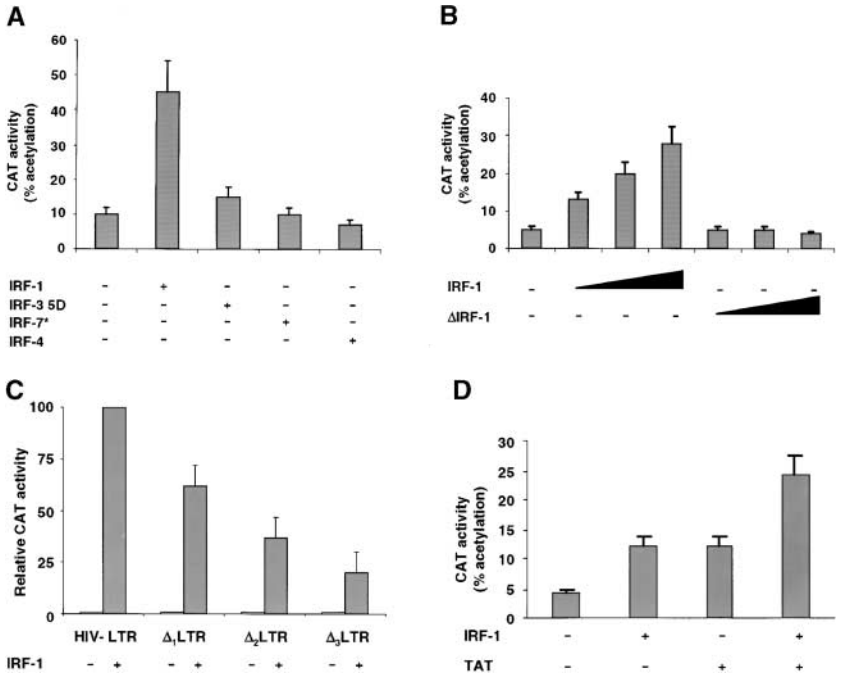

Figure 1. Effect of IRFs on HIV-1 LTR transactivation. (A) Jurkat cells were transiently cotransfected with the HIV-1 LTR-CAT (1 μg) and vectors (2 μg) expressing the indicated IRFs. IRF-3 5D and IRF-7* codify for the constitutively activated forms of IRF-3 and -7, respectively (references 40 and 41). After 24 h, CAT activity was evaluated as indicated in the Materials and Methods. (B) Dose–response effect of the wild-type IRF-1 or its mutant deleted in the activation domain (Δ IRF-1) on HIV-1 LTR-directed gene expression. Cells were transfected as in A except that 0.8 μg of HIV-1 LTR-CAT and increasing amounts of the vectors expressing IRF-1 or Δ IRF-1 (0.8, 1.6, 3.2 μg) were used. (C) Effect of IRF-1 on mutated HIV-LTR constructs. Cells were transiently transfected with wild-type HIV-LTR or Δ_1 LTR in which the ISRE region is deleted (reference 9), Δ_2 LTR in which the NF-κB sites are mutated or Δ_3 LTR in which both sites are deleted/mutated. IRF-1–expressing vector was cotransfected as indicated. Results are shown as percentages of the CAT activity of the wild-type HIV-LTR in IRF-1–transfected cells. (D) IRF-1 cooperates with Tat to enhance HIV-1 LTR-CAT activity. Cells were transiently cotransfected with the HIV-1 LTR-CAT (1 μg), Tat (5 ng), or IRF-1 (1 μg) expressing vectors, either alone or in combination, as indicated. CAT activity was determined after 24 h. The results quantified by an Instant Imager are reported as mean levels ± SE from three separate experiments.

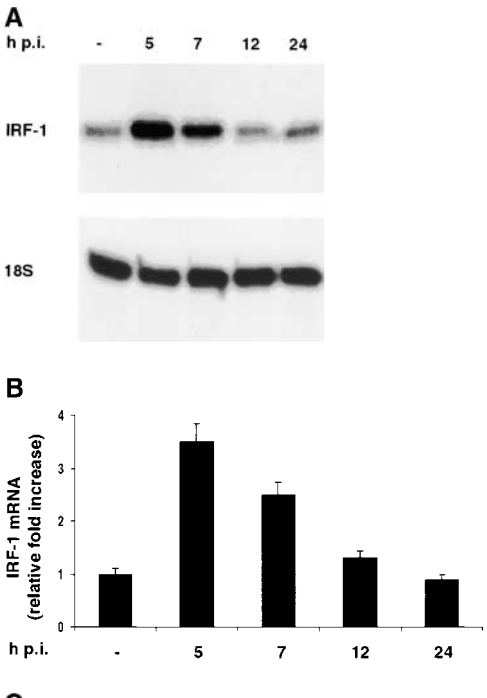

Figure 2. IRF-1 mRNA is induced early upon HIV-1 infection and before expression of Tat. (A) Jurkat cells were infected with the HIV-1 strain IIIB (5,000 cpm/ml) and, at the indicated time points, total RNA was extracted and analyzed by RNase protection with a IRF-1–specific antisense riboprobe. 18S rRNA was used as a control of RNA loading. (B) mRNA relative fold-increase after normalization to the 18S RNA, quantified by Instant Imager. Mean values from three separate experiments are shown. (C) Total RNA extracted at 5 and 24 h after infection shown in A was analyzed by RT-PCR for the doubly-spliced (tat/rev) transcript as described in Materials and Methods.

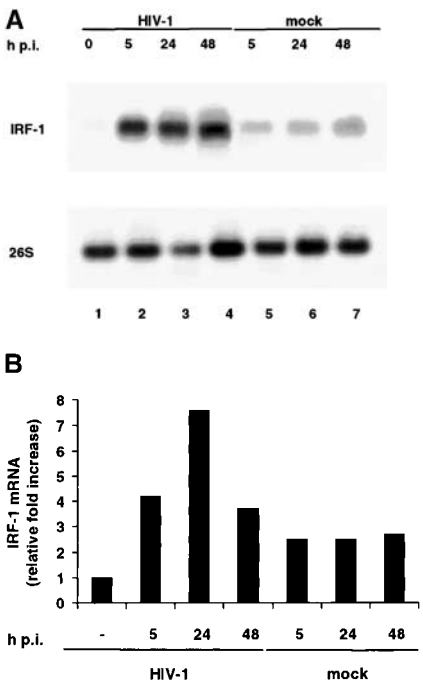

Figure 3. HIV-1 infection induces IRF-1 mRNA expression in primary CD4+ T lymphocytes. (A) Purified CD4+ T cells were infected with the HIV-1 IIIB strain (5,000 cpm/ml) and IRF-1 mRNA evaluated by semiquantitative RT-PCR analysis at the indicated time points after infection. A representative experiment out of three performed is shown. The increase in IRF-1 mRNA accumulation begun to be evident between 5 and 12 h after infection and peaked between 24 and 48 h after infection depending on the donor. 26S RNA was used for normalization as described in Materials and Methods. (B) mRNA relative fold-increase after normalization to the 26S RNA quantified by Instant Imager. (C) Total RNA extracted at the indicated time points as in A was analyzed by RT-PCR for the doubly-spliced (tat/rev) transcript as described in Materials and Methods.

fold and its expression peaked at 24 h after infection (sevenfold increase over basal levels). A progressive decrease was then observed as found with Jurkat cells. However, when the IRF-1 stimulation was maximal, no doubly spliced tat/rev RNA transcripts were detected (Fig. 3 C). A similar kinetic of IRF-1 induction was observed with three different healthy donors.

IRF-1–specific Binding Activity in Infected Cells. To test whether stimulation of IRF-1 upon HIV-1 infection was associated with the presence of a IRF-1–specific binding activity, EMSA was performed by incubating nuclear cell extracts from infected cells with an oligonucleotide representing the canonical IRF binding site (C13). As shown in Fig. 4 A, discrete complexes were detected both in uninfected and infected cells at all time points after infection, which contained both IRF-1 and IRF-2 or multimers of IRF-2 (52) as anti–IRF-1 and anti–IRF-2 antibodies supershifted the higher-mobility and lower-mobility shifted bands, respectively.

However, early after infection, a specific increase of IRF-1-containing complexes was observed (compare lane 6 versus lane 3), consistent with RNA expression data (Fig. 2). From 7 h after infection onward, IRF-1–specific complexes diminished reaching values comparable or below to those observed in uninfected cells. A control purified rabbit IgG and specific anti–IRF-3, -4, -7 antibodies did not affect the binding of any complex (data not shown). To determine the IRF-1–specific binding activity to the HIV-1 LTR, DNA affinity purification assays were performed with both Jurkat and primary CD4+ T cell extracts at 7 and 24 h after infection in the presence of a biotinylated HIV-ISRE probe. The isolated complexes were then examined by immunoblotting against IRF-1. As shown in

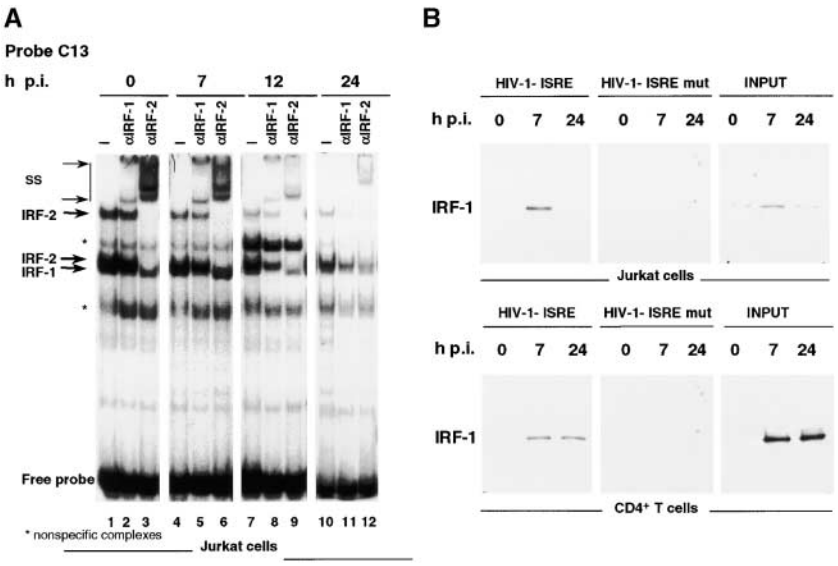

Figure 4. IRF-1 and IRF-2 bind the HIV-ISRE. (A) Nuclear cell extracts (20 μg) from Jurkat cells uninfected or infected with HIV-1 IIIB strain (5,000 cpm/ml) were prepared at different time points after infection and incubated with an oligonucleotide corresponding to the four tandem IRF-binding sites (C13). Supershift assays were performed in the presence of specific anti–IRF-1 and anti–IRF-2 antibodies as indicated. Binding complexes were resolved by PAGE and visualized by autoradiography. (B) DNA pull-down assays. Biotinylated oligodeoxynucleotides containing the wild-type or a mutated version of the HIV-ISRE (Materials and Methods), coupled to Streptavidin MagneSphere were incubated with nuclear extracts from Jurkat cells or primary $CD4^+$ T cells infected with the HIV-1 IIIB strain (5,000 cpm/ml). The bound proteins were eluted from the beads by boiling in sample buffer and analyzed by WB with antibodies against IRF-1. INPUT indicates the level of endogenous IRF-1 in the uninfected and infected nuclear cell extracts (20 μg) at the indicated time points determined by WB analysis.

Fig. 4 B, an increasing IRF-1 binding was evident in infected cells at 7 h after infection, which after 24 h returned to basal levels in Jurkat cells but was still present in $CD4^+$ T cells, according to the RNA expression data (Figs. 2 and 3). This corresponded to the presence of IRF-1 protein (INPUT, Fig. 4 B, right panels) in the same cell extracts. In addition, IRF-1–binding was highly specific since a mutated oligonucleotide (HIV-1-ISRE mut) or an unrelated one (data not shown) did not retain any protein from the same cell extracts.

Taken together, these results demonstrate that IRF-1 and IRF-2 bind to the ISRE-like motif of the HIV-1 LTR. However, early after virus infection, IRF-1 expression increases and this correlates with increasing protein levels and binding to specific LTR-target sequences.

Specific and High-Affinity Binding of IRF-1 and Tat

GST Pull-Down Experiments. To determine whether the cooperative effect of Tat and IRF-1 on HIV-1 LTR transactivation (Fig. 1 D) is mediated by physical interactions between the two proteins, GST pull-down assays were performed. IRF-1 was translated in vitro and tested for binding to a GST–Tat fusion protein. As shown in Fig. 5, the GST–Tat protein bound strongly to IRF-1, i.e., up to 30% of the IRF-1 input was bound to the immobilized Tat protein, whereas no binding was detected to control beads containing GST alone. In contrast, IRF-2, IRF-3, IRF-4, IRF-7, and IRF-8 did not bind to Tat. IRF-1 and Tat binding was also detected when a GST–IRF-1 fusion protein was incubated with labeled in vitro–translated Tat protein. In addition, the deletion of the COOH-terminal activation domain of IRF-1 strongly reduced the binding to Tat (Fig. 5, top panel).

Yeast Two-Hybrid Systems. To verify the Tat and IRF-1 interaction in vivo, the yeast two-hybrid system (53) was used. Although IRF-1 showed in this assay intrinsic transcriptional activation of the reporter gene (Table I), lacZ expression was significantly increased by Tat confirming an interaction between the two proteins. Deletions of the COOH-terminal region of IRF-1 abolished the effect confirming the involvement of this IRF-1 region in binding to Tat. In addition, due to the presence of the transcription activation domain of IRF-1 in the COOH-terminal, dele-

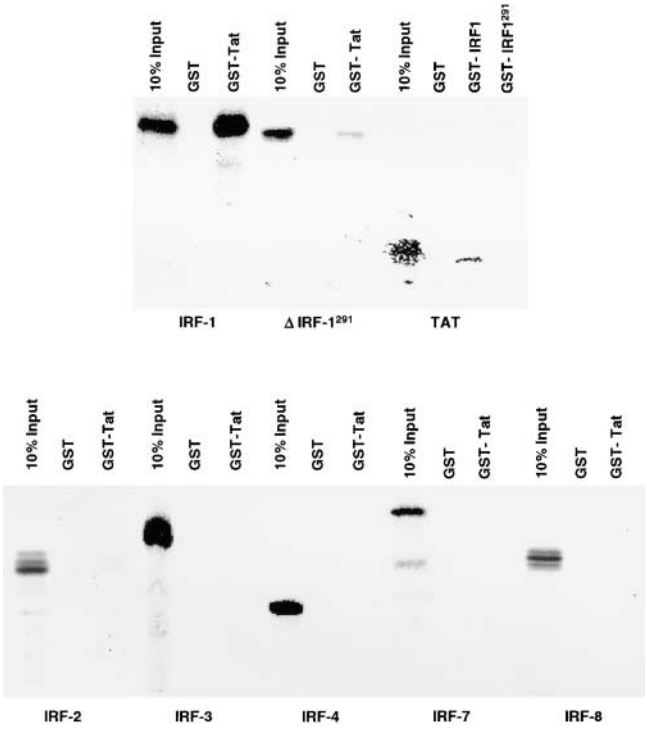

Figure 5. GST-pull down assays. The indicated IRFs (IRF-1, IRF-2, IRF-3, IRF-4, IRF-7, IRF-8, a COOH-terminal deleted mutant of IRF-1 [Δ IRF-1]), and Tat, were translated in vitro in the presence of ^{35}[S] methionine as indicated in Materials and Methods and incubated with recombinant GST–Tat or GST-IRF-1 fusion proteins immobilized on glutathione-sepharose. Input corresponds to 10% of the ^{35}[S]-labeled proteins used in the binding experiments. The complexes were resolved by PAGE and detected by autoradiography. Binding of ^{35}[S]-labeled proteins to beads containing only GST protein is also shown. Quantitation of incorporated radioactivity was performed by Instant Imager.

tions of this region also resulted in decreased intrinsic transcriptional activity as compared with the full length IRF-1. Altogether these results indicate that IRF-1 and Tat physically associate and that the COOH-terminal activation domain of IRF-1 is involved in this interaction.

Binding of Endogenous IRF-1 by Immobilized GST–Tat. To verify the binding of intracellular IRF-1 with Tat, Jurkat cells were treated or were not treated with IFN-γ for 4 h in order to optimally stimulate IRF-1 expression. Nuclear extracts were then incubated with equal amounts of the GST alone or the GST–Tat fusion protein. After extensive washing, associated proteins were resolved by SDS/PAGE and detected by WB. As shown in Fig. 6 A, a polyclonal antibody specific for IRF-1 detected a major band corresponding to IRF-1 in IFN-γ–induced cells (lane 4) but not in control cells (lane 7), where IRF-1 was only barely detectable. Beads containing a GST–Tat fusion protein were able to selectively bind IRF-1 (lane 6). Conversely, incubation of cell extracts with GST-control beads retained no proteins in controls (lane 8) as well as in cell extracts from IFN-γ–treated cells (lane 5). As control of specificity, the in vitro–translated IRF-1 (lane 1) was incubated with GST–Tat (lane 3).

Coimmunoprecipitation. To determine the in vivo interactions between IRF-1 and Tat, coimmunoprecipitation experiments were performed in 293 HEK cells cotransfected with the expressing vectors for IRF-1 and Tat. After cotransfection, anti–IRF-1 antibodies were used for immunoprecipitation followed by immunoblot with anti-Tat antibodies. As shown in Fig. 6 B, the Tat protein was readily detected in the anti–IRF-1 immunocomplexes obtained from cells cotransfected with both RcCMV/IRF-1 or RcCMV/Tat, whereas Tat was not detected in cells transfected with RcCMV/IRF-1 or RcCMV/Tat alone (Fig. 6 B). Vice versa, when immunoprecipitation was performed with anti-Tat antibodies followed by immunoblot with anti–IRF-1 antibodies, the IRF-1 protein coimmunoprecipitated with Tat in cells extracts of doubly transfected cells (data not shown). Altogether these results indicate that IRF-1 and Tat associate intracellularly.

Table I. *In Vivo Interaction between IRF-1 and Tat by the Two-Hybrid System*

	VP16	VP16-Tat
LexA	0.54 ± 0.1	0.53 ± 0.03
LexA–IRF-1	581 ± 27	819 ± 117
LexA–IRF-1 (1-291)	453 ± 80	539 ± 105
LexA–IRF-1 (1-234)	131 ± 65	121 ± 10

Yeast strain DBY1 was transformed with the yeast expression vectors Yeplac181G (Leu2) containing LexA or LexA-IRFs sequences, pRS314 (Trp1) containing VP16TAD or VP16TAD-Tat, and the LacZ reporter vector pSH18 (Ura3). Transformants were selected and analyzed as described in Materials and Methods. β-galactosidase activity is expressed as mean β-galactosidase units ± SD.

IRF-8 but not IRF-2 Represses the IRF-1-Tat–mediated Transactivation of the HIV-1 LTR. IRF-2 is the transcriptional repressor of IRF-1 and acts by competing for IRF-1 binding to target sequences on cellular genes. Since both IRF-1 and IRF-2 can bind the ISRE present in the HIV-1 LTR (reference 8, and this paper), experiments were performed to verify whether IRF-2 could repress the IRF-1 effect on the HIV-1 LTR. Jurkat cells were transiently cotransfected with the HIV-1 LTR-CAT vector and with the expression vectors for Tat, IRF-1, and IRF-2, respectively. As shown in Fig. 7 A, IRF-2 had no effect on LTR-directed transcription and was unable to inhibit IRF-1–mediated transactivation of the HIV-1 LTR, both in the presence or in the absence of Tat.

IRF-8 is another repressor of IRF-1 activity on cellular target genes. IRF-8 does not bind to DNA but acts mainly through complessing IRF-1 and/or IRF-2. As shown in Fig. 7 B, the expression of IRF-8 inhibited by 20% the Tat-mediated transactivation of the HIV-LTR in transient transfection assays. Therefore, to better evaluate

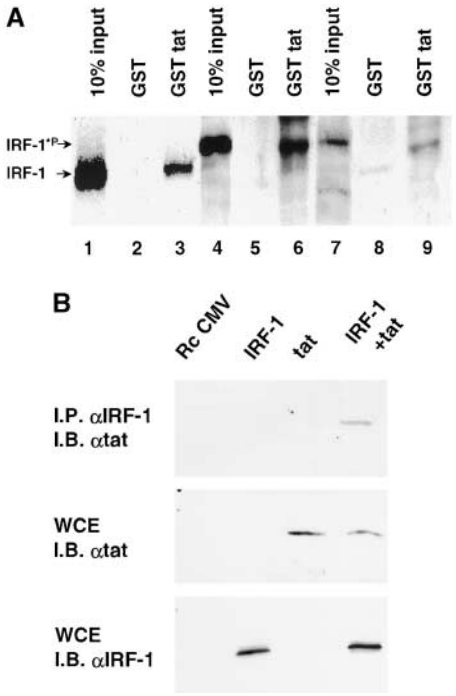

Figure 6. IRF-1 and Tat associate intracellularly. (A) In vitro–translated IRF-1 (lanes 1–3) and nuclear cell extracts from Jurkat cells treated with IFN-γ (lanes 4–6) or control medium (lanes 7–9) were incubated with purified GST–Tat fusion protein or GST alone. Bound proteins were then analyzed by WB using anti–IRF-1 polyclonal antibody as described in Materials and Methods. 10% of the extract used for binding assays is shown in lane 7 (untreated cells) and lane 4 (IFN-γ-treated cells). The slowly migrating IRF-1^{P*} band observed in IFN-γ–treated cell extracts is due to the phosphorylation induced by IFN-γ (reference 64). Extra bands in lanes 7–9 are not specific. (B) 293 HEK cells were transfected with the expression plasmids encoding IRF-1 or Tat, alone or in combination. Whole cell extracts (300 μg) were immunoprecipitated with anti–IRF-1 antibodies (αIRF-1). Immunoprecipitated complexes were separated by 10% SDS-PAGE and subsequently probed with anti-Tat antibodies (αTat) as indicated. Whole cell extracts (10 μg) were separated on 10% or 15% SDS-PAGE and probed with anti-Tat or anti–IRF-1 antibodies.

the IRF-8 inhibitory effect, Jurkat cells were stably transduced with IRF-8 and then transfected with the HIV-LTR CAT construct and the Tat-expression vector. After selection, transgene expression was assessed by RNase protection assay (Fig. 7 C). Bulk populations of transfected cells were chosen to avoid clonal variability. As shown in Fig. 7 D, the constitutive expression of IRF-8 reduced by ~50% both the IRF-1 and the Tat-directed HIV-1-LTR transcription.

Inhibition of HIV-1 Replication in IRF-8–expressing Cells. To evaluate the inhibitory effect of IRF-8 on virus replication, Jurkat cells stably expressing IRF-8 or control cells containing the vector alone were infected with 1,000 and 5,000 cpm/ml, corresponding to 0.001/0.005 $TCDI_{50}$ per cell of the HIV-1 IIIB strain virus. The accumulation of HIV-1 RNA species was then evaluated by semiquantitative RT-PCR (Fig. 8 A) at 24 and 48 h after infection. In control cells (lanes 1, 3, and 5), all HIV transcripts (unspliced, singly- or multi-spliced) were clearly detected after 2 d of infection. In contrast, in IRF-8–expressing cells (lanes 2, 4, and 6), a significant decrease of both spliced and unspliced viral RNA was observed at both infection doses, the doubly-spliced tat/rev being the more reduced. This correlated with an abolished or a reduced virus replication (Fig. 8 B). Specifically, at a low multiplicity of infection, p24 antigen production was, only barely detectable at 48 h after infection and undetectable at later time points, as compared with control cells. Similarly, a reduction of >3 logs was progressively observed, in cells infected with 5,000 cpm/ml of virus. This indicates that IRF-8 represses HIV-1 productive infection.

IRF-8 Blocks Activation of HIV-1 LTR Transcription by Interfering with IRF-1–Tat Binding. Since no direct activity of IRF-8 on LTR transcription nor a direct binding of IRF-8 to Tat was detected (Figs. 5 and 7), to investigate the molecular mechanism(s) responsible for IRF-8–mediated inhibition of HIV, GST-pull down assays were performed with in vitro–translated IRF-1 and IRF-8, and the labeled proteins were tested for binding to a GST–Tat fusion protein. As shown in Fig. 9, the binding of IRF-1 to the GST–Tat beads was clearly impaired in the presence of IRF-8, since the IRF-1 input retained on the GST–Tat beads was reduced by ~50% (compare lanes 2 and 3). On the other hand, the presence of IRF-2 did not affect the IRF-1 binding to immobilized Tat (compare lanes 3 and 4). These results indicate that the inhibitory effect exerted by IRF-8 is, at least in part, mediated by the competition of IRF-8 and Tat for the binding to IRF-1.

To further support this conclusion, Jurkat cells expressing IRF-8 were stably transduced also with an IRF-1–expressing vector. After selection, transgene expression was assessed by RNase protection analysis with RcCMV/IRF-8 cells (control) and with RcCMV/IRF-8/IRF-1 doubly transfected cells (Fig. 10 A). A polyclonal population of cells overexpressing both IRF-8 and IRF-1 was then infected with 1,000 and 5,000 cpm/ml and the production of the HIV p24 antigen evaluated. The dramatic reduction of p24 accumulation in IRF-8 constitutively expressing cells

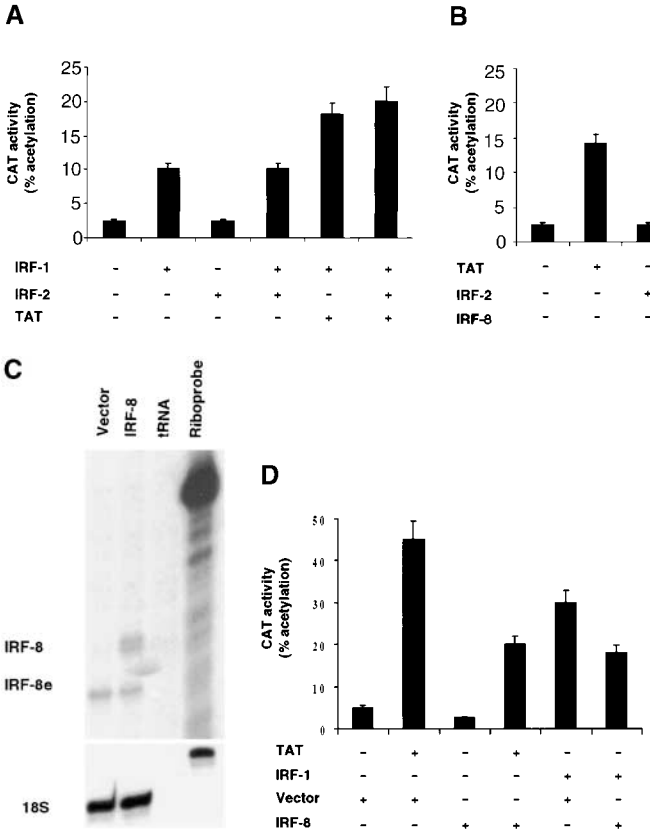

Figure 7. IRF-8 but not IRF-2 inhibits the IRF-1-mediated and Tat-mediated HIV-1 LTR activity. (A and B) Transient cotransfections were performed with the HIV-LTR CAT reporter construct (1 μg) and IRF-1, IRF-2, IRF-8 (1 μg), or Tat (5 ng) expression vectors, respectively, as indicated. CAT activity was quantified 48 h after transfection. (C) RNase protection assay with a IRF-8–specific antisense riboprobe on total RNA extracted from Jurkat cells transfected with an empty vector or an IRF-8–expressing vector. IRF-8 indicates the transcript of the transduced gene and IRF-8e the endogenous recognized transcript. 18S RNA was used as a control of RNA loading and tRNA as a control of specificity. (D) Jurkat cells constitutively expressing IRF-8 or the empty vector were transiently transfected with the Tat-expressing vector (20 ng) or IRF-1–expressing vector (1 μg) along with the HIV-LTR reporter construct and CAT activity quantified as described in B. The results quantified by an Instant Imager are reported as mean levels ± SE from three separate experiments.

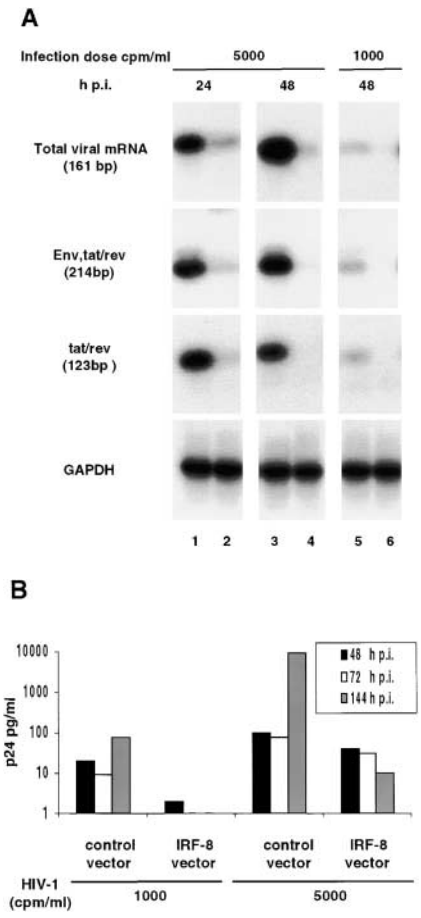

Figure 8. Inhibition of HIV-1 replication in IRF-8–expressing Jurkat cells. Jurkat cells stably transfected with the IRF-8 (lanes 2, 4, and 6) or the RcCMV (control vector) (lanes 1, 3, and 5) were infected with the HIV-1 IIIB strain at an infectious dose corresponding to 1,000 or 5,000 cpm/ml of RT activity. (A) Cells were collected after 24 and 48 h and total RNA analyzed by RT-PCR, as described in Materials and Methods. (B) HIV-p24 antigen production. After 48, 72 and 144 h, p24 antigen accumulation was determined in the cell supernatants as indicated in Materials and Methods.

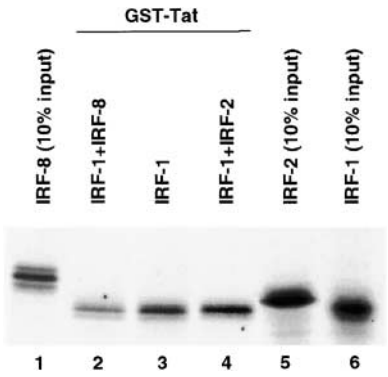

Figure 9. IRF-8 inhibits the binding of IRF-1 to immobilized Tat. Recombinant GST–Tat fusion protein was immobilized on glutathione agarose beads and incubated with the indicated ^{35}[S]-labeled IRFs as described in Materials and Methods. Input corresponds to 10% of the ^{35}[S]-labeled proteins used in the binding experiments; in lanes 1, 5, and 6 are shown the in vitro labeled IRF-8, IRF-2, and IRF-1, respectively. In lane 2, ^{35}[S]-labeled IRF-1 and IRF-8 were incubated together for 15 min at room temperature before the addition of GST–Tat fusion protein beads. Lane 3 shows the binding of ^{35}[S]-labeled IRF-1 alone to GST–Tat beads. In lane 4, ^{35}[S]-labeled IRF-1 and IRF-2 were preincubated together for 15 min at room temperature before the addition of GST–Tat fusion protein beads.

was reversed by at least 50–70% in the cells overexpressing also IRF-1 (Fig. 10 B). Thus, IRF-1 overcomes the inhibitory effect of IRF-8 on HIV-1 replication.

Discussion

HIV-1 transcription and replication is controlled by both viral and cellular factors, which act at the transcriptional, posttranscriptional, and/or translational levels. Both basal- and tissue-specific transcription factors that are essential for HIV-1 have been identified. In the present report we examined the role of the cellular IRFs in HIV-1 transcription and replication and their interactions with the viral transactivator Tat.

We identified IRF-1 as an essential factor for efficient HIV-1 gene expression especially in the early phase of viral replication and before expression of Tat. Several lines of evidence support this conclusion: (i) IRF-1 activates LTR-driven transcription in the absence of the viral transactivator Tat; (ii) IRF-1 is induced at very early time after virus infection and before expression of Tat; (iii) IRF-1 expression during infection correlates with a specific binding to the ISRE of the HIV-1 LTR; (iv) in the presence of low doses of Tat, IRF-1 increases Tat-mediated HIV-1 transactivation by a direct physical interaction with Tat through its transactivation domain; (v) IRF-8, a dominant negative regulator of IRF-1 activity, blocks HIV-1 transcription both in vitro and in vivo, and inhibition is released by overexpression of IRF-1.

The role of IRFs in the regulation of IFN and ISGs (54–56), as well as of genes expressed during inflammation, immune responses, hematopoiesis, cell proliferation, and differentiation has been clearly defined (10, 11). The recent identification of an ISRE on the HIV-1 LTR downstream the transcription start site together with the demonstration that sequences comprising the ISRE are essential for efficient HIV-1 transcription and virus replication (8, 9), allowed us to speculate that IRFs exert a role in HIV-1 transcription. Indeed we demonstrated that IRF-1, but not other IRFs, activates the HIV-1 promoter. IRF-1 may, thus, effectively activate transcription of Tat and, in turn, amplify HIV-1 transcription and virus replication. This can be particularly relevant at the initial phases of HIV-1 replication when viral transactivators are not yet synthesized or are present at subthreshold concentrations.

This has biological significance since IRF-1 is stimulated early after virus infection and before expression of Tat in both cell lines and primary CD4$^+$ T lymphocytes. The kinetic of IRF-1 induction closely resembles that described in cells infected by the vesicular stomatitis virus or Newcastle disease virus, where IRF-1 expression precedes IFN

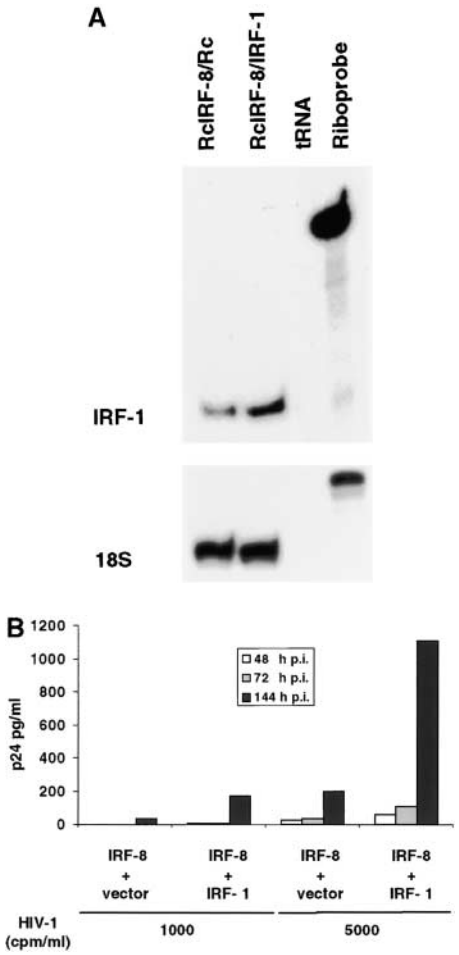

Figure 10. IRF-1 overexpression releases the inhibition of HIV-1 replication by IRF-8. Bulk populations of Jurkat cells stably transfected with IRF-8 were engineered to constitutively express IRF-1. (A) RNase protection with a IRF-1–specific antisense riboprobe on total RNA extracted from IRF-8–expressing cells, transfected with an empty vector (RcIRF-8/Rc), or with an IRF-1–expressing vector (RcIRF-8/IRF-1). 18S RNA was used as a control of RNA loading and tRNA as a control of specificity. (B) Cells were infected with the HIV-1 IIIB strain as in Fig. 8 and after 48, 72, and 144 h, HIV p24 antigen accumulation was determined in the cell supernatants as indicated in Materials and Methods.

type I production (7). Therefore, HIV-1 seems to have evolved a strategy to turn the IRF-1 activity to its own advantage, before massive IFNs production.

Our results point also to a potential role of IRF-1 during viral reactivation from latency. A stable reservoir of HIV-1 are latently infected resting $CD4^+$ T cells (57). Latent infection occurs in resting cells, whereas reactivation occurs only in activated T cells and is dependent on host transcription factors (58–60). IRF-1 that is present at discrete levels in activated but not in resting T cells (51) can thus contribute to viral reactivation even in the absence of Tat. Consistent with this, proinflammatory cytokines such as IFN-γ, IL-6, and TNF-α which lead to cell activation and drive HIV-1 replication (61) strongly activate IRF-1 (62, 63). In addition, the induced IRF-1 can still bind to Tat (Fig. 6) leading to further induction of LTR activation and virus replication. Therefore, we propose that IRF-1 exerts a key role in initiating and amplifying transcription from the HIV-1 LTR, increasing production of Tat, which, in turn, thereby amplifies LTR-directed gene expression. Consistent with this, IRF-1 binds to Tat and cooperates with suboptimal doses of Tat to activate transcription (Figs. 1 and 5). Of note both Tat and IRF-1 have been shown to functionally interact with general transcription factors such as TFIIB (12, 33) and coactivators or adaptors, such as the histone acethyltransferases p300/CBP and pCAF (13, 35–37). These interactions occur through different domains of the proteins thus, IRF-1 might modulate the HIV-1 LTR promoter activity also by acting as a bridge between Tat and component(s) of the basal transcriptional machinery and/or may participate to the Tat-holoenzyme complex according to the model proposed by Cujec et al. (23). It remains to clarify at what extent IRF-1 is critical for HIV-1 replication in T cells. To this purpose a strategy leading to inhibition of IRF-1 expression both basal- and virus-induced, but not interfering with cell viability, should be investigated.

In addition to activate HIV gene expression and replication the IRF system can also repress it specifically, since we showed that IRF-8 is able to impair the binding of IRF-1 to Tat in vitro and to drastically reduce HIV-1 replication in vivo and that this block is released by the simultaneous overexpression of IRF-1. It is, thus, conceivable that, since IRF-8 does not contain an activation/repression domain, excess of IRF-8 complessing IRF-1 may inhibit the IRF-1–induced LTR transcription and the binding of IRF-1 to Tat further impairing HIV-1 replication ultimately leading to a block of viral replication. These data, therefore, suggest that an increase in IRF-8 expression can be involved in the establishment of latency, whereas activation of IRF-1 expression functions as a positive regulator of HIV-1 transcription and replication. Thus the differential expression of these IRFs in activated versus non activated cells and in different cell types may determine productive infection and/or virus reactivation at different tissue sites.

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References

1. Cullen, B.R. 1991. Regulation of HIV-1 gene expression. *FASEB J.* 5:2361–2368.
2. Van Lint, C., J. Ghysdael, P. Paras, Jr., A. Burnyand, and E.

Verdin. 1994. A transcriptional regulatory element is associated with a nuclease-hypersensitive site in the *pol* gene of human immunodeficiency virus type. *J. Virol.* 68:2632–2648.
3. Jones, K.A., and B.M. Peterlin. 1994. Control of RNA initiation and elongation at the HIV-1 promoter. *Annu. Rev. Biochem.* 63:717–743.
4. Gaynor, R. 1992. Cellular transcription factors involved in the regulation of HIV-1 gene expression. *AIDS.* 6:347–363.
5. el Kharroubi, A., and E. Verdin. 1994. Protein-DNA interactions within DNase I-hypersensitive sites located downstream of the HIV-1 promoter. *J. Biol. Chem.* 269:19916–19924.
6. Gross, D.S., and W.T. Garrard. 1988. Nuclease hypersensitive sites in chromatin. *Annu. Rev. Biochem.* 57:159–197.
7. Harada, H., T. Fujita, M. Miyamoto, Y. Kimura, M. Maruyama, A. Furia, T. Miyata, and T. Taniguchi. 1989. Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes. *Cell.* 58:729–739.
8. Van Lint, C., C.A. Amella, S. Emiliani, M. John, T. Jie, and E. Verdin. 1997. Transcription factor binding sites downstream of the human immunodeficiency virus type 1 transcription start site are important for virus infectivity. *J. Virol.* 71:6113–6127.
9. Liang, C., X. Li, Y. Quan, M. Laughrea, L. Kleiman, J. Hiscott, and M.A. Wainberg. 1997. Sequence elements downstream of the human immunodeficiency virus type 1 long terminal repeat are required for efficient viral gene transcription. *J. Mol. Biol.* 272:167–177.
10. Nguyen, H., J. Hiscott, and P.M. Pitha. 1997. The growing family of interferon regulatory factors. *Cytokine Growth Factor Rev.* 8:293–312.
11. Taniguchi, T., K. Ogasawara, A. Takaoka, and N. Tanaka. 2001. IRF family of transcription factors as regulators of host defence. *Annu. Rev. Immunol.* 19:623–655.
12. Wang, I.M., J.C. Blanco, M.J. Tsai, and K. Ozato. 1996. Interferon regulatory factors and TFIIB cooperatively regulate interferon-responsive promoter activity in vivo and in vitro. *Mol. Cell. Biol.* 16:6313–6324.
13. Merika, M., A.J. Williams, G. Chen, T. Collins, and D. Thanos. 1998. Recruitment of CBP/p300 by the IFN β enhanceosome is required for synergistic activation of transcription. *Mol. Cell.* 1:277–287.
14. Hiscott, J., P. Pitha, P. Genin, H. Nguyen, C. Heylbroeck, Y. Mamane, M. Algarte, and R. Lin. 1999. Triggering the interferon response: the role of IRF-3 transcription factor. *J. Interferon Cytokine Res.* 19:1–13.
15. Wu, Y., and J.W. Marsh. 2001. Selective transcription and modulation of resting T cell activity by preintegrated HIV DNA. *Science.* 293:1503–1506.
16. Jeang, K.T., and A. Gatignol. 1994. Comparison of regulatory features among primate lentiviruses. *Curr. Top. Microbiol. Immunol.* 188:123–144.
17. Chang, H.-K., R.C. Gallo, and B. Ensoli. 1995. Regulation of cellular gene expression and function by the human immunodeficiency virus type 1 Tat protein. *J. Biomed. Sci.* 2:189–202.
18. Cullen, B.R. 1992. Mechanism of action of regulatory proteins encoded by complex retroviruses. *Microbiol. Rev.* 56: 375–394.
19. Peterlin, B.M., M. Adams, A. Alonso, A. Baur, S. Ghosh, X. Lu, and Y. Luo. 1993. Tat trans-activator. *In* Human Retroviruses. B.R. Cullen, editor. IRL Press, Oxford, UK. pp. 74–96.
20. Bohan, C.A., F. Kashanchi, B. Ensoli, L. Buonaguro, K.A. Boris-Lawrie, and J.N. Brady. 1992. Analysis of Tat transactivation of human immunodeficiency virus transcription in vitro. *Gene Expr.* 2:391–407.
21. Jones, K.A. 1997. Taking a new TAK on tat transactivation. *Genes Dev.* 11:2593–2599.
22. Jeang, K.T., R. Chun, N.H. Lin, A. Gatignol, C.G. Glabe, and H. Fan. 1993. In vitro and in vivo binding of human immunodeficiency virus type 1 Tat protein and Sp1 transcription factor. *J. Virol.* 67:6224–6233.
23. Cujec, T.P., H. Cho, E. Maldonado, J. Meyer, D. Reinberg, and B.M. Peterlin. 1997. The human immunodeficiency virus transactivation Tat interacts with the RNA polymerase II holoenzyme. *Mol. Cell. Biol.* 17:1817–1823.
24. Berkhout, B., A. Gatignol, A.B. Rabson, and K.-T. Jeang. 1990. TAR-independent activation of the HIV-1 LTR: evidence that Tat requires specific regions of the promoter. *Cell.* 62:757–767.
25. Alcami, J., T. Lain de Lera, L. Folgueira, M.A. Pedraza, J.M. Jacqué, F. Bachelerie, A.R. Noriega, R.T. Hay, D. Harrich, R.B. Gaynor, et al. 1995. Absolute dependence on κB responsive elements for initiation and Tat-mediated amplification of HIV transcription in blood CD4 T lymphocytes. *EMBO J.* 14:1552–1560.
26. Harrich, D., J. Garcia, R. Mitsuyasu, and R. Gaynor. 1990. TAR independent activation of the human immunodeficiency virus in phorbol ester stimulated T lymphocytes. *EMBO J.* 9:4417–4423.
27. Buonaguro, L., F.M. Buonaguro, G. Giraldo, and B. Ensoli. 1994. The human immunodeficiency virus type 1 Tat protein transactivates tumor necrosis factor β gene expression through a TAR-like structure. *J. Virol.* 68:2677–2682.
28. Brother, M.B., H.K. Chang, J. Lisziewicz, D. Su, L.C. Murty, and B. Ensoli. 1996. Block of Tat-mediated transactivation of tumor necrosis factor β gene expression by polymeric-TAR decoys. *Virology.* 222:252–256.
29. Roebuck, K.A., M.F. Rabbi, and M.F. Kagnoff. 1997. HIV-1 Tat protein can transactivate a heterologous TATAA element independent of viral promoter sequences and the trans-activation response element. *AIDS.* 11:139–146.
30. Kashanchi, F., G. Piras, M.F. Radonovich, J.F. Duvall, A. Fattaey, C.M. Chiang, R.G. Roeder, and J.N. Brady. 1994. Direct interaction of human TFIID with the HIV-1 transactivator Tat. *Nature.* 367:295–299.
31. Chiang, C.M., and R.G. Roeder. 1995. Cloning of an intrinsic human TFIID subunit that interacts with multiple transcriptional activators. *Science.* 267:531–536.
32. Yu, L., P.M. Loewenstein, Z. Zhang, and M. Green. 1995. In vitro interaction of the human immunodeficiency virus type 1 Tat transactivator and the general transcription factor TFIIB with the cellular protein TAP. *J. Virol.* 69:3017–3023.
33. Veschambre, P., A. Roisin, and P. Jalinot. 1997. Biochemical and functional interaction of the human immunodeficiency virus type 1 Tat transactivator with the general transcription factor TFIIB. *J. Gen. Virol.* 78:2235–2245.
34. Herrmann, C.H., and A.P. Rice. 1995. Lentivirus Tat proteins specifically associate with a cellular protein kinase, TAK, that hyperphosphorylates the carboxyl-terminal domain of the large subunit of RNA polymerase II: candidate for a Tat cofactor. *J. Virol.* 69:1612–1620.
35. Hottiger, M.O., and G.J. Nabel. 1998. Interaction of human immunodeficiency virus type 1 Tat with the transcriptional coactivators p300 and CREB binding protein. *J. Virol.* 72: 8252–8256.

36. Marzio, G., M. Tyagi, M.I. Gutierrez, and M. Giacca. 1998. HIV-1 Tat transactivator recruits p300 and CREB-binding protein histone acetyltransferases to the viral promoter. *Proc. Natl. Acad. Sci. USA*. 95:13519–13524.
37. Benkirane, M., R.F. Chun, H. Xiao, V.V. Ogryzko, B.H. Howard, Y. Nakatani, and K.-T. Jeang. 1998. Activation of integrated provirus requires histone acetyltransferase. p300 and P/CAF are coactivators for HIV-1 Tat. *J. Biol. Chem*. 273:24898–24905.
38. Ensoli, B., L. Buonaguro, G. Barillari, V. Fiorelli, R. Gendelman, R.A. Morgan, P. Wingfeld, and R.C. Gallo. 1993. Release, uptake and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation. *J. Virol*. 67:277–287.
39. Lin, R., A. Mustafa, H. Nguyen, D. Gewert, and J. Hiscott. 1994. Mutational analysis of interferon (IFN) regulatory factors 1 and 2. Effects on the induction of IFN-β gene expression. *J. Biol. Chem*. 269:17542–17549.
40. Lin, R., C. Heylbroeck, P.M. Pitha, and J. Hiscott. 1998. Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. *Mol. Cell. Biol*. 18:2986–2996.
41. Lin, R., Y. Mamane, and J. Hiscott. 2000. Multiple regulatory domains control IRF-7 activity in response to virus infection. *J. Biol. Chem*. 275:34320–34327.
42. Coccia, E.M., N. Del Russo, E. Stellacci, R. Orsatti, E. Benedetti, G. Marziali, J. Hiscott, and A. Battistini. 1999. Activation and repression of the 2-5A synthetase and p21 gene promoters by IRF-1 and IRF-2. *Oncogene*. 18:2129–2137.
43. Saura, M., C. Zaragoza, C. Bao, A. McMillan, and C.J. Lowenstein. 1999. Interaction of interferon regulatory factor-1 and nuclear factor κB during activation of inducible nitric oxide synthase transcription. *J. Mol. Biol*. 289:459–471.
44. Glisin, V., R. Crkvenjakov, and C. Byus. 1974. Ribonucleic acid isolated by cesium chloride centrifugation. *Biochemistry*. 13:2633–2637.
45. Coccia, E.M., E. Stellacci, M. Valtieri, B. Masella, T. Feccia, G. Marziali, J. Hiscott, U. Testa, C. Peschle, and A. Battistini. 2001. Ectopic expression of interferon regulatory factor-1 potentiates granulocytic differentiation. *Biochem. J*. 360: 285–294.
46. Borsetti, A., C. Parolin, B. Ridolfi, L. Sernicola, A. Geraci, B. Ensoli, and F. Titti. 2000. CD4-independent infection of two $CD4^-/CCR5^-/CXCR4^+$ pre-T-cell lines by human and simian immunodeficiency viruses. *J. Virol*. 74:6689–6694.
47. Kwon, H., N. Pelletier, C. DeLuca, P. Genin, S. Cisterna, R. Lin, M.A. Wainberg, and J. Hiscott. 1998. Inducible expression of IκBα repressor mutants interferes with NF-κB activity and HIV-1 replication in Jurkat T cells. *J. Biol. Chem*. 273:7431–7440.
48. Moscufo, N., F. Sverdrup, D.E. Breiding, and E.J. Androphy. 1999. Two distinct regions of the BPV1 E1 replication protein interact with the activation domain of E2. *Virus Res*. 65:141–154.
49. Dalton, S., and R. Treisman. 1992. Characterization of SAP-1, a protein recruited by serum response factor to the c-fos serum response element. *Cell*. 68:597–612.
50. Golemis, E.A., I. Serebriiskii, R.L. Finley, Jr., H.G. Kolomin, J. Gyuris, and R. Brent. 1999. Current Protocols in Molecular Biology. John Wiley and Sons, Inc., New York. 2001.1–2001.40.
51. Nelson, N., Y. Kanno, C. Hong, C. Contursi, T. Fujita, B.J. Fowles, E. O'Connell, J. Hu-Li, W.E. Paul, D. Jankovic, et al. 1996. Expression of IFN regulatory factor family proteins in lymphocytes. Induction of Stat-1 and IFN consensus sequence binding protein expression by T cell activation. *J. Immunol*. 156:3711–3720.
52. Fujii, Y., T. Shimizu, M. Kusumoto, Y. Kyogoku, T. Taniguchi, and T. Hakoshima. 1999. Crystal structure of an IRF-DNA complex reveals novel DNA recognition and cooperative binding to a tandem repeat of core sequences. *EMBO J*. 18:5028–5041.
53. Fields, S., and O. Song. 1989. A novel genetic system to detect protein-protein interactions. *Nature*. 340:245–246.
54. Maniatis, T., and H. Weintraub. 1992. Gene expression and differentiation. *Curr. Opin. Genet. Dev*. 2:197–198.
55. Taniguchi, T., H. Harada, and M. Lamphier. 1995. Regulation of the interferon system and cell growth by the IRF transcription factors. *J. Cancer Res. Clin. Oncol*. 121:516–520.
56. Kimura, T., K. Nakayama, J. Penninger, M. Kitagawa, H. Harada, T. Matsuyama, N. Tanaka, R. Kamijo, J. Vilcek, T.W. Mak, and T. Taniguchi. 1994. Involvement of the IRF-1 transcription factor in antiviral responses to interferons. *Science*. 264:1921–1924.
57. Siciliano, R.F. 1999. Latency and reservoirs for HIV-1. *AIDS*. 13:49–58.
58. Nabel, G., and D. Baltimore. 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature*. 326:711–713.
59. Tong-Starksen, S.E., P.A. Luciw, and B.M. Peterlin. 1987. Human immunodeficiency virus long terminal repeat responds to T-cell activation signals. *Proc. Natl. Acad. Sci. USA*. 84:6845–6849.
60. Greene, W.C. 1990. Regulation of HIV-1 gene expression. *Annu. Rev. Immunol*. 8:453–475.
61. Fauci, A.S. 1996. Host factors and the pathogenesis of HIV-induced disease. *Nature*. 384:529–534.
62. Fujita, T., L.F. Reis, N. Watanabe, Y. Kimura, T. Taniguchi, and J. Vilcek. 1989. Induction of the transcription factor IRF-1 and interferon-β mRNAs by cytokines and activators of second-messenger pathways. *Proc. Natl. Acad. Sci. USA*. 86:9936–9940.
63. Abdollahi, A., K.A. Lord, B. Hoffman-Liebermann, and D.A. Liebermann. 1991. Interferon regulatory factor 1 is a myeloid differentiation primary response gene induced by interleukin-6 and leukemia inhibitory factor: role in growth inhibition. *Cell Growth Differ*. 2:401–407.
64. Sharf, R., D. Meraro, A. Azriel, A.M. Thornton, K. Ozato, E.F. Petricoin, A.C. Larner, F. Schaper, H. Hauser, and B.-Z. Levi. 1997. Phosphorylation events modulate the ability of interferon consensus sequence binding protein to interact with interferon regulatory factors and to bind DNA. *J. Biol. Chem*. 272:9785–9792.