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Transcription Factor Binding Sites Downstream of the Human Immunodeficiency Virus Type 1 Transcription Start Site Are Important for Virus Infectivity

CARINE VAN LINT, CAROL ANN AMELLA, STEPHANE EMILIANI,
MALCOLM JOHN, TUN JIE, AND ERIC VERDIN*

The Picower Institute for Medical Research, Manhasset, New York 11030

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When transcriptionally active, the human immunodeficiency virus (HIV) promoter contains a nucleosome-free region encompassing both the promoter/enhancer region and a large region (255 nucleotides [nt]) downstream of the transcription start site. We have previously identified new binding sites for transcription factors downstream of the transcription start site (nt 465 to 720): three AP-1 sites (I, II, and III), an AP3-like motif (AP3-L), a downstream binding factor (DBF) site, and juxtaposed Sp1 sites. Here, we show that the DBF site is an interferon-responsive factor (IRF) binding site and that the AP3-L motif binds the T-cell-specific factor NF-AT. Mutations that abolish the binding of each factor to its cognate site are introduced in an infectious HIV-1 molecular clone to study their effect on HIV-1 transcription and replication. Individual mutation of the DBF or AP3-L site as well as the double mutation AP-1(III)/AP3-L did not affect HIV-1 replication compared to that of the wild-type virus. In contrast, proviruses carrying mutations in the Sp1 sites were totally defective in terms of replication. Virus production occurred with slightly delayed kinetics for viruses containing combined mutations in the AP-1(III), AP3-L, and DBF sites and in the AP3-L and DBF-sites, whereas viruses mutated in the AP-1(I,II,III) and AP3-L sites and in the AP-1(I,II,III), AP3-L, and DBF sites exhibited a severely defective replicative phenotype. No RNA-packaging defect could be measured for any of the mutant viruses as determined by quantification of their HIV genomic RNA. Measurement of the transcriptional activity of the HIV-1 promoter after transient transfection of the HIV-1 provirus DNA or of long terminal repeat-luciferase constructs showed a positive correlation between the transcriptional and the replication defects for most mutants.

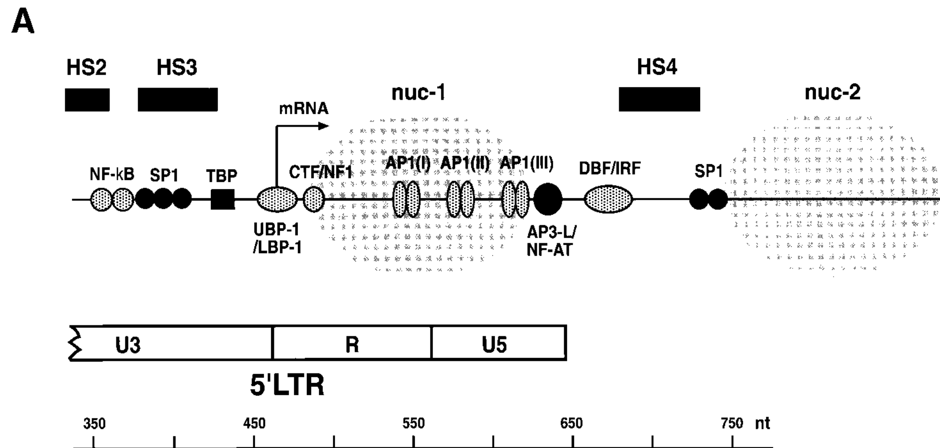
The replication rate of integrated human immunodeficiency virus type 1 (HIV-1) is controlled primarily at the level of transcription. This process is regulated by the interplay between *cis*-acting DNA elements located in the viral long terminal repeats (LTRs) and in the *pol* gene intragenic enhancer (79), by cellular transcription factors bound at these sites, and by the viral *trans*-regulatory protein Tat (reviewed in references 11, 20, 21, 38, and 39).

After integration into cellular genomic DNA, the HIV-1 provirus is packaged into chromatin and nucleosomes are deposited within the promoter region (80, 81). Independently of the site of integration, nucleosomes in the 5′ LTR are precisely positioned with respect to *cis*-regulatory elements (Fig. 1). In the transcriptionally silent provirus, these nucleosomes define two large nucleosome-free regions encompassing nucleotides (nt) 200 to 465 and 610 to 720. The first open chromatin region is associated with the promoter/enhancer in the U3 region and spans two distinct DNase I-hypersensitive sites (referred to as HS2 and HS3) (Fig. 1). The second open region is associated with a region overlapping the primer binding site immediately downstream of the 5′ LTR and spans a DNase I-hypersensitive site called HS4 (Fig. 1). These two open regions are separated by a single nucleosome, called nuc-1, encompassing nt 465 to 610 (80, 81). nuc-1 is specifically and rapidly disrupted during transcriptional activation of the HIV-1 promoter so that the transcriptionally active HIV promoter is characterized by a large open chromatin region encompassing nt 200 to 720 (13, 80, 81). The position of nuc-1 at the transcription start site and its disruption during transcriptional activation suggest that chromatin plays a crucial role in the suppression of HIV-1 transcription during latency and that nuc-1 disruption is necessary for transcriptional activation. These predictions are supported by recent experiments examining the transcription of the HIV promoter reconstituted into chromatin in vitro (60, 70).

In vivo and in vitro footprinting analysis of the region corresponding to nt 465 to 720, downstream of the transcription start site, has identified recognition sites for several constitutive and inducible transcription factors (13, 63): three AP-1 binding sites (site I [nt 541 to 547], site II [nt 572 to 578], and site III [nt 609 to 615]) which lie within the region protected by nuc-1, an AP3-like (AP3-L) motif (nt 617 to 626), a motif interacting with a nuclear factor called downstream binding factor (DBF) (nt 653 to 677), and two juxtaposed Sp1 binding sites (nt 724 to 743) (Fig. 1).

In this study, we have further characterized each of these binding sites and their role in the HIV replication cycle. We have found that the AP3-L site corresponds to an NF-AT site and that the DBF site corresponds to an interferon (IFN)-responsive factor (IRF) binding site. Point mutations have been introduced in each of these binding sites, alone or in combination, in the context of an intact HIV-1 provirus. Study of the replication of these mutant viruses shows that these sites play a critical role in HIV-1 transcription and replication and therefore define a new positive transcriptional regulatory element in the HIV-1 provirus.

* Corresponding author. Mailing address: Laboratory of Molecular Virology, The Picower Institute for Medical Research, 350 Community Dr., Manhasset, NY 11030. Phone: (516) 562-9495. Fax: (516) 365-5090. E-mail: verdin@picower.edu.



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+300 TCF-1α
AGCTGCATCCGGAGTACTACAAAGACTGCTGACATCGAGCTTTCTACAA

+350 NFκB-I NFκB-II Sp1-I Sp1-II
GGGACTTTCCGCTGGGGACTTTCCAGGGAGGTGTGGCCTGGGCGGGACTG

+400 Sp1-III
GGGAGTGGCGAGCCCTCAGATGCTACATATAAGCAGCTGCTTTTTGCCTG

+450 U3 R TAR
TACTGGGTCTCTCTGGTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCT

+500 AP1-I
AACTAGGGAACCCACTGCTTAAGCCTCAATAAAGCTTGCCTTGAGTGCTC
G G

+550 AP1-II
R U5
AAAGTAGTGTGTGCCCGTCTGTTGTGTGACTCTGGTAACTAGAGATCCCT
G G G

+600 AP1-III NFAT/AP3 U5 PBS
CAGACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGTGGCGCCCGAACAG
G G GTTT

+650 IRF/DBF
GGACTTGAAAGCGAAAGTAAAGCCAGAGGAGATCTCTCGACGCAGGACTC
CCC CCC

+700 Sp1 Sp1 SD
GGCTTGCTGAAGCGCGCACGGCAAGAGGCGAGGGGCGGCGACTGGTGAGT
TT TT TT
TT T T

+750 GAG
ACGCCAAAAATTTTGACTAGCGGAGGCTAGAAGGAGAGAG**ATG**GGTGCGA

+800
GAGCGTCGGTATTAAGCGGGGGAGAATTAGATAAATGGGAAAAAATTCGG

FIG. 1. Chromatin organization of the 5′ region of the HIV-1 genome. (A) DNase I-hypersensitive sites HS2, HS3, and HS4 (solid bars) are aligned with *cis*-acting elements and nucleosome positioning in the 5′ LTR. The location of the transcription initiation site at the U3-R junction is indicated by an arrow. (B) Transcription factor binding sites and the mutations introduced are shown in reference to the nucleotide sequence of the HIV LTR. Several landmarks (TAR, PBS [primer binding site], SD [splice donor], and GAG [beginning of the GAG open reading frame]) are shown for reference.

MATERIALS AND METHODS

Cell culture. Cell lines (obtained from the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, Bethesda, Md.) were grown at 37°C in a humidified 95% air–5% CO_2 atmosphere in RPMI 1640 medium (Gibco-BRL, Gaithersburg, Md.) supplemented with 10% fetal bovine serum (HyClone), 50 U of penicillin per ml, 50 μg of streptomycin per ml, and 2 mM glutamine. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (New York Blood Center, Melville, N.Y.) by density centrifugation on a Ficoll-Hypaque gradient (Pharmacia, Uppsala, Sweden).

Virus production assay. HIV-1 production was measured by determining p24 antigen secretion in culture supernatants by an enzyme-linked immunosorbent assay (NEN, Dupont). Reverse transcriptase (RT) activity was determined by a microassay (3).

Site-directed mutagenesis of the HS4 binding sites. A *Nar*I-*Sph*I fragment containing nt 1 to 1447 of the HIV-1 genome was obtained after partial digestion with *Nar*I and complete digestion with *Sph*I of pNL4-3(2) (AIDS Research and Reference Reagent Program). This fragment was subcloned into pUC19 digested with *Nar*I and *Sph*I to generate pEV47. This plasmid was used as a substrate for the mutagenesis of the HS4 binding sites (Fig. 1). Mutagenesis was performed on double-stranded plasmids by the Transformer site-directed mutagenesis method (CLONTECH, Palo Alto, Calif.). Six different mutations were generated with each of the following mutagenic oligonucleotides (mutations are highlighted in bold): EV244 (ΔDBF), 5′-CGCCCGAACAGGGACTTG**CCC**GCG**CCC**GTAAAGCCAGAGGAGATC-3′; EV271 (ΔAP3-L), 5′CAGACCCTTTTAGTCAGTG**GTTT**AAATCTCTAGCAGTGGCG-3′; EV270 [ΔAP-1(III)/AP3-L], 5′-CCTCAGACCCTT**G**TAG**G**CAGTG**GTTT**AAATCTCTAGCAGTG-3′; EV269 [ΔAP-1(I,II,III)/AP3-L], 5′CAATAAAGCTTGCCT**G**GAG**G**GCTCAAAGTAGTGTGTGCCCGTCTGT**G**GTG**G**GAC**G**CTGGTAACTAGAGATCCCTCAGACCCTT**G**TAG**G**CAGTG**GTTT**AAATCTCTAGCAGTG-3′; EV337 (ΔPS/SP1), 5′GAGGAGATCTCTCGA**A**G**A**AGGACTCGGCTTGCTGAAGCGCGCACGGCAAGA**TT**CGAGG**TT**C**TT**CGACTGGTGAGTACGCCAAAA-3′; and EV336 (ΔSP1), 5′AAGCGCGCACGGCAAGA**TT**CGAGG**T**GCG**T**CGACTGGTGAGTACGCCAAAA-3′. The oligonucleotide 5′-CTGAGAGTGCA**CCATGG**GCGGTGTGAAATACCG-3′, changing a unique *Nde*I restriction site in pEV47 into a *Nco*I site (highlighted in bold), was used for selection during mutagenesis. Mutated clones were identified by cycle sequencing (Applied Biosystems) and were fully resequenced between *Mro*I and *Bss*HII or between *Mro*I and *Sph*I (see below) after identification. The six mutated plasmids were designated pΔDBF, pΔAP3-L, pΔAP-1(III)/AP3-L, pΔAP-1(I,II,III)/AP3-L, pΔPS/SP1, and pΔSP1, respectively. In addition, three constructs containing combinations of the mutations described above were generated by subcloning a *Nar*I-*Nar*I fragment from pΔAP3-L, pΔAP-1(III)/AP3-L, and pΔAP-1(I,II,III)/AP3-L into pΔDBF digested with *Nar*I. The resulting plasmids were designated pΔAP3-L/DBF, pΔAP-1(III)/AP3-L/DBF, and pΔAP-1(I,II,III)/AP3-L/DBF, respectively.

Construction of infectious proviruses containing the HS4 mutations. The mutagenized fragments from the pEV47 recombinants were reintroduced into a circularly permuted infectious molecular clone of HIV-1, pILIC (a gift from A. Rabson). As a control, an unmutated *Mro*I-*Sph*I (nt 308 to 1447) fragment was purified from pEV47 and cloned into the unique *Mro*I-*Sph*I sites of pILIC8. We refer to this construct (a derivative of pILIC) as pHIV. A *Mro*I-*Bss*HII fragment (nt 308 to 711) from pΔDBF, pΔAP3-L, pΔAP-1(III)/AP3-L, and pΔAP-1(I,II,III)/AP3-L was introduced into the unique *Mro*I-*Bss*HII sites of pILIC19 to generate pHIVΔDBF, pHIVΔAP3-L, pHIVΔAP-1(III)/AP3-L, and pHIVΔAP-1(I,II,III)/AP3-L, respectively. A *Mro*I-*Sph*I fragment (nt 308 to 1447) from pΔPS/SP1 and pΔSP1 was introduced into the unique *Mro*I-*Sph*I sites of pHIV to generate pHIVΔPS/SP1 and pHIVΔSP1, respectively. A *Mro*I-*Bss*HII fragment (nt 308 to 711) from pΔAP3-L/DBF, pΔAP-1(III)/AP3-L/DBF, and pΔAP-1(I,II,III)/AP3-L/DBF was introduced into the unique *Mro*I-*Bss*HII sites of pHIV to generate pHIVΔAP3-L/DBF, pHIVΔAP-1(III)/AP3-L/DBF, and pHIVΔAP-1(I,II,III)/AP3-L/DBF, respectively.

Generation of viral stocks. Wild-type and mutant HIV-1 infectious DNAs were generated from the single-LTR-containing proviral constructs described above by *Bam*HI digestion and self-ligation. These concatemerized proviral DNAs (10 μg) were transfected into 10^7 JE6.1 cells (a clonal line of Jurkat cells) by using the DEAE-dextran procedure (44, 64). At 24 h posttransfection, the cultures were cocultivated with 10^7 SupT1 cells to allow rapid and efficient

TABLE 1. Oligonucleotides used in EMSA experiments

Name	Oligonucleotide sequence	Source and genomic position	Reference
AP-1(I)wt	5′-TGCCTTGAGTGCTCAAA-3′	HIV-1_{NL4-3} nt 536–552	This study
AP-1(I)mut	5′-TGCCTGGAGGGCTCAAA-3′	HIV-1_{NL4-3} nt 536–552	This study
AP-1(II)wt	5′-TCTGTTGTGTGACTCTG-3′	HIV-1_{NL4-3} nt 567–583	This study
AP-1(II)mut	5′-TCTGTGGTGGGACGCTG-3′	HIV-1_{NL4-3} nt 567–583	This study
AP-1(III)wt	5′-CCCTTTTAGTCAGTGTG-3′	HIV-1_{NL4-3} nt 604–620	This study
AP-1(III)mut	5′-CCCTTGTAGGCAGTGTG-3′	HIV-1_{NL4-3} nt 604–620	This study
AP3-L wt	5′-TCAGTGTGGAAAATCTCTAGCA-3′	HIV-1_{NL4-3} nt 613–634	This study
AP3-L mut	5′-TCAGTGGTTTAAATCTCTAGCA-3′	HIV-1_{NL4-3} nt 613–634	This study
AP-3_{SV40}	5′-CTACTGGGACTTTCCACACATC-3′	SV40 enhancer	56
NF-AT_{IL-2}wt	5′-AGAAAGGAGGAAAAACTGTTTCATACAGAAGGCGT-3′	IL-2 promoter	8
NF-AT_{IL-2}mut	5′-AGAAAGGACCTTAAACTGTTTCATACAGAAGGCGT-3′	IL-2 promoter	8
DBF wt	5′-AGGGACTTGAAAGCGAAAGTAAAGCCAGAG-3′	HIV-1_{NL4-3} nt 648–678	This study
DBF mut	5′-AGGGACTTGCCCGCGCCCGTAAAGCCAGAG-3′	HIV-1_{NL4-3} nt 648–678	This study
$ISRE_{ISG15}$wt	5′-GATCCTCGGGAAAGGGAAACCGAAACTGAAGCC-3′	ISG15 gene	9
$ISRE_{ISG15}$mut	5′-GATCCTCGGGAAAGGGAGGCCGAGGCTGAAGCC-3′	ISG15 gene	9
Sp1wt	5′-CGGCAAGAGGCGAGGGGCGGCGACTGGTGAG-3′	HIV-1_{NL4-3} nt 718–748	This study
Sp1mut1	5′-CGGCAAGATTCGAGGTTCTTCGACTGGTGAG-3′	HIV-1_{NL4-3} nt 718–718	This study
Sp1mut2	5′-CGGCAAGATTCGAGGTGCGTCGACTGGTGAG-3′	HIV-1_{NL4-3} nt 718–748	This study

recovery of progeny virus. Virus stocks were prepared from supernatants after filtration through a 0.45-μm-pore-size membrane when [^{3}H]RT levels exceeded 3,500 cpm/μl. Stocks were quantified by determining both RT activity and p24 concentration.

Viral infections. Infections were performed by incubating 0.5×10^6 cells with 500,000 cpm of RT activity of wild-type or mutant viruses (at 37°C for 2 h in 500 μl of culture medium). After infection, the cells were pelleted at $300 \times g$, washed three times with 1 ml of culture medium, resuspended in 1 ml of standard medium, and grown under standard conditions. Every 2 or 3 days, aliquots of 200 μl were removed from the infected cultures and replaced by normal medium. The aliquots were assayed for RT activity following centrifugation ($800 \times g$). The kinetics of viral replication were monitored by a [^{3}H]RT assay (3). For PBMC infections, PBMCs were isolated from normal donors, activated with phytohemagglutinin (Sigma, St. Louis, Mo.) (5 μg/ml) for 2 days, washed three times with RPMI, infected as described above, and maintained in complete RPMI 1640 medium supplemented with 20 U of interleukin-2 (IL-2) (Gibco-BRL) per ml.

Sequence analysis of HIV-1 genomic RNA. Viral particles from each stock were pelleted by ultracentrifugation (1,500,000 cpm of RT at $20,000 \times g$ for 2 h at 4°C) and digested with RNase-free DNase I (60 U/ml for 10 min at 4°C [Boehringer Mannheim]) in the presence of RNasin (Promega, Madison, Wis.) to remove contaminating DNA. HIV-1 genomic RNA was purified with Trizol (Gibco-BRL). Oligo(dT)-primed cDNA synthesis was performed by the Gene Amp RNA PCR kit method (Perkin-Elmer). cDNAs were amplified by PCR with a 5′ oligonucleotide primer corresponding to nt 489 to 510 (5′-AGCTCTCTG GCTAACTAGGGAAC-3′) and a 3′ primer corresponding to nt 793 to 814 (5′-TTAATACCGACGCTCTCGCACC-3′). PCR fragments were subcloned into the vector pCR2.1 (TA cloning kit; Invitrogen, San Diego, Calif.). After identification of recombinant clones, five inserts from each construct were sequenced with the PRISM dye terminator cycle-sequencing kit (Applied Biosystems, Foster City, Calif.). The nucleotide sequences of all five clones were identical and confirmed the presence of the original mutations.

Western immunoblot analysis. HIV-1 lysates were prepared by ultracentrifugation of each virus stock and resuspension of pellets in Laemmli buffer at a concentration of 30,000 cpm of RT/μl. Lysates were heated at 95°C for 5 min, separated by electrophoresis on an 8% polyacrylamide gel, transferred to nitrocellulose, and probed with a 1:2,000 dilution of purified human anti-HIV-1 immunoglobulin G (IgG) (National Institutes of Health AIDS Research and Reagent Program no. 192). A second antibody, horseradish peroxidase-conjugated goat anti-human IgG (Pierce, Rockford, Ill.) (diluted 1:10,000) was used for enhanced chemiluminescence detection (Amersham).

RNase protection analysis. HIV-1-specific transcripts were detected by RNase protection analysis after lysis of cells in guanidine thiocyanate (23) (lysate ribonuclease protection kit; U.S. Biochemical Corp.). An HIV-1-specific ^{32}P-labeled antisense riboprobe was synthesized in vitro by transcription of pGEM23 (a gift from M. Laspia) (43) with SP6 polymerase by standard methods (5). A glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific antisense probe (provided with the lysate ribonuclease protection kit) was synthesized by the same method and used on the same cell lysates. For HIV-1 genomic RNA analysis, viral particles from each stock were pelleted by ultracentrifugation (1,500,000 cpm of RT at $20,000 \times g$ for 2 h at 4°C) and lysed in guanidine thiocyanate. Viral RNA was then detected with the HIV-1 riboprobe described above.

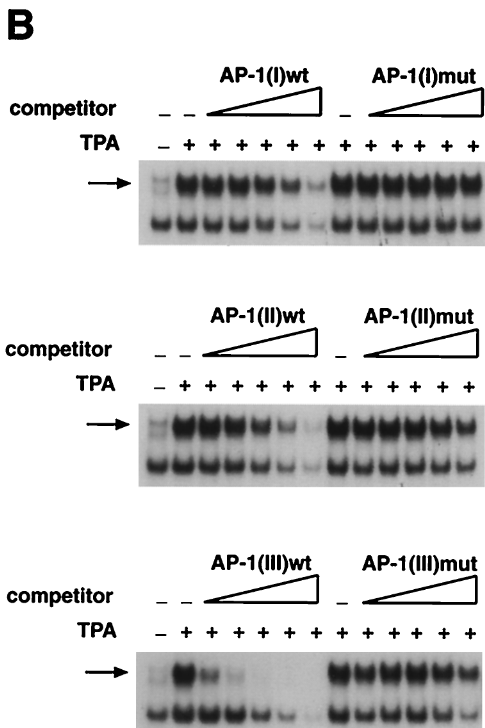
EMSAs. Nuclear extracts were prepared from nuclei by a rapid method described by Osborn (58). All buffers contained the protease inhibitors antipain (10 μg/ml), aprotinin (2 μg/ml), chymostatin (10 μg/ml), leupeptin (1 μg/ml), and pepstatin (1 μg/ml). Protein concentrations were determined by the method of Bradford (10) with bovine plasma gamma globulin as a standard. The DNA sequences of the coding strands of oligonucleotides used in this study are listed in Table 1. Electrophoretic mobility shift assays (EMSAs) were performed as previously described (79). Binding reactions with purified Sp1 were performed in the absence of nonspecific competitor DNA as specified by the manufacturer (Promega).

For supershift assays, polyclonal antibodies against Stat1 and Stat2 (gifts from Chris Schindler) or rabbit preimmune serum was added to the binding-reaction mixture and incubated for an additional 1 h at 4°C. The reaction mixture was centrifuged, and the supernatant was run on an acrylamide gel as previously described. Monoclonal anti-IRF-1 and anti-IRF-2 antibodies (Santa Cruz Biochemicals, Santa Cruz, Calif.) were used as recommended by the manufacturer with a purified rabbit IgG as the control (gift from Christine Metz).

Transient-transfection and luciferase assays. To reintroduce the HS4 mutations in a luciferase reporter system, PCR was used to amplify the promoter region (nt 1 to 792) from the wild-type (wt) and HS4 mutant proviral DNAs described above. *Kpn*I and *Xba*I sites were introduced into the 5′ and 3′ PCR primers, respectively, and the *Kpn*I-*Xba*I-restricted PCR fragments were cloned in pGL2-BASIC (Promega) digested with *Kpn*I and *Nhe*I. The 5′ primer oligonucleotide corresponding to nt 1 to 25 (coordinates with respect to the BRU provirus) contained an added *Kpn*I site (in bold) at the 5′ end (5′-CG**GGGTA CCT**^{nt1}GGAAGGGCTAATTCACTCCCAACG-3′). The 3′ primer oligonucleotide corresponding to nt 769 to 788 contained an added *Xba*I site (in bold) at the 5′ end (5′-GC**TCTAGAC**^{nt788}TCTCTCCTTCTAGCCTCCGC-3′). Amplification reactions were conducted with 100 ng of plasmid DNA as specified in the protocol provided with the high-fidelity Pfu DNA polymerase (Stratagene, La Jolla, Calif.) with a DNA thermal cycler 480 (Perkin-Elmer Cetus). Recombinant plasmids containing the wild-type or mutated HS4 binding sites were designated pLTRluc, pLTRΔDBF, pLTRΔAP3-L, pLTRΔAP1(III)/AP3-L, pLTRΔAP1(I, II,III)/AP3-L, pLTRΔAP3-L/DBF, pLTRΔAP1(III)/AP3-L/DBF, pLTRΔAP1 (I,II,III)/AP3-L/DBF, pLTRΔPS/SP1, and pLTRΔSP1. For transfection experiments, 3×10^6 exponentially growing Jurkat cells were cotransfected with 150 ng of the LTR-luciferase construct DNA mixed with 80 ng of pTat2ex DNA, a Tat-expressing vector (59), by the DEAE-dextran procedure as previously described (79). All transfection mixtures also contained 4 ng of pRL-TK in which a cDNA encoding Renilla luciferase is under the control of the herpes simplex virus thymidine kinase promoter region (Promega). At 24 h posttransfection, the cells were harvested and washed twice with phosphate-buffered saline, and luciferase enzyme activity was measured by using the dual-luciferase assay system (Promega) with a luminometer (Turner Designs luminometer [Promega]). Luciferase activities derived from the HIV promoter are normalized with respect to the Renilla luciferase activity by using the dual-luciferase reporter assay system (Promega). Eight independent transfections were performed with two independent plasmid DNA preparations.

RESULTS

Mutagenesis of DNA binding sites downstream of the transcription start. Mutations were designed to abolish binding of factors to their respective sites. The effect of the selected



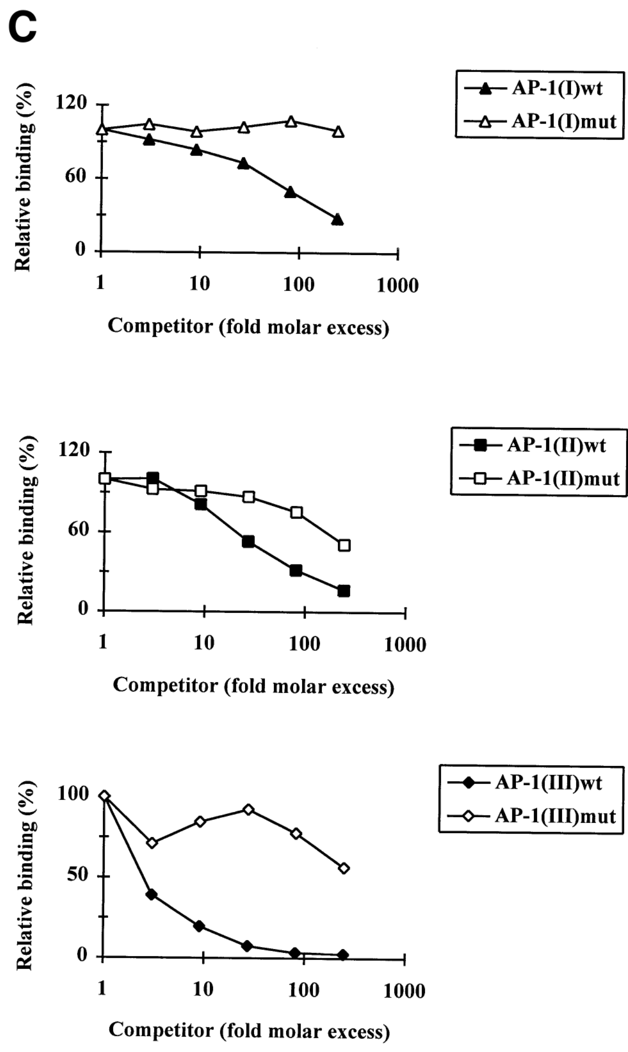


FIG. 2. Mutagenesis of the HS4 AP-1 binding sites. (A) Nucleotide sequences of the AP-1(I), AP-1(II), and AP-1(III) oligonucleotides are shown with underlined bases indicating the TPA-responsive element (TRE) and are aligned with a TRE consensus sequence. The positions of conserved T residues which were mutagenized into G residues are shown. (B) AP-1 wt and mutated oligonucleotides were tested in competition EMSAs. The AP-1(III)wt oligonucleotide was 5′-end labeled and incubated with 10 μg of nuclear extracts from uninduced (−) or TPA-induced (+) (10 ng/ml for 2 h) Jurkat cells either in the absence of competitor or in the presence of increasing concentrations (3-, 9-, 27-, 81-, and 243-fold molar excess) of unlabeled competitor oligonucleotide (as indicated at the top of each lane). Arrows indicate the appearance of AP-1 binding activity in nuclear extracts in response to TPA induction. (C) Quantification of EMSAs shown in panel B was performed with an InstantImager (Packard). The results are expressed as a percentage of binding in comparison to binding in the absence of competitor oligonucleotide.

mutations on binding affinity was analyzed by competition EMSAs.

(i) AP-1 sites. The specificity of binding of the AP-1 family of transcription factors has been extensively characterized, and mutations abolishing the binding of AP-1 to DNA have been described (62). Conserved thymidine residues at positions 2 and 6 were substituted with guanine residues in the three HS4 AP-1 sites (Fig. 2A). The effects of these 2-bp point mutations were analyzed by competition EMSAs with the AP-1(III)wt oligonucleotide as a probe and nuclear extracts from uninduced and tetradecanoyl phorbol acetate (TPA)-induced Jurkat cells. As expected, the appearance of AP-1 binding activity in nuclear extracts was observed in response to TPA (Fig. 2B). This retarded complex was inhibited by competition with an excess of the AP-1(I)wt, AP-1(II)wt, or AP-1(III)wt oligonucleotide (Fig. 2B), indicating binding of AP-1 to these sites as previously reported (13, 63). Determination of the molar excess of unlabeled AP-1(I)wt, AP-1(II)wt, and AP-1(III)wt oligonucleotide competitor required to achieve 50% competition (i.e., half-maximal binding) allowed the ranking of the three sites with respect to their affinity for AP-1: AP-1(III) > AP-1(II) > AP-1(I) (Fig. 2C). In contrast, the AP-1-specific retarded band was not competed by oligonucleotides containing the base substitutions described above [AP-1(I)mut, AP-1(II)mut, and AP-1(III)mut] (Fig. 2B and C), demonstrating that

the selected mutations abolished binding. Thus, although the three AP-1 sites of the HS4 region have different binding affinities, each of the mutations abrogated binding of AP-1 to its respective site.

(ii) AP3-like site. Competition experiments with an oligonucleotide containing the consensus AP-3 site from the simian virus 40 (SV40) enhancer (54, 55, 68) showed competition of the factors binding to the HIV AP3-L site (data not shown). However, the AP-3 site did not compete as efficiently as the homologous AP3-L oligonucleotide, indicating the presence of a low-affinity AP-3 binding site. This observation suggested that the factor(s) present in this complex might be distinct from AP-3. To address this possibility, EMSAs were performed with both the HIV-1 AP3-L and the SV40 AP-3 sites as probes with nuclear extracts from resting or stimulated cells (Fig. 3A). Incubation of the AP3-L_{HIV} probe with nuclear extract from Jurkat cells showed that complexes bound to this probe increased markedly in intensity in response to costimulation with anti-CD3 and anti-CD28 antibodies (Fig. 3A, compare lane 1 with lanes 5 and 6) or in response to anti-CD3 stimulation alone (compare lane 1 with lanes 3 and 4) but not in response to TPA treatment (lane 2), whereas the complex observed with the AP-3_{SV40} probe was induced by TPA and to a much lesser degree by CD3 or CD3-plus-CD28 stimulation (lanes 1 to 6). Comparison of binding specificities with the same two probes (AP3-L_{HIV} and AP-3_{SV40}) and nuclear extracts from human cell lines of different origins (SupT1, Jurkat, and A3.01 [$CD4^+$ T-lymphocytic cell lines], HeLa [epithelial cell line], U937 and KG-1 [promonocytic/macrophage cell lines], and Raji and Namalwa [B-lymphocytic cell lines]) showed distinct patterns of factors binding the two different probes (lanes 7 to 15). Factors binding to the AP3-L motif are preferentially expressed in lymphocytes (T and B cells), whereas the SV40 AP-3 probe did not recognize any factors in uninduced extracts with the exception of KG-1 and RAJI nuclear extracts. We conclude from these experiments that distinct factors bind to the HIV-1 AP3-L and the SV40 AP-3 sites.

(iii) The AP3-L site binds an ionomycin-inducible factor corresponding to NF-AT. Computer analysis of the DNA sequence of the AP3-L motif revealed regions with close homologies to binding sites for other known transcription factors: AP-3 (54), the CD28-responsive element (16), NF-IL6 (71), NF-κB (reviewed in reference 14), and the nuclear factor of activated T cells (NF-AT) (8) (Fig. 3B). We performed supershift assays with specific antibodies for each of the members of the NF-κB family (p50, p52, p65, c-Rel, and RelB [a gift from U. Siebenlist]) and competition EMSAs with consensus binding sites corresponding to the CD28-responsive element, NF-IL6, and NF-κB. These experiments indicate that the AP3-L motif does not contain a recognition site for any of these transcription factors (data not shown).

When we used TPA-ionomycin-treated nuclear extracts from A3.01 cells in gel shift experiments, we observed the binding of an inducible factor(s) to the AP3-L probe (Fig. 4A). A similar retarded band was observed with extracts from cells treated with ionomycin alone (data not shown). This binding was specific as demonstrated by competition experiments with the same unlabeled oligonucleotide (Fig. 4A) and the lack of competition when a mutant oligonucleotide containing four point mutations centered on the AP3-L binding site was used (Fig. 4A and D). Binding of this ionomycin-inducible factor to the AP3-L probe was efficiently competed by an NF-AT binding site derived from the interleukin-2 promoter and not competed by the same site containing a point mutation known to abrogate NF-AT binding (8, 34) (Fig. 4A). To identify directly the factors binding to the HIV AP3-L site, we used two dif-

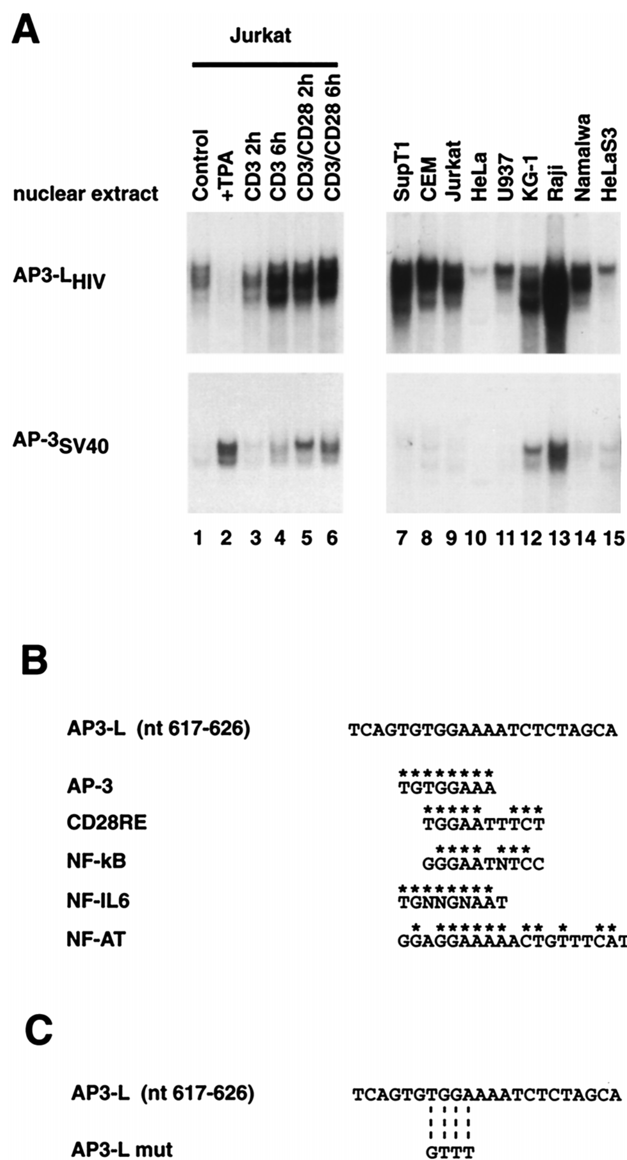


FIG. 3. Characterization of the factors binding the AP3-L motif. (A) AP3-L_{HIV} or AP3-L_{SV40} oligonucleotide probes were incubated with nuclear extracts from human cell lines of different origins (indicated above the lanes), and the binding reaction products were analyzed by EMSA. When indicated, Jurkat cells were treated with TPA (10 ng/ml for 6 h), with anti-CD3 antibodies (1 μg/ml for 2 or 6 h), or with anti-CD3 plus anti-CD28 antibodies (1 μg/ml of each for 2 or 6 h). (B) Alignment of the AP3-L site nucleotide sequence with the consensus binding sites for AP-3 (54), CD28RE (16), NF-κB (14), NF-IL6 (71), and NF-AT (8). Conserved residues are indicated by an asterisk. (C) Mutagenesis of the AP3-L motif was realized by replacing a TGGA motif centrally located in the footprinted area with the sequence GTTT.

ferent antibodies directed against NF-AT1 (anti-67.1 and antiR59 [30, 53]; a gift from A. Rao). Addition of anti-NF-AT1 antibodies to binding reaction mixtures containing either uninduced or TPA-ionomycin-inducible extracts and the AP3-L probe resulted in the retardation of the TPA-ionomycin-inducible complex (Fig. 4B). No supershifted complex was observed with control antibodies or when the antibodies were added to the probe alone (Fig. 4B). Last, we examined the ability of the purified NF-AT1 DNA-binding domain (DBD) to bind to the HIV AP3-L probe. Comparison of binding of increasing amounts of the NF-AT1 DBD (49) to probes corresponding to

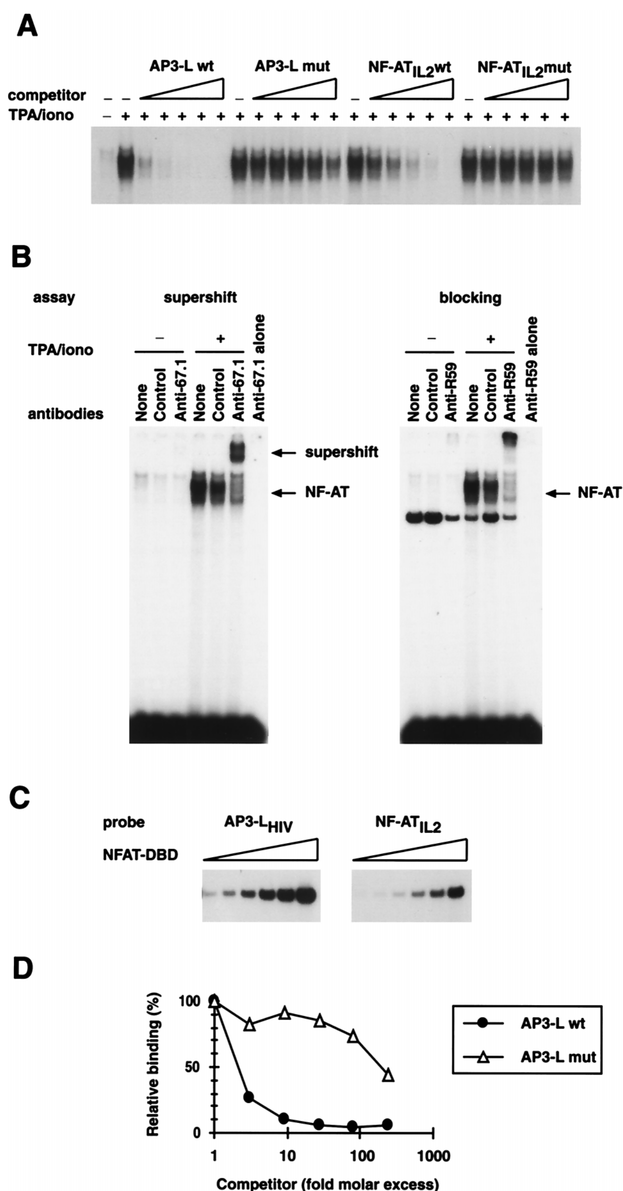


FIG. 4. The AP3-L motif is a NF-AT binding site. (A) Competition experiments. AP3-L wt and mutated oligonucleotides were tested in competition EMSAs. The AP3-L wt oligonucleotide was 5′-end labeled and used as a probe. This probe was incubated with 10 μg of nuclear extracts from TPA-ionomycin (iono)-treated A3.01 cells in the absence of competitor or in the presence of increasing concentrations (3-, 9-, 27-, 81-, and 243-fold molar excess) of the homologous oligonucleotide, of an oligonucleotide containing four substitutions in the AP3-L motif (AP3-L mut [Fig. 3C]), of an oligonucleotide containing the NF-AT_{IL-2} site, or of a mutated version known to abolish NF-AT binding. (B) Supershift assays. Untreated or TPA-ionomycin-treated A3.01 nuclear extracts were incubated with a radiolabeled AP3-L wt oligonucleotide. Two NF-ATp-specific antisera, anti-67.1 and anti-R59 (30, 53) (a gift from A. Rao), were added either at the beginning (anti-R59) or at the end (anti-67.1) of the binding reaction. Preimmune rabbit serum was used as a negative control. The possibility that the antibody itself binds to DNA was ruled out by performing the binding reaction in the absence of nuclear extract (anti-67.1 alone, anti-R59 alone). (C) Recombinant NF-AT binds to the AP3-L motif. Purified recombinant NF-AT DBD was added in increasing concentrations (0.125, 0.25, 0.5, 1, 2, and 4 ng) to radiolabeled probes corresponding to the AP3-L binding site and to the NF-AT_{IL-2} site. (D) Competition graph. The gel shown in panel A was quantified with using a phosphorimager (Instant Imager; Packard). Complex formation in the absence of unlabeled competitor DNA was taken as 100%.

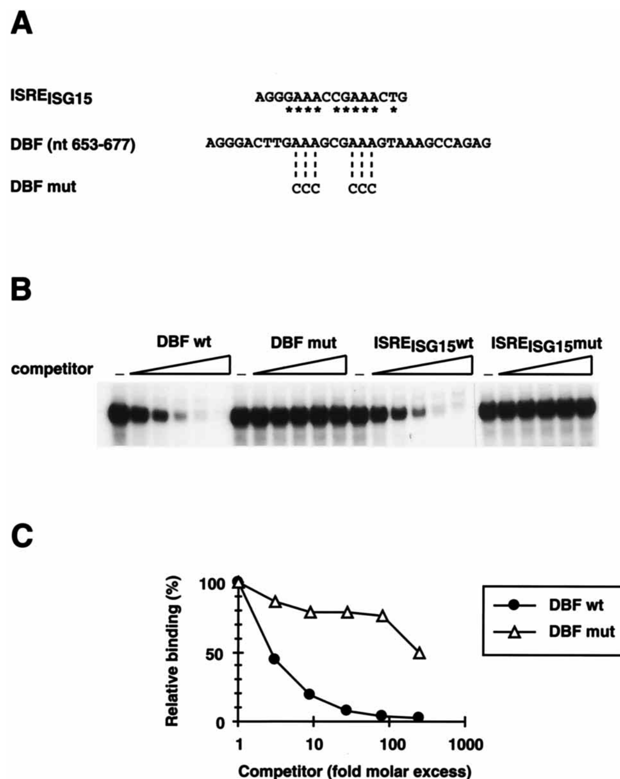


FIG. 5. Mutagenesis of the HS4 DBF binding site. (A) The nucleotide sequence of the DBF wt oligonucleotide is aligned with the ISRE from the ISG15 gene. Conserved residues are indicated by an asterisk. Bases that are changed in the mutated version of this oligonucleotide (DBF mut) are indicated. (B) DBF wt and mutated (mut) oligonucleotides were tested in competition EMSAs. The DBF wt oligonucleotide was 5′-end labeled and used as probe. This probe was incubated with 10 μg of nuclear extracts from A3.01 cells in the absence of competitor or in the presence of increasing concentrations (3-, 9-, 27-, 81-, and 243-fold molar excess) of the homologous DBF wt oligonucleotide, of the DBF mut oligonucleotide, of the $ISRE_{ISG15}$ oligonucleotide ($ISRE_{ISG15}$wt), or of a mutant version derived from it ($ISRE_{ISG15}$mut). (C) Levels of complex formation of EMSAs shown in panel B were quantified by radioimaging analysis (InstantImager). Complex formation in the absence of unlabeled competitor DNA was taken as 100%.

the AP3-L site or the NF-AT_{IL-2} site showed relatively higher binding to the HIV AP3-L probe than to the NF-AT_{IL-2} site (Fig. 4C). These experiments demonstrate that the HIV AP3-L site corresponds to a bona fide high-affinity NF-AT binding site.

(iv) DBF site. To abolish binding of factors to the DBF site (nt 648 to 677), we substituted two conserved central groups of A residues for C residues (Fig. 5A). These mutations abolished the ability of the oligonucleotide to compete the binding of factors to the DBF wild-type (wt) probe, whereas unlabeled DBF wt oligonucleotide was an efficient competitor, therefore confirming the loss of DBF binding to the mutated site (Fig. 5B and C).

We previously noted a strong homology of the DBF site to the IFN-stimulated regulatory element (ISRE) (13, 76) (Fig. 5A). This sequence serves as a recognition site for members of the IFN regulatory factor (IRF) family, which includes IRF1, IRF2, and ICSBP, and members of the STAT family of transcription factors (reviewed in references 33 and 67). Under basal conditions, the ISRE is occupied by the constitutively expressed IRF factors. In response to IFN-α stimulation, the ISRE becomes occupied by an additional complex, called

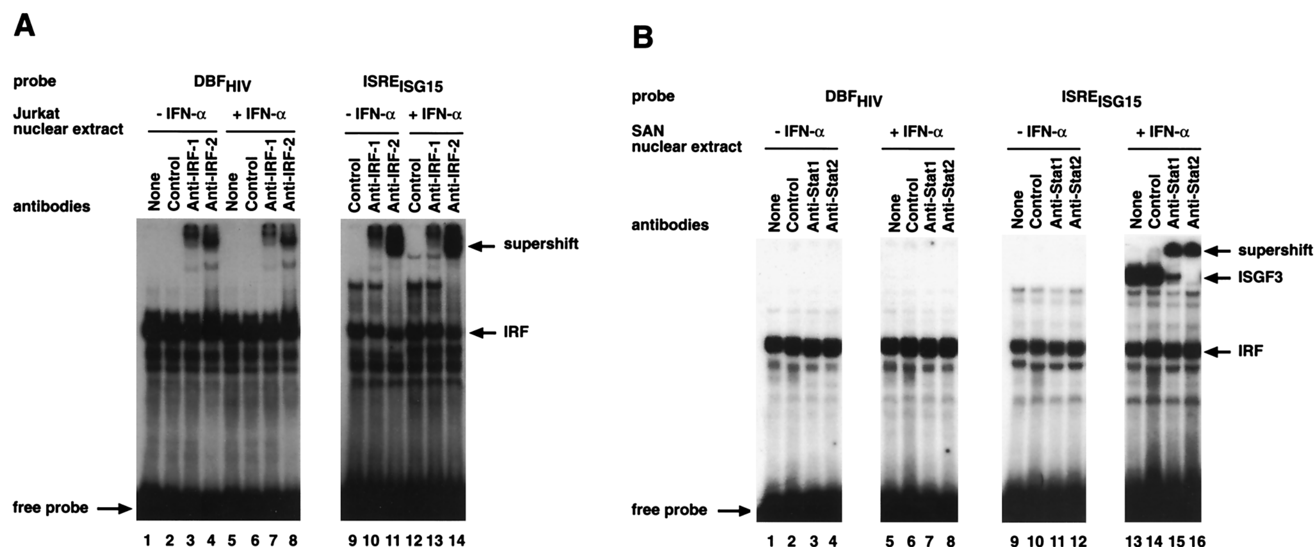


FIG. 6. The HIV-1 DBF site binds IRFs. (A) $DBFwt_{HIV}$ (lanes 1 to 8) or $ISRE_{ISG15}$wt (lanes 9 to 14) oligonucleotide probes were incubated with nuclear extracts from uninduced (−IFN-α) or IFN-induced (+IFN-α) Jurkat cells. Supershift assays were performed in the absence of antibody (lanes 1 and 5) or in the presence of anti-IRF-1 antibody (lanes 3, 7, 10, and 13), of anti-IRF-2 antibody (lanes 4, 8, 11, and 14), or of purified rabbit IgG as a control (lanes 2, 6, 9, and 12). (B) $DBFwt_{HIV}$ (lanes 1 to 8) or $ISRE_{ISG15}$wt (lanes 9 to 16) oligonucleotide probes were incubated with nuclear extracts from uninduced (−IFN-α) or IFN-induced (+IFN-α) (50 U/ml for 30 min) SAN cells. Supershift assays were performed in the absence of antibody (lanes 1, 5, 9, and 13) or in the presence of anti-Stat1 antibody (lanes 3, 7, 11, and 15), of anti-Stat2 antibody (lanes 4, 8, 12, and 16) or of preimmune serum as a control (lanes 2, 6, 10, and 14).

ISGF3, composed of STAT1, STAT2, and the p48 protein (24, 56). To compare the specificity of the HIV-1 DBF site with that of a classical ISRE, the ISRE from the ISG15 gene was used as a competitor in gel retardation experiments. As shown in Fig. 5B, the DBF retarded band was competed by an excess of unlabeled $ISRE_{ISG15}$wt oligonucleotide as efficiently as by the homologous DBF wt oligonucleotide. In contrast, the $ISRE_{ISG15}$mut oligonucleotide containing mutations abolishing IRF binding did not have any inhibitory effect on complex formation (Fig. 5B). In addition, reverse experiments in which a labeled probe corresponding to the $ISRE_{ISG15}$ oligonucleotide was used and competed by the DBF wt oligonucleotide confirmed these observations (data not shown). We conclude from these experiments that the DBF wt and $ISRE_{ISG15}$wt oligonucleotides are recognized by related and/or identical proteins.

To confirm the identity of the factor(s) present in the DBF-retarded complex, we performed supershift assays with antibodies specific for individual IRF proteins (Fig. 6). The DBF_{HIV} oligonucleotide was used as probe in EMSAs with nuclear extracts from untreated and IFN-α-treated Jurkat cells (Fig. 6A, lanes 1 to 8). Addition of both anti-IRF1 and anti-IRF2 antibodies generated a supershifted complex in uninduced extracts (lane 3 and 4). The same pattern was observed with IFN-α-induced Jurkat nuclear extracts (lanes 5 to 8). A similar pattern of binding and supershifted complexes was observed when the $ISRE_{ISG15}$ oligonucleotide was used as a probe (lanes 9 to 14). These observations are consistent with previous observations that the IRF factors are constitutively expressed in lymphoid cell lines. To examine the ability of the DBF site to bind the IFN-α-induced ISGF3 complex, anti-Stat1 and anti-Stat2 antibodies were used in supershift assays with nuclear extracts from SAN cells, a cell line in which the ISGF3 induction is readily visualized (35) (Fig. 6B). Using the DBF probe, we found that the pattern of binding observed with uninduced SAN nuclear extracts (Fig. 6A, lane 1) was identical to that observed with IFN-α-induced extracts (Fig. 6A, lane 5). The retarded complex did not contain ISGF3 since it was not affected by anti-Stat1 and anti-Stat2 antibodies (Fig. 6B, lanes 3 to 7 and 4 to 8, respectively). In contrast, when similar experiments were performed with the $ISRE_{ISGF15}$ probe, a slowly migrating ISGF3 band was induced upon IFN-α treatment (Fig. 6B, compare lanes 9 and 13). Anti-Stat1 and anti-Stat2 antibodies abolished this ISGF3 band, and a supershifted complex was observed (Fig. 6B, lanes 15 and 16, respectively), while preimmune serum did not interfere with complex formation (Fig. 6B, lane 14).

Taken together, our results demonstrate that the DBF site present in the HIV-1 leader region is homologous to the ISRE and specifically binds the IRF-1 and IRF-2 proteins.

(v) Sp1 sites. Footprinting analysis of the HIV-1 leader region with purified Sp1 identified strong binding sites in a GC-rich sequence extending from nt 725 to 746 (13). This region contains three motifs with close homology to the Sp1 consensus sequence (Fig. 7A). A 2-bp mutation (GG to TT) that interferes with Sp1 binding (37) was introduced into each of these potential Sp1 sites. The effect of this mutation, designated Sp1mut1, on Sp1 binding affinity was analyzed by competition EMSAs with the Sp1wt oligonucleotide as probe (Fig. 7B and C). Incubation of this probe with affinity-purified human Sp1 resulted in a retarded complex. This complex was inhibited by competition with an excess of the Sp1wt oligonucleotide but to a much lesser degree by the same oligonucleotide containing the 6-bp substitution, demonstrating that the Sp1mut1 mutation decreased the specific binding of Sp1 to the affected sites (Fig. 7B).

The RNA leader sequence of HIV-1 is organized in a complex stem-loop secondary structure encompassing nt 457 to 1180 that plays a critical role in packaging of the viral genome in particles (6, 27). According to this model, four of the six G residues which were mutated in Sp1mut1 appear unimportant for base pairing, and their mutation might therefore not affect RNA packaging. In contrast, two G residues (G_{734} and G_{736}) are involved in base pairing, and their disruption might affect the packaging of the viral genome. For this reason, a second mutation of the Sp1 sites, designated Sp1mut2, was generated

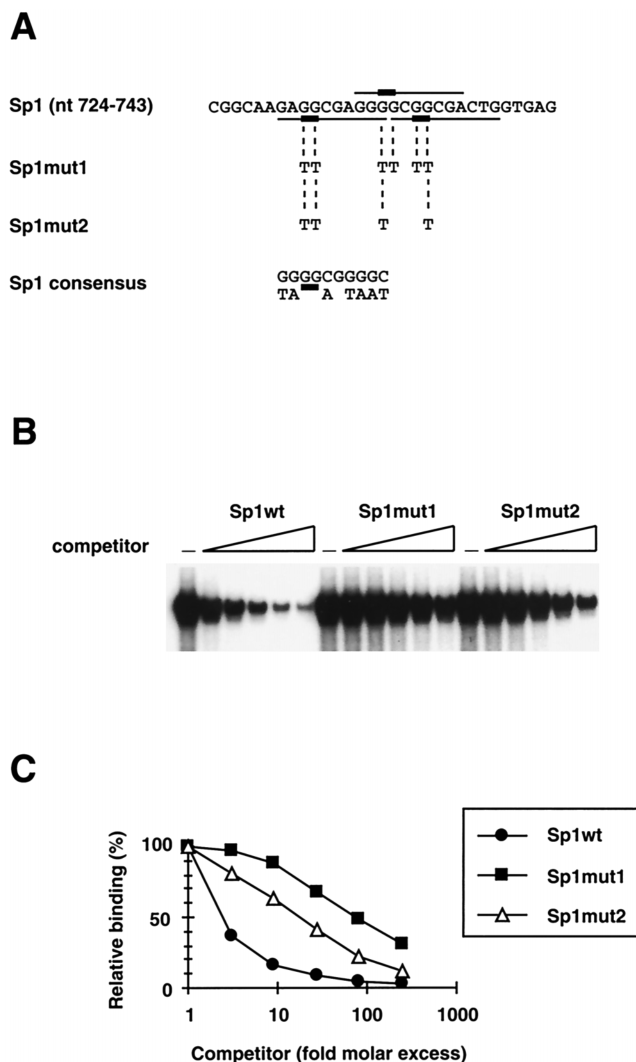


FIG. 7. Mutagenesis of the HS4 Sp1 binding sites. (A) Nucleotide sequence of the Sp1wt oligonucleotide, with underlined bases indicating the three potential Sp1 binding sites. The recognition core sequence 5′-GG-3′ of each site is indicated by a thicker bar. For Sp1mut1 and Sp1mut2 oligonucleotides, only the bases that are changed compared with the wt sequence are indicated. The Sp1 consensus DNA binding site is shown. (B) The effects of Sp1mut1 and Sp1mut2 mutations were tested in competition EMSAs. Sp1wt oligonucleotide probe was incubated with 0.05 fpu of affinity-purified Sp1 (Promega) in the absence of competitor or in the presence of increasing concentrations (3-, 9-, 27-, 81-, and 243-fold molar excess) of the homologous Sp1wt oligonucleotide, of the Sp1mut1 oligonucleotide or of the Sp1mut2 oligonucleotide. (C) Levels of complex formation of EMSAs shown in panel B were quantified by radioimaging analysis (InstantImager). Complex formation in the absence of unlabeled competitor DNA was taken as 100%.

so that G_{734} and G_{736} were not modified (Fig. 7A). As observed with the Sp1mut1 oligonucleotide, competition EMSAs showed that the Sp1mut2 oligonucleotide was significantly less efficient as a competitor than was the Sp1wt oligonucleotide (Fig. 7B and C), demonstrating that this mutation also greatly decreased binding to the Sp1 sites.

Generation of HIV-1 proviruses containing individual or combinations of mutated binding sites. To address the biological significance of each of the above binding sites in the HIV-1 life cycle, the mutations described above were introduced individually or in combination into an infectious clone of HIV-1.

In vitro site-directed mutagenesis of the DNA binding sites was performed with a plasmid containing the 5′ portion of the HIV-1 genome (nt 1 to 1447) and oligonucleotides containing the mutated sites as described in Materials and Methods. The mutation in pHIVΔPS/SP1 corresponds to Sp1mut1 and to two additional substitutions ($C_{690} \rightarrow A$, $C_{692} \rightarrow A$) designed to restore base pairing in the packaging-signal secondary stem-loop structure. The mutation in pHIVΔSP1 corresponds to Sp1mut2 and should not impair the packaging signal (see above). After site-directed mutagenesis and confirmation of the mutations by sequencing, fragments containing these mutations (*Mro*I-*Bss*H2 for all mutations except Sp1; *Mro*I-*Sph*I for the Sp1 mutations) were subcloned back into the corresponding sites of a derivative of pILIC referred to as pHIV (see Materials and Methods).

Replication properties of mutant viruses. (i) Generation of wt and mutant HIV-1 stocks by transfection-cocultivation. wt and mutant HIV-1 infectious proviruses were generated from the single-LTR-containing constructs by *Bam*HI digestion and self-ligation. To obtain stocks of infectious viruses and to obtain an initial measurement of the ability of mutant viruses to replicate, these proviruses were transfected into Jurkat cells. Transfected cells were cocultivated with SupT1 cells 1 day following transfection. Progeny virus production in coculture supernatants was then monitored by measuring the level of p24 gag antigen over a 50-day period (Fig. 8). Cell-free supernatants were harvested at the peak of viral production to generate virus stocks for subsequent infectivity studies.

Transfection-cocultivation with wt and mutant HIV proviral DNAs resulted in virus production detected at different times following transfection (Fig. 8). On the basis of their growth characteristics, the nine HS4 mutant proviruses were classified into four replicative phenotypes: (i) mutant proviruses pHIVΔDBF, pHIVΔAP3-L, and pHIVΔAP-1(III)/AP3-L demonstrated a replication phenotype similar to that of the wt provirus pHIV; (ii) virus production occurred with slightly delayed kinetics with mutants pHIVΔAP3-L/DBF and pHIVΔAP-1(III)/AP3-L/DBF in comparison to wild-type pHIV (detection 24 days following transfection); (iii) proviruses pHIVΔAP-1(I,II,III)/AP3-L and pHIVΔAP-1(I,II,III)/AP3-L/DBF exhibited a severely reduced replication phenotype since virus could be recovered only 44 to 48 days posttransfection, and in addition, lower concentrations of viral antigen were produced by these proviruses (Fig. 8); and (iv) no virus production was detected during a 60-day observation period following transfection with the proviruses pHIVΔPS/SP1 and pHIVΔSP1, indicating that proviruses carrying mutations in the HS4 Sp1 sites were totally defective in terms of replication.

To assess the possibility of reversion to a wt phenotype as an explanation for the growth of the mutant viruses, HIV-1 genomic RNA from each virus stock was purified and analyzed by RT-PCR with a primer pair that amplified 325 bp of the 5′ portion of the genome (nt 489 to 814) containing the mutated binding sites. PCR fragments were subcloned, and five individual clones for each mutant LTR were resequenced. This analysis confirmed the presence of the original mutations in the HS4 region (data not shown).

(ii) Infections of human PBMCs and T-cell lines with wt or mutant HIV-1 stocks. To study the effects of the HS4 mutations on viral growth kinetics, we infected phytohemagglutinin-stimulated PBMCs with wt and mutant HIV-1 stocks. Infection with wt virus resulted in rapid and vigorous virus production, with RT activity reaching a peak on days 4 to 6 postinfection, followed by a rapid drop reflecting a rapid reduction in viable cell numbers (Fig. 9A, left graph). Mutant viruses HIVΔDBF, HIVΔAP3-L, and HIVΔAP-1(III)/AP3-L replicated efficiently with replication kinetics and levels of virus production that

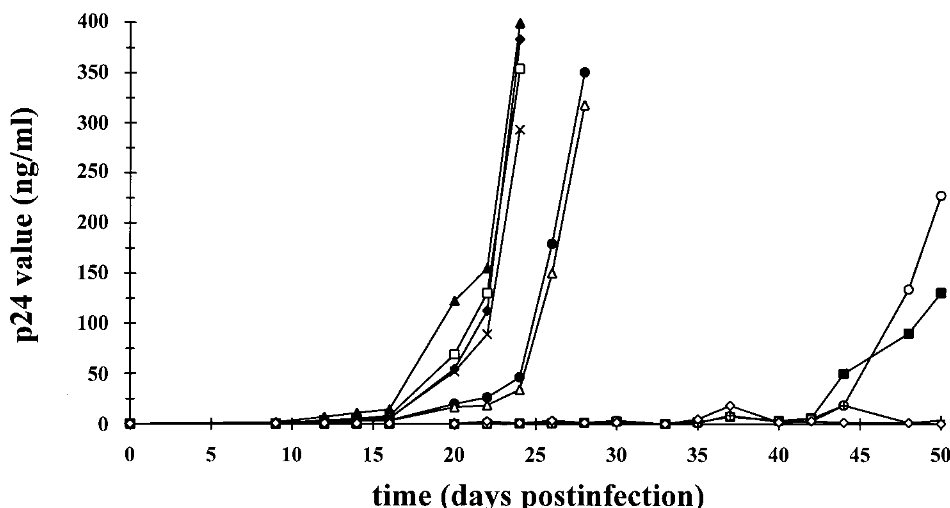


FIG. 8. Effect of HS4 mutations on HIV-1 replication in T-lymphoid cells. Infectious molecular clones of HIV-1 containing the HS4 mutations were transfected into Jurkat cells. Culture supernatants were harvested after different times (0 to 50 days) and tested for virus production by measuring secreted p24 antigen. Results for pHIV (◆), pHIVΔDBF (□), pHIVΔAP3-L (▲), pHIVΔAP-1(III)/AP3-L (×), pHIVVΔAP-1(I,II,III)/AP3-L (■), pHIVΔAP3-L/DBF (●), pHIVΔAP-1(III)/AP3-L/DBF (△), pHIVΔAP-1(I,II,III)/AP3-L/DBF (○), pHIVΔPS/SP1 (/), and pHIVΔSP1 (◇) are shown. One representative experiment of three is shown.

were similar to those of the wt control virus, indicating that individual mutation of the DBF or AP3-L site and the double mutation AP-1(III)/AP3-L did not affect HIV-1 replication in PBMCs (Fig. 9A, left). Virus production was observed with mutant viruses HIVΔAP3-L/DBF and HIVΔAP-1(III)/AP3-L/DBF to the same level as with wt HIV, with slightly delayed replication kinetics (the peak of infection was on day 8) (Fig. 9B, left). In contrast, infection of PBMCs with mutant viruses HIVΔAP-1(I,II,III)/AP3-L and HIVΔAP-1(I,II,III)/AP3-L/DBF produced very low RT release (about 8 times less than the amount released from cultures infected with the wt HIV at the peak of virus production), demonstrating severely reduced growth kinetics (Fig. 9C, left). These data are in good agreement with those obtained after transfection-cocultivation assays.

Similar results were obtained when the growth properties of mutant viruses on the T-lymphocyte cell lines Jurkat (Fig. 9, right panels) and SupT1 (data not shown) were assayed. However, although HIVΔAP-1(I,II,III)/AP3-L/DBF demonstrated delayed kinetics and produced lower concentrations of viral antigen than did the wt in Jurkat and SupT1, this mutant was less defective for replication in T-cell lines than it was in stimulated PBMCs. These differences may be due to different levels of specific transcription factors in different cell types examined. Such cell-type-specific differences in the replication properties of HIV-1 have been reported by others studying Tat activation response element (TAR) and LTR mutant viruses (25, 42).

Thus, the integrity of the DNA binding sites downstream of the HIV-1 transcription start site is critical for HIV-1 replication in human T cells, indicating a positive regulatory function for this region. Our findings strongly suggest a crucial role of the AP-1(I) and AP-1(II) sites on HIV-1 replication [compare viruses HIVΔAP-1(III)/AP3-L with HIVΔAP-1(I,II,III)/AP3-L and HIVΔAP-1(III)/AP3-L/DBF with HIVΔAP-1(I,II,III)/AP3-L/DBF].

Mutations do not affect virus RNA packaging. As discussed above, the RNA leader sequence of HIV-1 is assumed to adopt a stable secondary structure that plays a role in packaging of the viral genome in particles (6, 27). Therefore, each of the HS4 mutations could, in principle, be deleterious to virus replication by impairing RNA packaging. To test such a possibility, we used RNase protection assays to quantify HIV RNA from equivalent amounts of viral particles from each mutant virus stock (Fig. 10A). This experiment showed that the wt and mutant viruses contained the same amount of packaged RNA (Fig. 10A). As an internal control, we confirmed the presence of HIV-1-specific proteins in each of the mutant viruses. Lysates from equivalent amounts of RT activity from the wild-type and mutant virus stocks were prepared, and Western blot analyses were performed with purified human anti-HIV-1 IgG. Similar levels of p24 (Fig. 10B) and of the other viral structural proteins (data not shown) were detected in all lysates. These results demonstrate that the reduced replication phenotypes we observed with mutant viruses were not due to defects in RNA packaging. Since we were unable to generate virus stocks with the SP1 mutants, the effect of these mutations on packaging of the HIV-1 genome into particles could not be assessed.

Downstream binding sites play a positive regulatory role on HIV-1 transcription. To assess the transcriptional regulatory function of the HS4 binding sites, the single-LTR-containing proviral constructs carrying the HS4 mutations were transiently transfected into Jurkat cells and total RNA was purified 24 and 48 h posttransfection. Steady-state levels of HIV-1 mRNA were measured by the RNase protection assay with an HIV-1 promoter-specific probe (Fig. 11A). RNase protection analysis of the same cellular extracts with an antisense probe for the GAPDH gene was performed as an internal control to correct sample-to-sample variations in mRNA levels (Fig. 11A). As shown in Fig. 11B, individual mutation of the DBF or AP3-L site as well as the double mutation AP-1(III)/AP3-L reduced the viral RNA level (to 64, 47, and 51% of the wt level, respectively), although these mutations had no effect on HIV-1 replication. Mutation of the AP3-L/DBF and of the AP-1(III)/AP3-L/DBF sites resulted in a dramatic decrease of LTR-mediated transcription, resulting in as little as 24 and 18% of the wt expression, respectively (Fig. 11B). These transfection results contrast with our infection experiments, in which the same mutations only slightly delayed HIV-1 replication (see above), suggesting that other *cis*-acting elements in the HIV genome compensate for the deleterious effects of these muta-

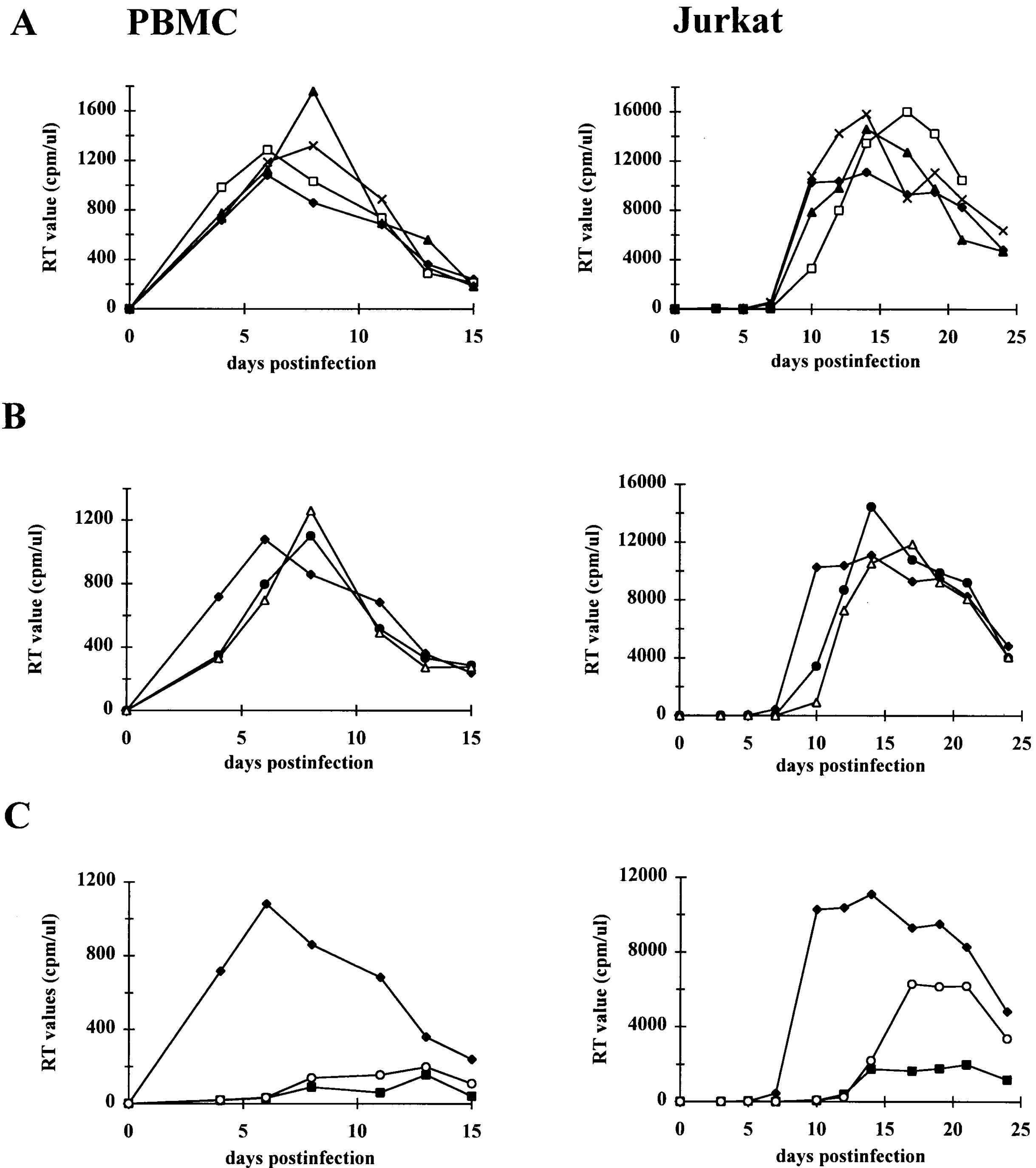


FIG. 9. Mutations in the HS4 binding sites affect HIV-1 growth kinetics. PHA-activated human PBMCs and Jurkat cells were infected with equivalent amounts of RT activity of wt or mutant HIV infectious stocks as described in Materials and Methods. Culture supernatants were harvested at different times following infection and analyzed for RT activity. Results for HIV (◆), HIVΔDBF (□), HIVΔAP3-L (▲), HIVΔAP-1(III)/AP3-L (×), HIVVΔAP-1(I,II,III)/AP3-L (■), HIVΔAP3-L/DBF (●), HIVΔAP-1(III)/AP3-L/DBF (△), and HIVΔAP-1(I,II,III)/AP3-L/DBF (○) are shown. The means of duplicate samples are presented. Infections were performed three times with identical results. A representative experiment is shown.

tions on viral transcription. Mutation of AP-1(I,II,III)/AP3-L and of AP-1(I,II,III)/AP3-L/DBF sites also resulted in decreased HIV-1 mRNA levels (28 and 26% respectively [Fig. 11B]). These data are in agreement with our infection studies in which these mutant viruses demonstrated a severely reduced replication phenotype. As noted above, mutations of the Sp1 sites were lethal for the virus and were therefore expected to demonstrate the most significant effects on HIV-1 transcription. However, transfection of pHIVΔPS/SP1 and pHIVΔSP1 had almost no effect on the viral mRNA level (Fig. 11), indicating that the Sp1 sites had no positive function on the HIV-1 promoter under these experimental conditions. Similar results were obtained when a construction containing the HIV LTR carrying the same mutations was cotransfected with a Tat ex-

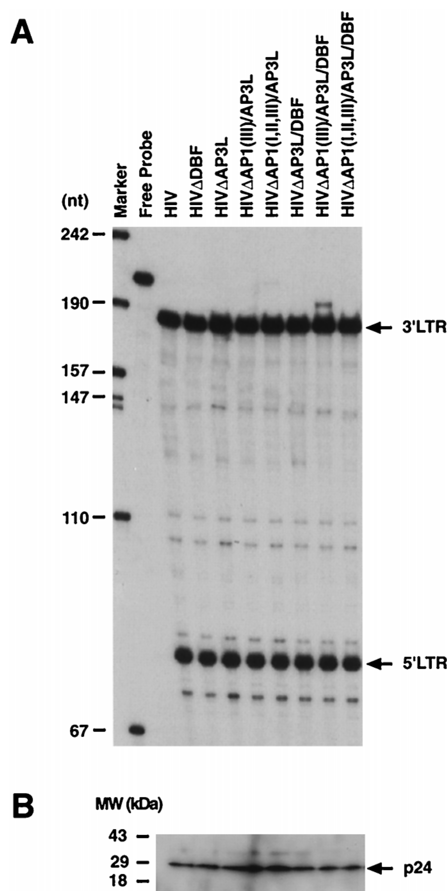


FIG. 10. HS4 mutations do not affect HIV genomic packaging. (A) Equivalent amounts of viral particles (assessed by the RT assay) from each mutant HIV stock were pelleted by ultracentrifugation. After lysis of viral particles in guanidine thiocyanate, HIV-1 RNA was detected by RNase protection analysis with an antisense HIV-1 riboprobe which protects two bands (200 and 83 nt) corresponding to the 3′ and 5′ LTR, respectively. Lane Marker contains pSK−/*Apa*II markers, whose sizes (in nucleotides) are indicated on the left. Intensities of RNA bands were quantified by radioimaging analysis (InstantImager). (B) The ultracentrifuged viral stocks described in panel A were lysed in Laemmli buffer, analyzed by Western blotting with anti-HIV-1 immunoglobulin (donated by Alfred Prince, AIDS Research and Reference Reagent Program), and detected by enhanced chemiluminescence with a horseradish peroxidase-conjugated goat anti-human IgG. MW, molecular mass (indicated in kilodaltons).

pression vector (59) (Fig. 11C), except for pLTRΔAP-1(I,II, III)/AP3L, which showed normal transcriptional activity, whereas it showed defective activity in the complete virus transfection assay. This discrepancy could be due to the role of other HIV-encoded proteins that might play a role in transcriptional regulation or to pleiotropic effects at the level of RNA stability or dimerization. Identical results were obtained with other cell lines (Raji, SupT1) and in the absence of Tat (data not shown).

These observations demonstrate that the positive regulatory function of the downstream binding sites takes place in part at the level of transcription.

DISCUSSION

A cluster of binding sites for several transcription factors (AP-1, AP3-L/NF-AT, DBF/IRF, and SP1) has been identified downstream of the HIV-1 transcription start site. In the present study, we have characterized each of these binding sites and have identified minimal point mutations that eliminated the binding of the factors to their respective sites. The AP3-L site is shown to bind an ionomycin-inducible factor corresponding to NF-AT, and the DBF site binds IRF1 and IRF2 factors. HIV-1 proviruses containing individual or combinations of the mutated sites were generated, and their growth kinetics on human PBMCs and T-lymphocyte cell lines were compared with those of wt HIV-1. Individual mutation of the DBF or AP3-L site, as well as the double mutation AP-1 (III)/AP3-L, did not affect HIV-1 replication. Proviruses carrying mutations in the Sp1 sites were found to be defective for virus replication. Virus production occurred with slightly delayed kinetics with viruses containing mutations in AP-1(III)/AP3-L/DBF sites and in AP3-L/DBF sites. Viruses mutated in AP-1(I,II,III)/AP3-L sites and in AP-1(I,II,III)/AP3-L/DBF sites exhibited severely reduced replication. RNase protection assays from equivalent amounts of viral particles from each mutant HIV stock showed no RNA-packaging defect. Furthermore, point mutations in the HS4 region nearly completely inhibited HIV-1 LTR-directed transcription, suggesting that *cis*-acting elements within this region are required for optimal promoter activity.

Role of transcription factor binding sites downstream of the transcription start site in HIV-1 replication. (i) AP-1 sites. Functionally important AP-1 sites have been identified in the regulatory regions of cellular genes and of retroviruses, including human T-cell leukemia virus type 1, human foamy virus, and feline immunodeficiency virus type 1 (36, 52, 74). Furthermore, AP-1 binding sites have also been identified in the genome of the lentivirus visna virus, where they play a critical role in basal activity and transactivation of the viral LTR by the virus-encoded TAT protein (19, 29).

HIVΔAP3-L and HIVΔAP-1(III)/AP3-L both exhibit a replication phenotype similar to wt HIV, and HIVΔAP3-L/DBF and HIVΔAP-1(III)/AP3-L/DBF both display slightly delayed replication, suggesting that, in vivo, the AP-1(III) site may not be critical for HIV-1 replication, although this site binds AP-1 with a stronger affinity than does either AP-1(I) or AP-1(II). In contrast, we have observed a severe delay in replication with HIVΔAP-1(I,II,III)/AP3-L and with HIVΔAP-1(I,II,III)/AP3-L/DBF in both transfection and infection assays. These mutations affect viral replication at the transcriptional level, as indicated by our transfection studies. These results therefore indicate a crucial role of AP-1(I) and AP-1(II) sites in HIV-1 transcription and replication.

While these AP-1 sites were initially characterized by in vitro footprinting assays with purified c-jun protein (13), it is important to stress that we have not yet identified the factors that bind to these sites under physiological conditions. The AP-1 family of transcription factors is composed of representatives from the jun and fos family that can homo- or heterodimerize (4, 15, 41, 65, 73, 84). In addition, jun proteins can heterodimerize with ATF/CREB proteins, thereby further increasing the potential diversity of factors bound to AP-1 sites (7, 22). Different specificities in terms of DNA binding can therefore be generated depending on the partners in the complex. Preliminary characterization of the factors binding to sites I and III suggested that these sites interact with factors distinct from jun and fos (63). Further characterization of the factors binding to these AP-1 sites in the HIV-1 leader sequence will shed new light on their role in HIV-1 transcriptional regulation.

(ii) AP3-L/NF-AT motif. On the basis of sequence homology and gel retardation experiments, we have identified the AP3-L site as an NF-AT binding site. Interestingly, uninduced nuclear extracts from several lymphoid cell lines contained factors binding to the AP3-L probe, even though the NF-AT binding activity is typically dependent on T-cell activation signals (Fig.

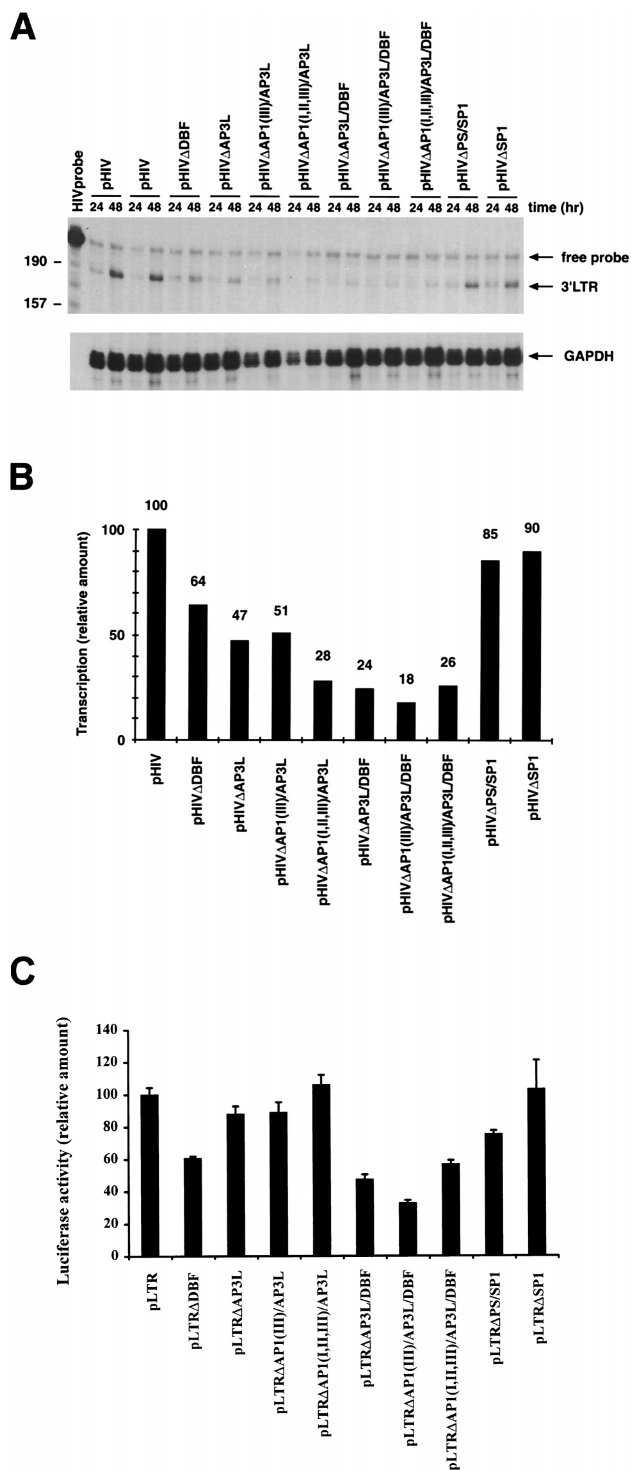


FIG. 11. Effects of the HS4 mutations on HIV-1 transcription. (A) The transcriptional activity of the HIV-1 DNA proviral constructs was determined following transient transfection into Jurkat cells and analysis by an RNase protection assay 24 and 48 h after transfection. Names of the proviral constructs transfected are indicated at the top of each lane. Cellular lysates were incubated with two specific probes corresponding to the HIV-1 LTR and to the GAPDH promoter. The figure shows only the 200-nt protected band corresponding to the HIV-1 3′ LTR and the broad band (5 nt wide) corresponding to the protected GAPDH probe. Undigested HIV-1 probe is shown for reference. DNA size markers are shown on the left for reference. (B) RNA bands from the experiment shown in panel A were quantified by PhosphorImager scanning of the gel. HIV-1 RNA levels measured 48 h posttransfection were normalized relative to the levels of GAPDH RNA. The results are presented as histograms indicating the relative transcription with respect to that of wild-type plasmid pHIV, which was assigned a value of 100%. (C) Plasmids containing either wt or mutated HIV LTR driving the firefly luciferase were cotransfected with an expression vector for Tat and with a control plasmid in which the herpes simplex virus thymidine kinase is driving the renilla luciferase gene expression. Luciferase activities (firefly and renilla) were measured in cellular extracts. Results are expressed as $\text{Luciferase}_{\text{firefly}}/\text{Luciferase}_{\text{renilla}}$. The average of eight different transfections (± standard error of the mean) with two different DNA preparations are shown.

3A). These factors present in uninduced T cells could correspond to newly identified members of the NF-AT family of transcription factors such as NFAT3 or NFATx/NFAT4/NFATc3 (31, 32, 51). Individual mutation of the AP3-L/NF-AT site or of the DBF site did not affect HIV-1 replication, whereas the simultaneous mutation of both sites (AP3-L/DBF) slightly delayed replication, suggesting that these sites may functionally substitute for each other in positively regulating HIV-1 transcription. Another NF-AT binding site, which is likely to functionally complement the site that we mutated in the R region, has been identified in the U3 region of the HIV-1 LTR (70). Functional redundancy is a common feature of viral and cellular transcriptional regulatory regions and has been extensively studied in the context of the SV40 enhancer (17, 28, 57, 83). Virus revertants arising after the mutation of enhancer elements contained duplications of the remaining elements, implying that different parts of an enhancer can functionally substitute for each other (28).

(iii) DBF/IRF site. We have demonstrated that the DBF site presents a sequence homology to the IFN-stimulated response element and binds a complex that contains both the IRF-1 and IRF-2 proteins. The IRF-1 protein was originally identified as a protein that binds to the ISRE of IFN-α/β-stimulated genes (24, 45). It is present in low concentrations under basal conditions and is induced at the transcriptional level in response to a variety of cytokines including IFN-γ, IFN-α, IL-1, IL-6, tumor necrosis factor alpha and leukemia inhibitory factor (1, 18). In agreement with our observations, Thornton et al. recently demonstrated that the DBF site binds factors specific for known ISREs (76). These authors have shown that cells expressing a dominant negative factor of the IRF family are nonpermissive for HIV-1 infection, suggesting that infection by HIV-1 is, at least in part, regulated by an IRF-dependent transcriptional pathway(s) (76). However, in contrast to their observations, we were unable to demonstrate binding of the ISGF3 complex to the HIV element. Our binding experiments therefore define the DBF site as a site uniquely bound by members of the IRF family of transcription factors and not by the ISGF3 complex. We have not examined in this report the possibility that this site functions as an IFN-α/β-stimulated response element and therefore confers IFN responsiveness to the HIV-1 promoter. Additionally, since IRF-1 is also induced in response to IFN-γ, IL-1, IL-6, and tumor necrosis factor alpha (1, 18), the DBF site might serve, in cooperation with NF-κB, TCF-1α, and NF-AT, to increase the responsiveness of the HIV-1 promoter to extracellular activation signals. Experiments are under way to test this hypothesis.

(iv) Sp1 sites. Whereas mutations of the Sp1 sites in the HS4 region have no effect on HIV-1 promoter activity in transient-transfection assays (reference 13 and see above) we observed that proviruses containing the same mutations are defective for virus replication. Three possible explanations can be proposed to explain these results. (i) Transient-transfection experiments may not reflect the regulation found in vivo with the intact provirus since transiently transfected DNA is not assembled

into physiological chromatin. Similar discrepancies between transient transfection studies and in vivo functional studies have been reported previously for HIV (25, 42, 82). (ii) The Sp1 mutations might disrupt RNA packaging and cause a replication defect independent of transcription. Indeed, our lack of understanding of the folding of the RNA structure involved in RNA packaging in terms of tertiary or quaternary RNA interactions might have hampered our efforts not to disrupt a biologically important structure. (iii) The HIV leader sequence is involved in other RNA functions which include dimerization, translation initiation, and efficiency. It is therefore conceivable that the Sp1 mutations affect one of these functions and, as a consequence, HIV-1 replication.

Similar concerns exist for other mutations studied in this report. Indeed, the HIV leader RNA is organized in a large structure that plays many critical roles in the HIV life cycle (27). These include packaging and dimerization of the RNA genome and initiation of reverse transcription (see references 26, 27, 40, and 66 for examples). In addition, disruption of this RNA structure might result in decreased stability of HIV RNA. While we have excluded a packaging defect for the mutants described here, except for Sp1 mutations, other pleiotropic effects of these mutations cannot be excluded. Such pleiotropic effects are in fact likely in the case of mutant HIVΔAP1 (I,II,III)/AP3L, since this mutant showed delayed replication kinetics as a virus and little to no effect at the transcriptional level in transient-transfection assays. In the case of other viruses, a close correlation exists between the replication kinetics of the virus and the effect of the mutations in transient-transfection reporter assays, and it is therefore likely that the replication defects observed are transcriptional in nature.

We have previously demonstrated that a single nucleosome positioned at the transcription start site (nuc-1) is disrupted during transcriptional activation of the HIV-1 promoter (78, 81). The molecular mechanism for the positioning and disruption of nuc-1 is at present unclear. Three distinct mechanisms potentially contribute to the ordering of nucleosomes relative to nuclease-hypersensitive sites in other systems: (i) sequence-directed nucleosome positioning, (ii) statistical positioning which relies on the generation of boundaries by nonhistone proteins causing nucleosomal arrays to phase themselves relative to these boundaries, and (iii) active positioning of a nucleosome by its direct or indirect interaction with a *trans*-acting factor (reviewed in reference 72). Since the AP-1(III), AP3-L, and DBF sites lie at the 3′ boundary of nuc-1, these sites could play an important role in the positioning of nuc-1 at its 3′ boundary. This could occur either indirectly through a boundary effect or directly through an interaction between a nucleosomal component and one of these factors. The Sp1 sites lie at the 5′ boundary of nuc-2 and are thus likely to play a role in its positioning. Recent experiments in which an LTR containing mutations in most of the HS4 binding sites [AP-1(III), AP3-L, DBF, and Sp1] was stably integrated into HeLa cells have shown reduced transcriptional activity of the HIV promoter accompanied by the disappearance of nuclease-hypersensitive site IV (12). These experiments demonstrate that these sites collectively contribute to the establishment of a nucleosome-free region corresponding to HS4 and are in good agreement with the results reported here. A study of the chromatin organization of the leader sequence of HIV-1 with the mutants described in this paper will further define which factors are critical for the establishment of the native chromatin organization of integrated HIV-1. The transcription factors AP-1 and NF-AT are both induced in response to T-cell activation signals, as is the disruption of nuc-1. It is therefore conceivable that these factors play a role in the disruption by competing with nuc-1 histones for binding to DNA.

Our observations indicate that the HS4 binding sites characterized here constitute a novel enhancer that functions independently of, or in concert with, other factors binding to the HIV-1 LTR to activate HIV-1 transcription. Several studies have shown that mutated proviruses with no functional NF-κB binding sites are still competent in terms of viral replication (44, 48, 64), indicating that NF-κB binding sites can be complemented by other *cis*-acting elements located in the viral genome. The binding sites studied in this report could play such a role, alone or in conjunction with *cis* elements of the 5′ LTR. Binding of these factors downstream of the HIV-1 transcription start site could cause additional cellular specificity, increase the strength of the promoter-enhancer unit located in the LTR, or provide a mechanism to broaden the viral response to extracellular stimuli and activate transcription under a wider variety of cellular conditions. Interestingly, the integrated Moloney murine leukemia provirus contains a DNase I-hypersensitive site immediately downstream of the 5′ LTR (61), in a position similar to the DNase I-hypersensitive site 4 of HIV-1. This region of the provirus contains a *cis*-acting element responsible for the selective suppression of viral transcription in embryonic carcinoma cells (46, 47, 77). Since Sp1 has been shown to mediate the formation of DNA loops between Sp1 proteins bound at two different sites on a DNA molecule (50, 75), we previously proposed that Sp1 proteins bound at the promoter (37) and Sp1 proteins bound in the HS4 region interact with each other and bring in close proximity the two nucleosome-free regions and the factors interacting with them (81). Such a loop may thus be important for positioning sites in HS4 close to the promoter and therefore provides a structural framework in which the interactions described above could take place. The observations reported here demonstrate a crucial role in HIV-1 infectivity and transcriptional regulation for the nuclease-hypersensitive region located downstream of the transcription start site. Demonstration of a positive regulatory element in the transcribed region of the HIV genome introduces an additional factor into an already complex network of regulators affecting the degree of HIV gene expression.

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