Nuclear Factors That Bind Two Regions Important to Transcriptional Activity of the Simian Immunodeficiency Virus Long Terminal Repeat

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Previous studies identified two regions in the U3 region of a molecular clone of simian immunodeficiency virus, SIV_{mac142}, that are important to transcriptional activity under conditions of induction as well as basal-level expression (B. Renjifo, N. A. Speck, S. Winandy, N. Hopkins, and Y. Li, *J. Virol.* 64:3130–3134, 1990). One region includes the NF- κ B binding site, while the other lies just 5' of this site between nucleotides –162 and –114 (the –162 to –114 region). The fact that the NF- κ B site mutation attenuated transcriptional activity in uninduced T cells and fibroblasts where activated NF- κ B would not be present suggested that a factor(s) other than NF- κ B could be acting through this site. In this study, we have identified a factor which binds to a *cis* element overlapping the NF- κ B site. This factor, which we call simian factor 3 (SF3), would play a role in regulation under conditions of basal level expression, whereas under conditions of induction, NF- κ B would act via this region. SF3 may also bind to an element in the –162 to –114 region. In addition, we have identified two other factors that bind the –162 to –114 region. One, which we designated SF1, is a ubiquitous basal factor, and the other, SF2, is a T-cell-predominant phorbol myristate acetate-inducible factor. Through identification of nuclear factors that interact with the U3 region of the SIV_{mac142} long terminal repeat, we can gain insight into how this virus is transcriptionally regulated under conditions of basal-level expression as well as conditions of T-cell activation.

The simian immunodeficiency viruses (SIVs) are a diverse group of lentiviruses, some of which are able to induce in monkeys a disease that is similar to human AIDS (6, 31, 32). SIVs are the closest known relatives of human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2), with similar genome organizations as well as many shared biological properties, including tropism for CD4⁺ cells such as T cells and monocytes/macrophages and cytopathic effects and syncytium formation in vitro (5, 25). For these reasons, as well as the fact that, to date, no animal inoculated with HIV has developed an AIDS-like disease, infection of monkeys by SIV is being intensively studied as the best candidate for an animal model for AIDS. Contributing significantly to this model's usefulness was the demonstration that a molecular clone (SIV_{mac239}) is pathogenic in macaques (28).

The long terminal repeats (LTRs) of retroviruses contain promoter and enhancer elements essential to transcriptional activity and regulation. LTRs have been shown to play an important role in both disease induction and specificity in some type C retroviral systems (4, 8, 29, 47). Since increased viral replication is associated with disease progression of HIV (22), LTR sequences may also influence the disease course of AIDS. A number of proteins, both cellular and viral, have been shown to act through *cis* elements in the HIV LTR. Some of these factors have been shown by in vitro assays to play a role in regulation of viral production (30, 33, 34). These factors include inducible transcription factors such as nuclear factor κ B (NF- κ B) (10, 27, 39), nuclear factor of activated T cells (NFAT-1) (45), and AP-1

(14), constitutively active transcription factors such as Sp1 (24) and upstream stimulatory factor (USF) (2, 15), and a virally encoded protein, Tat (7, 13).

In order to determine the role of transcriptional signals in the pathogenesis of SIV, we have been identifying *cis* elements and cellular transcription factors that interact with them in the LTRs of molecular clones of SIV with the ultimate goal of mutating these sequences and determining the effects of the mutations on pathogenesis in vivo. The majority of the work was done with the LTR of a molecular SIV clone (SIV_{mac142}) derived from a virus isolated from a rhesus macaque which died of an AIDS-like disease (3). As determined by sequence analysis, the SIV_{mac142} LTR contains elements similar to those found in the HIV-1 and HIV-2 LTRs, including a TATA box (nucleotide –32 to –27), three motifs related to the Sp1 consensus binding site (–52 to –80), and an NF- κ B binding site (–96 to –105). By functional analysis, the LTR was also found to contain a *trans*-acting responsive (TAR) element which is capable of being transactivated by the HIV-1 Tat protein (42).

We previously identified additional *cis* elements important to both basal and TPA (12-*O*-tetradecanoylphorbol-13-acetate)-inducible transcription of the virus through a deletion and mutational analysis of the SIV_{mac142} LTR (42). In our studies, one of two highly detrimental mutations affecting transcriptional activity in phorbol myristate acetate (PMA)-induced and uninduced T cells (Jurkat) as well as fibroblasts (Rat-1) was the NF- κ B binding site mutation (–GGGACTT TCC– to –GGGACTTTAA–). NF- κ B is an inducible factor which is usually constitutively active only in mature B cells (43, 44) and macrophages (19). It was therefore surprising that the NF- κ B site mutation we constructed affected the basal activity of the LTR in uninduced T cells and fibro-

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blasts. This result suggested that a constitutively active factor may act via this site in addition to the inducible factor, NF- κ B. The second region shown to be important to both basal and PMA-inducible transcriptional activity of the SIV_{mac}142 LTR lies between nucleotides -162 and -114 (the -162 to -114 region). Deletion from -162 to -114 caused a fivefold reduction in transcriptional activity in fibroblasts.

In this study, our goal was to identify the factors which bind to these two functionally important regions, the -162 to -114 and NF- κ B sequences. We have identified two factors which bind in the -162 to -114 region; one contributes to the basal activity through this region, and the other contributes to the inducibility. We also identified a constitutive factor which binds an element overlapping the NF- κ B site whose binding would have been disrupted by the NF- κ B site mutation studied previously.

MATERIALS AND METHODS

Extracts and cell lines. Jurkat T cells were grown in RPMI 1640 supplemented with 10% inactivated fetal calf serum, 200 mM glutamine, and 100 μ g each of penicillin and streptomycin per ml. Rat-1 cells were grown in Dulbecco modified Eagle medium with 10% inactivated fetal calf serum and 100 μ g each of penicillin and streptomycin per ml.

Some nuclear extracts were made by the method of Dignam et al. (9). Others were prepared by a miniprep procedure as follows. Approximately 5×10^7 cells were pelleted and washed once with ice-cold phosphate-buffered saline. The cells were resuspended in a 10% Nonidet P-40 solution in TE (10 mM Tris hydrochloride [pH 8], 1 mM EDTA) and allowed to sit on ice for 5 min. The nuclei were pelleted and resuspended in approximately 60 μ l of buffer C (9) by dispersing the pellet up and down with a Pasteur pipette. The nuclear debris was then removed by centrifugation, and the supernatant was saved. Protein concentrations were determined by the Bio-Rad protein assay.

Probes and competitors. The probes and competitors used were double-stranded synthetic oligonucleotides. Complementary synthetic oligonucleotides were made with an Applied Biosystems 380B DNA synthesizer. Double-stranded oligonucleotide probes were generated by end labeling one strand with [γ - 32 P]ATP (ICN) by using polynucleotide kinase (New England Biolabs) and annealing this strand with the complementary strand. The resulting double-stranded probes were gel purified in 15% native polyacrylamide gels and eluted from gel slices overnight at room temperature in 1 M NaCl-20 mM Tris hydrochloride (pH 8)-1 mM EDTA (pH 8). Double-stranded oligonucleotide competitors were prepared by annealing the complementary strands in TE at a final double-stranded DNA concentration of 50 ng/ μ l. The sense-strand sequence (5' to 3') of each oligonucleotide and its designation are as follows (lowercase letters designate linker sequences): SIV(-148 to -114), gatccCCTTCTTGA AATGGCTGACAGGAAGGAAACg; SF1 site, gatccCCTT CTTGAAATGGCTGACATTAATg; SF2 site, gatccATTT CTTACAGGAAGGAACTAGCTg; NF- κ B/SF3(142), GC TGAGACAGCAGGGACTTTCCACAAGGGGAT; NF- κ B/SF3(239), AAACAGCAGGGACTTTCCACAAGG; and NF- κ B-(239), AAACAGCACTCACTTTCCACAAGGG.

Plasmids. The vector used in these studies for in vivo transcriptional analyses, pMoCAT, consists of a pUC13 vector into which the bacterial chloramphenicol acetyltransferase (CAT) gene, the polyadenylation signal from pSV2CAT, and the Moloney murine leukemia virus pro-

moter have been subcloned (46). Blunt-ended double-stranded synthetic oligonucleotides were subcloned upstream of the Moloney murine leukemia virus promoter into the *Xba*I site of pMoCAT. Clones were sequenced by dideoxy-chain termination with Sequenase.

Gel binding. For Tris-borate gels, binding reactions were carried out and gels were electrophoresed by using the specifications described by Manley et al. (36).

For Tris-glycine gels, binding reactions were carried out in a mixture of 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.5), 1 mM EDTA, 150 mM NaCl, 5 mM dithiothreitol, and 10% glycerol for 15 min at room temperature. Typical reaction mixtures contained 10,000 cpm of end-labeled probe, 10 μ g of extract, 10 ng of a heterologous single-stranded oligonucleotide, and 75 ng of poly(dI-dC) in a total volume of 20 μ l. The binding reaction mixtures were electrophoresed through a 6% polyacrylamide gel (acrylamide/bisacrylamide ratio, 29:1) containing 8.5 mM Tris (pH 8.5), 65 mM glycine, and 0.35 mM EDTA. The running buffer contained 5 mM Tris (pH 8.5), 40 mM glycine, and 0.2 mM EDTA. The gels were run at 12 V/cm at room temperature.

Methylation interference. Methylation interference assays were performed as described by Manley et al. (36) with the following modification. In some cases, the protein-DNA complex and free DNA bands were cut from the preparative binding gels, and the DNA was eluted by casting the gel slices into 1% agarose gels and electrophoresing them onto DEAE paper (Schleicher & Schuell) (11). DNA was eluted from the paper in 500 μ l of 20 mM Tris (pH 8)-0.1 mM EDTA-1 M NaCl for 1 h at 68°C.

DNA transfection and CAT assays. Jurkat T cells (10^7 per sample) were transfected with 0.5 to 2 μ g of test plasmid and 8 to 8.75 μ g of carrier DNA (salmon sperm DNA) by the DEAE-dextran method (20). Rat-1 cells (2×10^6 per sample) were transfected with the same quantities of DNAs by the calcium phosphate method (16). Eighteen hours posttransfection, the cultures were treated with 10^{-7} M PMA. Cells were harvested 48 h posttransfection. CAT assays were performed as described previously (18). The amount of protein used per assay was standardized by the Bio-Rad protein assay. From 50 to 200 μ g of protein was used per sample, depending on the experiment.

PCR mutagenesis and plasmid construction. The primers used were as follows (5' to 3'; mutations are underlined): PCR1 (nucleotide -297 to -279), GTGGGATGACCCCTTG GTTA; PCR2 (+260 to +242), GGGTCCTAACAGAC CAGGG; PCR3 (-110 to -77), GACAGCATCTACTTTC CACAAGGG; and PCR4 (-77 to -110), CCCTTGTG GAAAGTAGATGCTGTC. Polymerase chain reaction (PCR) mutagenesis was performed as described previously (23) with the following modifications. The target plasmid for mutagenesis (pCS142RI.4) contained the 3' LTR of SIV_{mac}142 in pUC-CAT. The final PCR product was restricted with *Nhe*I and *Hinc*II to form a 380-bp DNA fragment corresponding to the 3' portion of the SIV LTR fragment to be subcloned. This 380-bp fragment was purified from an agarose gel by electrophoresis onto DEAE paper. DNA was eluted from the paper in 500 μ l of 20 mM Tris (pH 8)-1 mM EDTA-1 M NaCl for 2 h at 68°C. The DNA was extracted with phenol and chloroform twice, ethanol precipitated, rinsed with 70% ethanol, dried, and resuspended in TE. A three-part ligation was performed with the *Nhe*I- and *Hinc*II-restricted PCR product, a *Hind*III-*Nhe*I restriction fragment corresponding to the 5' portion of the SIV LTR, and

FIG. 1. Identification of SF1 and SF2 binding by electrophoretic mobility shift assay of oligonucleotide SIV(-148 to -114) in the Tris-borate buffer system. The binding reaction mixtures contained 50,000 cpm of end-labeled oligonucleotide DNA as a probe and were incubated with EL4 nuclear extract (20 μ g) and, in some cases, unlabeled double-stranded oligonucleotide competitor DNAs. The arrows indicate the two complexes designated SF1 (lower complex) and SF2 (upper complex). Competitors: lane 1, no competitor; lane 2, oligonucleotide SIV(-148 to -114) (50 ng); lane 3, oligonucleotide SF1 (12.5 ng); lane 4, oligonucleotide SF2 (12.5 ng); lane 5, nonspecific competitor oligonucleotide NF- κ B/SF3(142) (50 ng).

HindIII- and SmaI-restricted pUC-CAT to form plasmid pSIV(NF- κ B-, SF3+)CAT.

The entire subcloned PCR product was sequenced by the dideoxy-chain termination method with Sequenase.

RESULTS

Two nuclear factors, SF1 and SF2, that bind to the -162 to -114 region. The importance of the -162 to -114 region to the transcriptional activity of the SIV LTR shown in our previous studies led us to study the *trans*-acting factors that bind this region by electrophoretic mobility shift assay. Initial experiments using the -162 to -114 region as a probe and a mouse T-cell nuclear extract (EL4) indicated the presence of two specific complexes. The same binding pattern was seen when a synthetic oligonucleotide corresponding to nucleotides -148 to -114 [SIV(-148 to -114)] was used as a probe. Methylation interference data obtained with SIV(-148 to -114) identified two potential binding sites for nuclear factors in this region (data not shown). Oligonucleotides corresponding to each of these sites individually (designated the simian factor 1 [SF1] and SF2 sites) were synthesized and used as competitors in this assay with SIV(-148 to -114) used as a probe. As shown in Fig. 1, each oligonucleotide competed for one of the two protein-DNA complexes. No shift which corresponded to both factors binding simultaneously was seen.

To define specific guanine contact residues on the DNA for these two factors, methylation interference assays were performed. When the two strands of the synthetic oligonucleotides used in the binding gels were analyzed, specific

FIG. 2. Methylation interference experiments to define the binding sites of SF1 and SF2 within the -148 to -114 region of the SIV_{mac}142 LTR. Preparative reaction mixtures contained 1.2×10^6 cpm of end-labeled oligonucleotide probe, 320 μ g of EL4 nuclear extract, and 12 μ g of poly(dI-dC) in a final volume of 80 μ l. Free DNA and protein-DNA complex bands are indicated by F and C, respectively, above the lanes. Arrows indicate bands in the sequence ladder involved in the methylation interference pattern. Methylation of G residues indicated by asterisks results in inhibition of specific complex formation. (A) Analyses of the SF1 and SF2 complexes formed with the SF1 and SF2 oligonucleotides, respectively. The noncoding strand of the SF2 complex is not included, since no guanine residues on this strand were involved in binding of the complex. (B) Summary of the results of these methylation interference experiments. The region from nucleotide -148 to -114 of the SIV_{mac}142 LTR is shown.

binding sites for these nuclear factors were delineated as 5'-CTGAC-3' (SF1; nucleotide -135 to -131), and 5'-AGGAAGG-3' (SF2; -130 to -124) (Fig. 2).

Nuclear extracts from cell lines representing both hematopoietic and nonhematopoietic lineages were tested for the presence of the SF1 and SF2 complexes. A Tris-glycine buffer system was used for these experiments, since the intensities of the SF1 and SF2 complexes were greater and their mobilities were more distinct in this buffer system than in the Tris-borate buffer system, allowing for easier identification of the complexes. When the SF1 site oligonucleotide was used as a probe, specific complexes of identical mobility were seen in each extract except the Rat-1 cell nuclear extract, with no significant difference in abundance, leading us to conclude that SF1 is a ubiquitous nuclear factor (Fig. 3A). The Rat-1 cell nuclear extract did contain a shift corresponding to SF1, as determined by methylation interference assays (data not shown), although this shift showed a higher mobility than in other extracts. On the other hand, a strong SF2 complex signal was detected only in the T-cell extracts (EL4 and Jurkat) (Fig. 3B). Methylation interference assays for the SF1 and SF2 complexes were performed only with the EL4 and Rat-1 cell nuclear extracts. Therefore, we cannot exclude the possibility that some of the complexes seen in the non-T-cell nuclear extracts in Fig. 3B correspond to SF2 complexes. However, these bands all have considerably lower intensities than the SF2 complexes

FIG. 3. Tissue distribution of the SF1 (A) and SF2 (B) complexes determined by using the SF1 and SF2 oligonucleotides, respectively, as probes in electrophoretic mobility shift assays in a Tris-glycine buffer system. In each reaction, 10,000 cpm of end-labeled oligonucleotide was incubated with 10 μ g of protein from various nuclear extracts. Arrows indicate the specific complexes. The first of the three lanes shown for each extract contains no competitor, the second contains specific competitor oligonucleotide (oligonucleotide SF1 for panel A and oligonucleotide SF2 for panel B) (15 ng), and the third contains nonspecific competitor (oligonucleotide SF2 for panel A and oligonucleotide SF1 for panel B) (15 ng). Lanes 17 and 18 contain nonspecific and specific oligonucleotides, respectively, in panel A. The arrowhead in panel A indicates the mobility of SF1 complex in Rat-1 extract. The cell lines used to derive the extracts are indicated above the lanes. EL4 is a mouse T-cell line, Jurkat is a human T cell line, Rat-1 is a rat fibroblast cell line, TSA9 is a CFU-E stage erythroleukemia cell line, WEHI is a B-cell line, and NIH 3T3 is a mouse fibroblast cell line.

in the T-cell extracts. Therefore, these data suggest that SF2 is a T-cell-predominant factor.

Activity of SF1 and SF2 sites in transient expression assays. To determine whether SF1 and SF2 contribute to the basal enhancer activity and/or inducibility that was observed in the -162 to -114 region in our previous studies (42), the oligonucleotides corresponding to the individual binding sites (designated the SF1 and SF2 sites) were subcloned into a pUC-CAT plasmid containing the promoter region from

FIG. 4. Relative CAT activities of pMo3(SF1)CAT and pMo2(SF2)CAT in uninduced Jurkat T cells and in Jurkat T cells induced with 10^{-7} M PMA. CAT activity is shown relative to that of uninduced pMoCAT in Jurkat T cells, which was arbitrarily assigned a value of 1.0. Each value represents the average of three independent experiments, and in each experiment, plasmids were transfected into three independent cultures.

Moloney murine leukemia virus (pMoCAT). The resulting plasmids were designated pMo3(SF1)CAT, which contains three tandem copies of the SF1 binding site in the antisense orientation, and pMo2(SF2)CAT, which contains two tandem copies of the SF2 binding site in the sense orientation. These constructs were transfected into Jurkat T cells. The CAT activity seen with the Moloney murine leukemia virus promoter alone (pMoCAT) was considered the background activity. pMo3(SF1)CAT showed an approximately eightfold average increase in conversion over background in both induced and uninduced T cells, consistent with a role for the SF1 factor in basal enhancer activity. The level of pMo2(SF2)CAT activity was only 1.6-fold over background in uninduced cells. However, in PMA-induced T cells, it showed approximately a threefold increase in conversion over background (twofold increase over its basal activity), suggesting a role for this element in the additional PMA inducibility seen in this region (Fig. 4). This level of inducibility is the same as that seen in our previous studies using the entire -162 to -114 region subcloned upstream of pMoCAT (42).

A constitutive factor which binds to the NF- κ B site. Our previous study indicated that the NF- κ B region played a role in transcriptional activity of the SIV_{mac}142 LTR in uninduced T cells and fibroblasts. Using an electrophoretic mobility shift assay, we searched for factors in uninduced T cells which could bind to this region. A synthetic oligonucleotide [designated NF- κ B/SF3(142)] corresponding to the SIV_{mac}142 NF- κ B binding site was used as a probe. The 5'-end-labeled oligonucleotide probes were incubated with nuclear extracts from both uninduced Jurkat T cells or Jurkat T cells that had been induced with PMA for 5, 10, or 20 min. With the PMA-induced extracts, a shift shown to be NF- κ B by methylation interference is observed (Fig. 5 and 6A). In the uninduced extract, a different complex with a slightly higher mobility is formed (Fig. 5). This complex can be inhibited by an oligonucleotide containing mutations in the guanine residues necessary for binding by NF- κ B (5'-CTCACTTTCC-3') but cannot be inhibited by an oligonucleotide containing the mutations used in our original studies (5'-GGGACTTTAA-3') (data not shown). A synthetic oligonucleotide corresponding to the SIV_{mac}239 NF- κ B site [designated NF- κ B/SF3(239) and differing from NF- κ B/SF3(142) by a single base pair upstream of the NF- κ B site] was also

FIG. 5. Comparison of the nuclear factors binding the NF- κ B/SF3(142) oligonucleotide in uninduced Jurkat T-cell nuclear extract (lanes 1 to 3) and in Jurkat T-cell nuclear extract from cells induced with 10^{-7} M PMA in a Tris-glycine buffer system (lanes 4 to 8). The reaction mixtures contained 10,000 cpm of end-labeled oligonucleotide probe, 10 μ g of protein, and, in some cases, 15 ng of unlabeled double-stranded oligonucleotide competitor DNA. Arrows indicate the SF3 (lanes 1 to 3) and NF- κ B (lanes 4 to 8) complexes. Lanes 4 to 8 show complexes formed by using nuclear extract from Jurkat T cells that had been induced with PMA for 5 min (lane 4), 10 min (lane 5), or 20 min (lanes 6 to 8). Competitors: lanes 1, 4, 5, and 6, no competitor; lanes 2 and 7, specific competitor oligonucleotide [NF- κ B/SF3(142)] (15 ng); lanes 3 and 8, nonspecific competitor oligonucleotide (Sp1 site from adenovirus E1B promoter; nucleotide -58 to -30) (15 ng).

used in these assays as a probe with the same results (data not shown).

As expected, when a methylation interference assay was performed with an uninduced T-cell extract, no interference pattern consistent with that of NF- κ B was seen. However, a methylation interference pattern different from that of NF- κ B was observed. The binding site for this constitutive factor was delineated as 5'-CTTTCCAC-3'. The methylation interference pattern is seen on the antisense strand (5'-GTGGAAAG-3') overlapping the NF- κ B site with only partial protection of the most 5' and 3' guanine residues (Fig. 6B). We have named this nuclear factor SF3.

Enhancer activity attributable to the SF3 site in transient expression assays. The NF- κ B site mutation used in our previous studies (5'-GGGACTTTAA-3') should have disrupted not only binding of NF- κ B but also, as suggested by the methylation interference assay described above, binding of the constitutive factor SF3. Because this mutation affected activity of the LTR in uninduced T cells and fibroblasts (Rat-1), we hypothesized that the drop in activity seen with this binding site mutation was attributable to the inability of SF3 to bind to its site. To test this hypothesis, we constructed a mutant LTR which could no longer bind NF- κ B but could bind SF3. This was done by mutating the first three guanine residues of the NF- κ B site to TCT (5'-TCTACTTTCC-3') by PCR mutagenesis. This mutation has been shown to abrogate binding of NF- κ B (12, 19, 39,

FIG. 6. Methylation interference analysis of NF- κ B and SF3 binding to the NF- κ B/SF3(142) oligonucleotide in Jurkat T-cell nuclear extract from cells induced with 10^{-7} M PMA (A) and uninduced Jurkat T-cell nuclear extract (B). The preparative reaction mixtures contained 5×10^5 cpm of end-labeled oligonucleotide probe, 50 μ g of nuclear extract, 10 ng of single-stranded oligonucleotide DNA, and 670 ng of poly(dI-dC). F and C, arrows, and asterisks are defined in the legend to Fig. 2.

40), but it would not be expected to affect binding of SF3, since these guanine residues are not contact points for this factor. The mutated LTR was then subcloned into pUC-CAT, forming pSIV(NF- κ B-, SF3+)CAT, and transient

FIG. 7. Representative CAT assay performed with Rat-1 cells to compare activity of pSIV(NF- κ B-, SF3-)CAT and pSIV(NF- κ B-, SF3+)CAT. Lanes: 1, pSIVCAT; 2, pSIV(NF- κ B-, SF3-)CAT; 3, pSIV(NF- κ B-, SF3+)CAT. Numbers above the lanes are averages from one experiment. Five independent experiments were performed, with consistent results. In each experiment, plasmids were transfected into three independent cultures. In a typical experiment, absolute conversion ranged between 12 and 30% for pSIVCAT.

A.

B.

FIG. 8. (A) Summary of the cellular nuclear factors thus far identified which may be involved in transcriptional regulation of SIV_{mac}142. Binding of SF3 to its upstream site is represented with an oval with a broken border, since the criterion for the presence of this site is competition in electrophoretic mobility shift assays and, in this study, function and methylation interference data were not included, as they were for the other factors shown here. (B) Summary of sites through which nuclear factors act under conditions of induction versus basal-level expression in both T cells and fibroblasts. Data are from binding studies, activity studies, or both. In the cases in which methylation interference assays have been performed, asterisks indicate the guanidine residues required for binding. Only factors which are hypothesized to play a role in transcriptional regulation of SIV under the stated conditions are shown. For example, although SF2 is present and can bind to its site in uninduced T cells, its binding is not shown in uninduced conditions since SF2 is active only under conditions of induction.

transfection experiments were performed with Rat-1 fibroblasts. The activity of pSIV(NF-κB-, SF3+)CAT was compared with that of pSIV(NF-κB-, SF3-)CAT, which contains the original NF-κB site mutation (5'-GGGACTT TAA-3') and with that of pSIVCAT (38), which contains the wild-type LTR sequence. In our previous studies, mutation of the NF-κB and SF3 sites [pSIV(NF-κB-, SF3-)CAT] reduced transcription by 10-fold in Rat-1 cells (37). In the present studies, transcription from pSIV(NF-κB-, SF3-)CAT was only two- to sevenfold lower than transcription from pSIVCAT; the reason for this variability is unknown. However, as predicted, pSIV(NF-κB-, SF3+)CAT was considerably more active than pSIV(NF-κB-, SF3-)CAT, with an average of 80% of pSIVCAT activity (Fig. 7), indicating a possible role for SF3 in basal transcription from the SIV LTR.

DISCUSSION

We have defined two *cis* elements in the SIV_{mac}142 LTR which may play important roles in the basal transcriptional activity of the virus (the SF1 and SF3 sites), as well as one which contributes to PMA inducibility (the SF2 site). Two of these elements lie exclusively within the -162 to -114 region of U3, which has been shown to be important to the transcriptional activity of the LTR in fibroblasts. SF1 is a basal factor which has a ubiquitous tissue distribution. SF2 could be the factor responsible for the PMA inducibility mediated through this region and appears to be expressed predominantly in T cells. Although SF2's activity is inducible, its binding is constitutive since no difference in binding patterns over this site with PMA-induced versus uninduced T-cell nuclear extracts in electrophoretic mobility shift as-

says is seen. An element similar in sequence and location to the SF2 site has been identified in the HIV-2 LTR (37). As seen with the SF2 site, this element binds an inducible factor (NF-CD3R) which is not dependent upon induction for its binding activity. In addition, the SF2 site shows sequence homology to the leukemia virus factor t (LVt) binding site, which is located in a highly conserved region in the enhancer of mammalian type C retroviruses (17). The LVt binding site cross-competes for binding with the SF2 site in electrophoretic mobility shift assays using the Tris-glycine buffer system (data not shown). There is evidence that LVt is also expressed primarily in T cells (35). Therefore, it appears that SF2 may be used by other viral systems as well.

In addition to SF1 and SF2, we identified a factor, SF3, in uninduced T cells which binds an element found on the antisense strand overlapping the NF- κ B site. By sequence homology, this element is a consensus core site (5'-TGTGG A/T A/T A/T G-3'). However, a number of core sequences from type C retroviral enhancers failed to compete for binding of SF3 in electrophoretic mobility shift assays. The simian virus 40 enhancer core C site (48) which binds activating protein 3 (AP3) is 100% homologous to the SF3 binding site and does compete for binding of the SF3 complex. We think it is unlikely that SF3 is AP3, however, since an oligonucleotide corresponding to the -148 to -114 region of SIV_{mac}142, which includes the SF1 and SF2 sites but does not contain a consensus AP3 site, competes for binding of SF3 in electrophoretic mobility shift assays (data not shown). These experiments were performed with partially purified protein from calf thymus. This protein was identified as SF3 on the basis of its competition profile in electrophoretic mobility shift assays and methylation interference assays. By sequence analysis of the -148 to -114 region, a potential SF3 binding site was found overlapping the SF2 binding site (5'-AGGAAGGAAA-3').

The SF3 binding site also shows homology to the purine-rich binding site for the *ets* proto-oncogene (26). When an oligonucleotide containing the *ets-1* binding site from Moloney murine sarcoma virus (21) was used as a competitor in electrophoretic mobility shift assays, partial inhibition of the SF3 complex was observed (data not shown). Therefore, SF3 may be a member of the *ets* family of transcription factors, some of which have ubiquitous tissue distributions (1).

Data from this analysis help to form a clearer picture of the factors that may be involved in transcriptional regulation of the SIV LTR (Fig. 8A). A more refined picture can be drawn when the factors which bind in different cell types and under conditions of induction as opposed to basal-level expression are differentiated. Figure 8B shows the factors we have identified so far which act through the -140 to -86 region of the SIV_{mac}142 LTR. The factors we have shown to demonstrate activity in PMA-induced T cells are compared with those factors which may act under basal-level conditions or in fibroblasts. Under conditions of basal-level expression, where no activated NF- κ B is present, SF3 may be able to act through the NF- κ B site. We hypothesize that SF3 also binds in the -162 to -114 region, on the basis of results of electrophoretic mobility shift assays using partially purified protein from calf thymus, and contributes to the importance of this region to transcriptional activity as well. These results can be extrapolated to explain data obtained previously in our deletion analysis (42). In CAT assays, although deletion of the -162 to -114 region has a detrimental effect on basal expression in fibroblasts, it has very little effect on expression in PMA-induced T cells. The activity of the -162 to

-114 region in induced T cells is detected only when the NF- κ B site is mutated (41). Our interpretation is that this may be due to the transcriptional roles of SF1 and SF2, which can be observed under these experimental conditions only when NF- κ B activity is no longer a factor. It is difficult to deduce from these results whether the same effect would be observed *in vivo*. Elucidation of the relationship between the interaction of these factors and viral growth awaits further studies. Mutation of these elements in a pathogenic molecular clone (SIV_{mac}239) will be an important *in vivo* test of the role of basal factors versus inducible factors in the disease progression of immunodeficiency syndromes.

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