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# Comparison of the Transcriptional Activity of the Long Terminal Repeats of Simian Immunodeficiency Viruses $SIV_{mac}251$ and $SIV_{mac}239$ in T-Cell Lines and Macrophage Cell Lines

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**The U3 regions of the long terminal repeats (LTRs) of simian immunodeficiency viruses $SIV_{mac}251$ and $SIV_{mac}239$ were analyzed for basal transcriptional activity and for interaction with cellular factors in the T-cell line HUT-78 and the monocyte/macrophage cell line U937. A number of 5′ deletions and mutations were made in the U3 regions of the two LTRs, and these constructs were placed upstream of a plasmid containing the bacterial chloramphenicol acetyltransferase reporter gene. The nucleotide sequences between −225 and +18 were sufficient to maintain full transcriptional activity of both LTRs in HUT-78 and U937 cells. Nucleotide sequence analysis revealed several differences between $SIV_{mac}251$ and $SIV_{mac}239$ within this region. Analysis of deletion mutants revealed that an additional removal of bases, from −124 to −225, had little effect on the transcriptional activity of the clone 239 LTR, whereas this deletion resulted in a significant reduction of activity in the clone 251 LTR. DNase protection assays using nuclear extracts from HUT-78 and U937 cells showed that bases within this region bound cellular factors. In addition, the NF-κB site was protected in DNase assays with HUT-78 cells and 12-*O*-tetradecanoylphorbol-13-acetate-treated U937 cells. An additional DNase footprint was detected in $SIV_{mac}239$, at −52 to −38, just upstream of the TATA box. This site overlaps the 3′ half of the 3′-most Sp-1 site and is downstream of 11 bases that are found in $SIV_{mac}239$ but not $SIV_{mac}251$. Thus, differences in the sequences in the U3 region of the LTRs of $SIV_{mac}251$ and $SIV_{mac}239$ have been identified which appear to alter the transcriptional activity of these promoters as well as changing the interaction of cellular proteins with sequences in the LTRs.**

The simian immunodeficiency viruses (SIVs) are primate lentiviruses that are serologically and genetically related to the human immunodeficiency viruses HIV-1 and HIV-2 (3, 8, 12, 19, 25). SIVs have been isolated from naturally infected Old World primates, including African green monkeys ($SIV_{agm}$) (8), sooty mangabeys ($SIV_{sm}$) (13), and mandrills ($SIV_{mnd}$) (48). There has been no disease associated with these strains of SIV in their natural hosts. However, $SIV_{mac}$ has been isolated from captive rhesus macaques (Old World Asian primates), and this virus causes a disease very similar to AIDS in humans (6, 7, 31, 32). $SIV_{mac}$, like HIV-1 and HIV-2, infects T4 lymphocytes and monocytes/macrophages in vivo and causes cytopathic effects in infected T-cell lines in vitro (6, 26, 41). These similarities in disease and cell tropism make $SIV_{mac}$ one of the best animal models with which to study the pathogenesis of AIDS.

A number of closely related strains of $SIV_{mac}$ have been isolated and have different pathogenic characteristics in monkeys. $SIV_{mac}251$ was isolated from a rhesus monkey with malignant lymphoma (6). This virus was passaged in a monkey that died 85 days after infection, and $SIV_{mac}239$ was isolated from this animal. Infectious molecular clones have been obtained for both $SIV_{mac}251$ and $SIV_{mac}239$ (38). The complete nucleotide sequence of the molecular clone $SIV_{mac}251$ (251) has been determined (3, 11), and the infectious molecular clone $SIV_{mac}239$ (239) has been shown to have sequence differences by restriction enzyme analysis (38). Infection of rhesus monkeys with virus from the 239 infectious molecular clone resulted in AIDS-like disease and death of infected animals in less than a year (27).

Nucleotide sequences in the U3 region of retroviral long terminal repeats (LTRs) are responsible for transcriptional regulation of the virus and thus play a role in the pathogenic potential of the virus (4, 29, 47). In addition, sequences in the visna virus LTR have been shown to be responsible, in part, for cell and tissue tropism of the virus in vivo in transgenic mice (46). Thus, sequences in the LTRs of the immunodeficiency viruses are likely to be important for the cell-specific regulation of the viral life cycle. The HIV LTR contains a negative regulatory element, two copies of an NF-κB binding sequence, three nonconsensus Sp-1 binding sites, a TATA box, and the transactivating region, all of which are believed to be important for transcriptional regulation in a variety of cell types (24, 36, 37, 42, 51). In T-cell lines, transcriptional activation of HIV is mediated by the NF-κB sites (34, 37). In addition, the two NF-κB sequences have been shown by DNase protection assays to interact with cellular proteins in phorbol ester-treated HeLa cells and activated T-cell lines, implicating both the protein kinase C and protein kinase A pathways in transcriptional activation (52). The role of the Sp-1 sites in HIV-1 in T-cell lines is less clear (24, 52). However, deletion of NF-κB sites produced a virus that could infect the T-cell lines MT4 and A3.01, suggesting a role for the Sp-1 sites in transcription (30). These studies show that the LTR of HIV contains multiple sets of sequences that bind cellular factors and control transcription.

In this study, transcriptional regulation of the LTRs of the

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$SIV_{mac}$ strains 251 and 239 by cellular factors has been investigated. The nucleotide sequence of the LTR of 239 was determined and compared with that of 251. The LTRs of both 239 and 251 contain only one NF-κB binding site, (A)GGGACTTTCC. This site is identical to the 5'-most NF-κB site in the HIV-1 LTR (37), which has been shown to interact with cellular factors from lymphoid cells and phorbol ester-treated HeLa cells but not from monocytes or unstimulated HeLa cells (52). In contrast, the Sp-1 sites are not as well conserved between HIV and SIV; these sites also differ between the two SIV strains. The 251 LTR contains two Sp-1 sites (18), and 239 has a duplication of sequences such that an 11-bp insertion within the 5' Sp-1 site creates a third Sp-1 site (discussed below).

To investigate whether the biological differences between $SIV_{mac}251$ and $SIV_{mac}239$ were in part determined by the different nucleotide sequences in the LTR or the interaction of those sequences with cellular factors, the LTRs of the two strains were analyzed for basal transcriptional activity and interaction with cellular factors. The cell lines used in these studies were the human monocytic cell line U937, which differentiates into macrophagelike cells when stimulated with phorbol esters (43), and the human T-cell line HUT-78. Basal levels of activity of each LTR construct linked to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene were found to be dependent on the cell type and activation state of the cell. Deletion analysis showed that sequences important for basal activity in these cells lie between −225 and +18. Transient transfection assays with deletion mutants showed that the region from −124 to −225 was essential for the activity of the 251 LTR but less important for the LTR of the 239 strain. DNase protection assays identified a region from −155 to −137 that interacted with cellular proteins, and site-directed mutagenesis showed the importance of these sequences for transcription. The nucleotide sequences from −52 to −38 in the 239 LTR were protected in the DNase assay. This region includes part of the 3'-most Sp-1 site. Interaction of cellular factors with the nucleotide sequences at −155 to −137 and the NF-κB sites were induced by treating U937 cells with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), while in HUT-78 cells, cellular factors that interacted with the LTRs of both strains were present prior to TPA stimulation of the cells. Thus, the LTRs of 251 and 239 differ in nucleotide sequence, the sequences required for basal transcriptional activity, and also those sequences which interact with cellular factors.

## MATERIALS AND METHODS

**Cell lines.** The U937 cell line was obtained from Tom Folks. HUT-78 and U937 cells were maintained according to standard culture technique, using RPMI medium with 10% fetal bovine serum (GIBCO) and 2 mM glutamine (GIBCO).

**Construction of LTR subclones.** Polymerase chain reaction (PCR) (44) was used to amplify specific sequences of the LTRs of $SIV_{mac}239$ and $SIV_{mac}251$. The DNA templates for the reaction were the infectious proviral lambda clones of 251 and 239, obtained from the laboratory of Ronald Desrosiers (38). PCR primers complementary to the 5' end of the LTR were synthesized with a *Xho*I restriction enzyme site at the 5' terminus. The 3' oligonucleotide primers were synthesized with either a *Bam*HI (+18) or *Sal*I (+367) restriction enzyme site at the 3' terminus (Fig. 1). Combinations of these primers were used to amplify sequences of both 239 and 251 from −469 to +367, −225 to +18, and −123 to +18. The −469 to +367 DNAs were digested with *Xho*I and *Nar*I, cutting the amplified 239 and 251 sequences at the 5' end and at position +310. Plasmid pUC-CAT (20) containing the CAT reporter gene was digested with *Xho*I and *Acc*I (*Nar*I and *Acc*I leave cohesive ends). The amplified LTR sequences and plasmid pUC-CAT were individually purified by agarose gel electrophoresis and ligated together with T4 DNA ligase, and the DNA was transformed into *Escherichia coli* DH5. The two clones containing the nearly full U3 region were designated 239:fl-CAT and 251:fl-CAT. To make the deletion clones shown in Fig. 1, the amplified DNA from −225 to +18 for d1 and the amplified DNA from −123 to +18 for d2 were digested with *Xho*I and *Bam*HI as was the pUC-CAT vector, and clones were obtained as described above. All clones were sequenced by the dideoxy method of Sanger et al. (45).

**Transfections and CAT assays.** HUT-78 and U937 cells were transfected by the DEAE-dextran and dimethyl sulfoxide shock method (33). Replicates of $10^7$ cells were transfected with 10 μg of the respective LTR-CAT constructs and either incubated for 46 to 48 h in RPMI medium containing 10% fetal bovine serum (unstimulated) or incubated under the same conditions with the addition of TPA (Sigma Chemical Co.) to a final concentration of $1.6 \times 10^{-7}$ M at the 23- to 24-h time point. The cells were lysed, and cell extracts were assayed for CAT activity as previously described (21). Extracts were incubated for 5 h in the presence of [$^{14}$C]chloramphenicol (DuPont, NEN Research Products) and acetyl coenzyme A. The acetylated form of chloramphenicol was separated from the unacetylated form by thin-layer chromatography, and percent conversion was determined by scintillation counting of the spots. The CAT assay was linear for the incubation time and protein concentrations used for both HUT-78 and U937 cell extracts.

**RNase protection assay.** Replicates of $10^7$ HUT-78 cells were transfected with plasmids 251:d1-CAT and 251:d2-CAT. Half of the cells transfected with the d1 construct were stimulated with TPA at the 24-h time point, and total cellular RNA was isolated as described by Chirgwin et al. (5), with modifications (21). The RNase protection assays were done as previously described (49). A *Hin*dIII-to-*Eco*RI DNA fragment of the initial 252 bp of the pUC-CAT CAT gene was subcloned into pGEM-3Z (Promega). That construct was linearized with *Hin*dIII, and a labeled antisense RNA was transcribed with T7 polymerase (Promega) and [α-$^{32}$P]UTP (DuPont, NEN Research Products). Labeled RNA (50,000 cpm) was hybridized overnight at 50°C with 40 μg of total cellular RNA from each of the transfections, the RNA was treated for 45 min at 37°C with RNase A, and the reactions were run out on a 6% acrylamide-urea gel. Gels were exposed to X-ray film, and laser densitometry was performed to determine approximate band intensities.

**DNase assays.** Nuclear extracts (9) were prepared from HUT-78 cells 48 h after refeeding, with or without $1.6 \times 10^{-7}$ M TPA stimulation at 24 h, as described above. U937 nuclear extracts were prepared 48 h after refeeding without TPA stimulation or with $1.6 \times 10^{-7}$ M TPA stimulation at 4, 24, or 48 h prior to harvesting. Assays were performed under previously reported conditions (14). The 251:d1-CAT, 251:d2-CAT, and 239:d1-CAT constructs were used to make $^{32}$P-end-labeled DNA fragments. Each construct was digested with either *Xho*I (for labeling the plus strand) or *Bam*HI (for labeling the minus strand); the 5' ends were dephosphorylated by using calf intestinal phosphatase (Pharmacia) and end labeled by incubating the linearized plasmids with T4 kinase (Bethesda Research Laboratories) at 37°C for

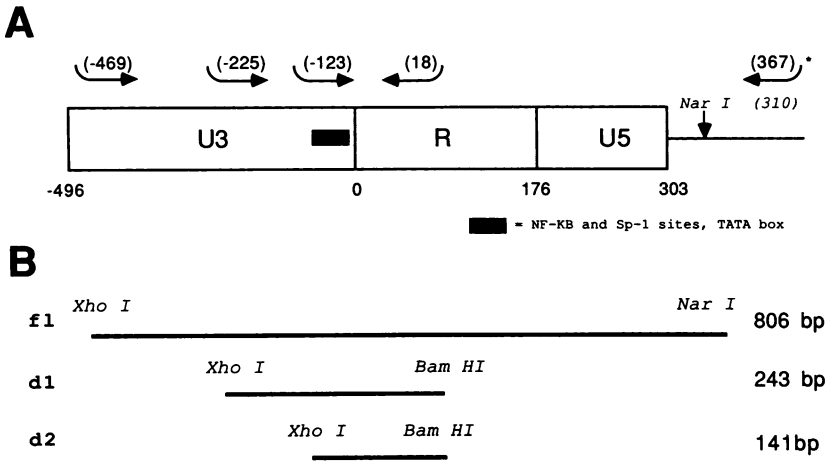


FIG. 1. SIV LTR constructs made by PCR amplification. (A) Oligonucleotides used for the PCR amplification of the SIV LTR sequences and their positions within the $SIV_{mac}251$ LTR. Arrows on the right indicate oligonucleotides synthesized complementary to the antisense strand; arrows on the left indicate oligonucleotides complementary to the sense strand. (B) Sequences amplified from both $SIV_{mac}251$ and $SIV_{mac}239$ as shown and subcloned upstream of the bacterial CAT reporter gene of a pUC-CAT vector. The *Xho*I and *Bam*HI sites were added on the oligonucleotides to facilitate directional cloning.

40 min in the presence of [$\gamma$-$^{32}$P]ATP. The end-labeled LTR fragment was separated from the plasmid by digestion with *Bam*HI (plus strand) or *Xho*I (minus strand) and electrophoresis on a nondenaturing 8% polyacrylamide gel. Nuclear extracts were preincubated with the labeled fragments and treated with DNase I (Bethesda Research Laboratories) at 0.3 to 0.4 $\mu$g/ml for 60 s. Control reactions were incubated with 50 $\mu$g of bovine serum albumin (BSA) before DNase was added. Reactions were electrophoresed on an 8% acrylamide-urea gel.

**Mutation of d1 sequences.** Site-specific mutations were made according to principles described by Lathe et al. (28) for the addition of DNA linkers to plasmids. Both plasmids 251:d1-CAT and 239:d1-CAT were digested with *Stu*I, creating blunt ends at −137 and −179. The 42-bp fragment was purified away from the plasmid by agarose gel electrophoresis. The sequences were replaced with complementary 42-base oligonucleotides, one strand of which is shown in Fig. 7. A 25-$\mu$g sample of each of the complementary oligonucleotides was resuspended in 100 $\mu$l of 2× SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0), boiled for 3 min, and allowed to slow cool for 1 h at room temperature to anneal. The DNA was ethanol precipitated, washed, dried, and run out on a 9% nondenaturing acrylamide gel. DNA was eluted by crushing the gel and incubating it overnight at 37°C in 0.5 M ammonium acetate–1 mM EDTA–10 mM magnesium acetate–0.1% sodium dodecyl sulfate. After incubation, the solution was spun at 2,000 rpm for 5 min through a Centrex column (Schleicher & Schuell, Inc.), and the DNA was ethanol precipitated. The unphosphorylated 42-bp fragment was then ligated in 100-fold molar excess to the 5′ phosphorylated blunt ends of the *Stu*I-digested d1-CAT plasmid (either 251 or 239 construct) and gel purified. The plasmid was heated to 65°C to remove the noncovalently linked strand of the oligonucleotides and slowly cooled to 4°C to reanneal complementary sequences that had been ligated to the opposite 5′ end. The DNA was transformed into *E. coli* DH5 and screened, using the *Hpa*I site (see Fig. 7) for insertion of the 42 bp, and sequenced by the dideoxy method (45) to determine the orientation.

## RESULTS

These studies were done to analyze the nucleotide sequences in the LTRs of the 239 and 251 strains of $SIV_{mac}$ that were important for the basal transcriptional activity in T cells and monocytes/macrophages. Cellular factors that affect basal transcription of the virus promoter are important for the early phase of transcription prior to the synthesis of the viral transactivating factor, *tat*. Thus, differences in the nucleotide sequence of viral LTRs could account for differences in replication of the virus in specific cell types in vivo.

**PCR subcloning and nucleotide sequence analysis of products.** Subcloning of the full (fl) and deleted (d1 and d2) LTRs of 251 and 239 was accomplished by using PCR. The amplified fragments shown in Fig. 1 were placed upstream of the bacterial CAT reporter gene. The 251:d2 and 239:d2 clones contain the enhancer elements important for the transcription from the HIV LTR, including the potential NF-κB site, Sp-1 sites, and the TATA box. However, the 251:d2-CAT construct had unexpectedly low basal activity compared with the full-length LTR constructs. Thus, additional upstream sequences were included in the d1 constructs to identify other sequences involved in transcriptional activation. In addition, none of these deletion constructs (d1 and d2) contain the portion of the R region analogous to the transactivating region of HIV which is required for the transcriptional enhancement mediated by the *tat* protein (18). All clones were sequenced to ensure that no nucleotide changes were introduced by the *Taq* polymerase. Sequences of the 251 clone were compared with the published sequence (3, 11). Within the d1 and d2 regions, all clones contained the published sequence, while outside this region, several point mutations were observed. Since the sequence of 239 was unavailable at the time of the experiments, the DNA from separate PCR reactions were cloned, and individual clones were sequenced. Identical sequences were found in four of five clones from different reactions, and all four constructs had four point differences and an insertion of 11 bp after base −61 compared with construct 251 (Fig. 2). Subsequent comparison of the sequences of

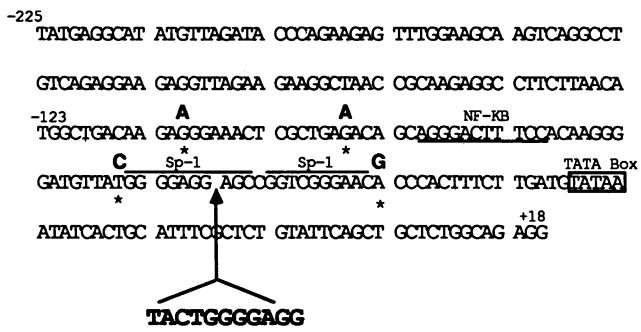


FIG. 2. Comparison of the 251 and 239 d1 constructs. The sequence of $SIV_{mac}251$ from −225 to +18 (d1) is shown. The NF-κB sequence is underlined, the putative Sp-1 sites are overlined, and the TATA box is boxed. Differences found in $SIV_{mac}239$ have an asterisk beneath them, and the corresponding base in 239 is in bold print above the base in 251. The 11-bp insertion in 239 is in bold, with an arrow indicating its placement relative to the 251 LTR.

these clones with the complete nucleotide sequence of 239 showed that these clones were identical to the original $SIV_{mac}239$ (27).

**Transcriptional analysis.** The wild-type and deleted LTR-CAT constructs of 239 and 251 were then analyzed for transcriptional activity in T cells and monocyte/macrophages. Transient expression assays for CAT activity were carried out with protein lysates from unstimulated and TPA-stimulated cells (Tables 1 and 2) (described in Materials and Methods). In HUT-78 cells, all of the LTR-CAT constructs were induced by TPA stimulation (Table 1). Lower levels of basal activity were observed for both the 251 and 239 full-length (fl) constructs than for the d1 constructs. When the HUT-78 cells were stimulated with TPA, the difference between the fl and d1 constructs for both 239 and 251 was less significant. The 251:fl-CAT and 251:d1-CAT constructs had slightly higher levels of activity than did 239:fl-CAT and 239:d1-CAT, respectively. Thus, it was surprising that 251:d2-CAT had considerably lower activity (three- to fourfold) than did 239:d2-CAT. In addition, 251:d2CAT was 8- to 10-fold less active (in unstimulated and stimulated HUT-78 cells) than was 251:d1-CAT. 239:d2-CAT, on the other hand, had levels of activity comparable to those of 239:fl-CAT.

TABLE 1. Activities of LTR-CAT constructs in HUT-78 cells

| CAT construct | CAT activity[a] (% conversion) | Relative value[b] | Fold induction |
|---|---|---|---|
| Wild-type sequences | | | |
| 239:fl | 1.3 | 0.62 | |
| 239:fl + TPA[c] | 4.2 | 1.2 | 3.2 |
| 239:d1 | 2.1 | 1.0 | |
| 239:d1 + TPA | 3.6 | 1.0 | 1.7 |
| 239:d2 | 2.5 | 1.2 | |
| 239:d2 + TPA | 4.0 | 1.1 | 1.6 |
| 251:fl | 1.2 | 0.20 | |
| 251:fl + TPA | 6.1 | 0.60 | 5.1 |
| 251:d1 | 6.1 | 1.0 | |
| 251:d1 + TPA | 10.4 | 1.0 | 1.7 |
| 251:d2 | 0.63 | 0.10 | |
| 251:d2 + TPA | 1.3 | 0.13 | 2.1 |
| Mutant sequences | | | |
| 239:d1Δ | 2.2 | 1.1 | |
| 239:d1Δ + TPA | 4.5 | 1.2 | 2.0 |
| 251:d1Δ | 1.3 | 0.21 | |
| 251:d1Δ + TPA | 2.6 | 0.25 | 2.0 |

[a] Values are from a representative experiment. Repeats of experiments yielded similar trends. This experiment was done by using 70 μg of each extract, and incubation was for 5 h. Values are normalized to 100 μg.

[b] Determined by dividing the activity of that sample by the activity of the d1-CAT construct of the same strain.

[c] After being transfected with the LTR-CAT constructs, cells were split into two groups. One set of replicates was stimulated at 24 h by adding TPA to a final concentration of $1.6 \times 10^{-7}$ M. All cells were then harvested at 48 h posttransfection.

TABLE 2. Activities of LTR-CAT constructs in U937 cells

| CAT construct | CAT activity[a] (% conversion) | Relative value[b] | Fold induction |
|---|---|---|---|
| Wild-type sequences | | | |
| 239:fl | 0.30 | 0.31 | |
| 239:fl + TPA[c] | 1.6 | 0.47 | 5.3 |
| 239:d1 | 0.96 | 1.0 | |
| 239:d1 + TPA | 3.4 | 1.0 | 3.5 |
| 239:d2 | 2.5 | 2.6 | |
| 239:d2 + TPA | 5.1 | 1.5 | 2.0 |
| 251:fl | 0.79 | 0.53 | |
| 251:fl + TPA | 3.0 | 0.50 | 3.8 |
| 251:d1 | 1.5 | 1.0 | |
| 251:d1 + TPA | 6.0 | 1.0 | 4.0 |
| 251:d2 | 0.44 | 0.29 | |
| 251:d2 + TPA | 2.7 | 0.45 | 6.1 |
| Mutant sequences | | | |
| 239:d1Δ | 0.97 | 1.0 | |
| 239:d1Δ + TPA | 1.6 | 0.47 | 1.6 |
| 251d1Δ | 0.71 | 0.47 | |
| 251d1Δ + TPA | 1.5 | 0.25 | 2.1 |

[a] Values are from a representative experiment. Repeats of experiments yielded similar trends. This experiment was done by using 60 μg of each extract, and incubation was for 5 h. Values are normalized to 100 μg.

[b] Determined by dividing the activity of that sample by the activity of the d1-CAT construct of the same strain.

[c] After being transfected with the LTR-CAT constructs, cells were split into two groups. One set of replicates was stimulated at 24 h by adding TPA to a final concentration of $1.6 \times 10^{-7}$ M. All cells were then harvested at 48 h posttransfection.

In U937 cells, the constructs were generally more inducible by TPA stimulation (Table 2). This may be due in part to the lower transcriptional activity of all of the constructs in the unstimulated U937 cells. TPA treatment of the U937 cells resulted in CAT activity of the LTR constructs that was comparable to that in TPA-stimulated HUT-78 cells. The difference in CAT activity between 251:d1-CAT and 251:d2-CAT was not as dramatic in the U937 cells as in the HUT-78 cells, suggesting that the sequences deleted in the d2 construct are less important for transcriptional activity in macrophages. However, there was still a significant difference (about sixfold) in CAT activity between the 239:d2-CAT and the 251:d2-CAT constructs in unstimulated U937 cells. In contrast, when the U937 cells were treated with TPA, the difference in activity between the constructs is only twofold. Thus, TPA treatment may induce factors that activate sequences in the 251:d2-CAT that compensate for the loss of the upstream sequences.

**Steady-state levels of CAT RNA in transfected cells.** To determine whether the CAT assays reflected the RNA levels present in the cells, RNase protection assays were performed to measure steady-state levels of RNA in the HUT-78 cells. RNA was isolated at 48 h posttransfection for plasmid 251:d1-CAT with and without TPA stimulation, along with plasmid 251:d2-CAT in unstimulated cells (Fig. 3). The result shown in Fig. 3 was quantified by laser densitometry. Ratios of the protected RNA for 251:d1-CAT

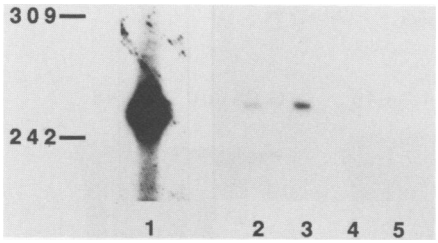


FIG. 3. RNase protection assay. Labeled antisense CAT RNA (500 cpm) (the first 252 bases) was run on a 6% acrylamide-urea gel and exposed to X-ray film (lane 1); 50,000 cpm of the labeled CAT antisense RNA was hybridized to 40 μg of total cellular RNA from HUT-78 cells transfected with 10 μg of 251:d1-CAT (lanes 2 and 3), 10 μg of 251:d2-CAT (lane 4), and no DNA (lane 5) before digestion with RNase A for 45 min at 37°C, running out on a 6% acrylamide-urea gel, and exposure to X-ray film. Intensifying screens were used to visualize the bands. The HUT-78 cells used to obtain the cellular RNA for the reaction shown in lane 3 were stimulated with TPA after 24 h. Sizes in base pairs are indicated on the left.

unstimulated, 251:d1-CAT stimulated, and 251:d2-CAT-transfected cells were 1.0 to 3.4 to 0.20, respectively. This correlates with the levels of CAT activity in both unstimulated and TPA-stimulated cells.

**DNase protection assays.** To localize the sequences in the LTR that interact with cellular proteins, DNase protection assays were performed on the 239:d1 and 251:d1 constructs. Nuclear extracts were prepared from both HUT-78 and U937 cells, and DNase protection assays were performed. Titration of the amount of extract showed that 100 μg was necessary to protect regions of the DNA strands; thus, 100 μg was used for all subsequent assays. Assays were repeated to correct for any variations that may occur; the results shown in Fig. 4 and 5 are representative. The TATA box was partially protected by nuclear extracts from both cell types on the negative strand of 251 and 239 at −28 to −24 (Fig. 4 and 5). The NF-κB site was protected in both 251 and 239 between −95 and −77 in both unstimulated and stimulated HUT-78 extracts. In addition, the 251:d1 fragment had a protected region upstream at −115 to −98. Nuclear extracts from U937 cells, on the other hand, did not contain factors

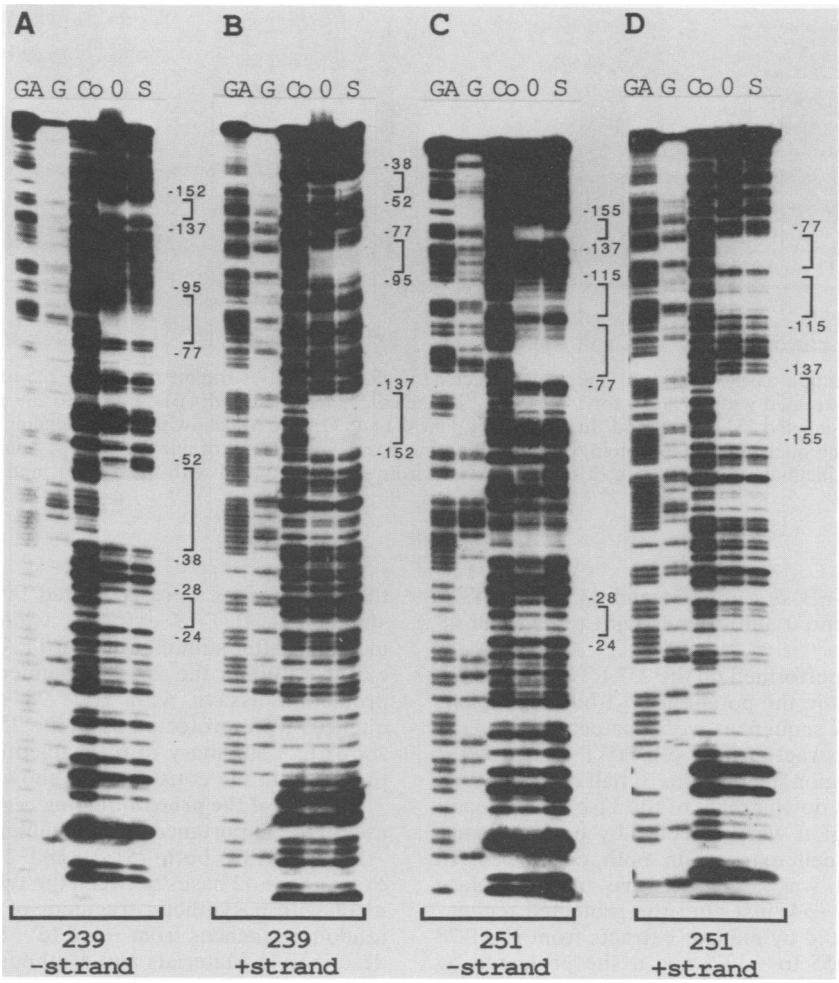


FIG. 4. DNase I protection assay using HUT-78 nuclear extracts. Labeled LTR DNA fragments from −225 to +18 were incubated with nuclear extracts and treated with DNase I for 1 min. (A) $^{32}$P-labeled 239 minus strand; (B) $^{32}$P-labeled 239 plus strand; (C) $^{32}$P-labeled 251 minus strand; (D) $^{32}$P-labeled 251 plus strand. In each panel, lanes GA and G are Maxam-Gilbert sequencing reactions, Co is a reaction incubated with BSA and no nuclear extract protein, 0 is a reaction incubated with 100 μg of nuclear extract from unstimulated cells, and S is a reaction incubated with 100 μg of nuclear extract from cells stimulated with TPA 24 h prior to preparation.

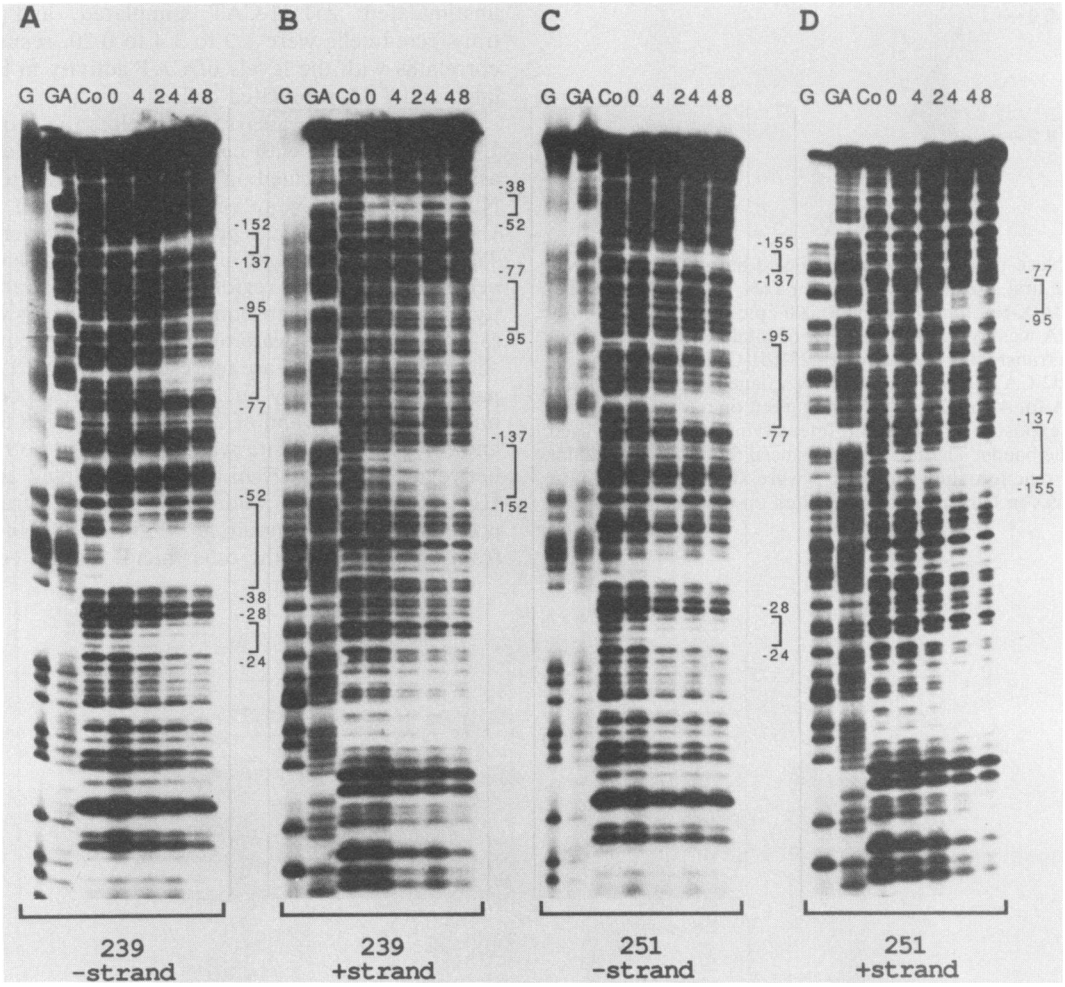


FIG. 5. DNase I protection assay using U937 nuclear extracts. Labeled LTR DNA fragments from −225 to +18 were incubated with nuclear extracts and then treated with DNase I for 1 min. (A) $^{32}$P-labeled 239 minus strand; (B) $^{32}$P-labeled 239 plus strand; (C) $^{32}$P-labeled 251 minus strand; (D) $^{32}$P-labeled 251 plus strand. In each panel, lanes G and GA are Maxam-Gilbert sequencing reactions, Co is a reaction incubated with BSA and no nuclear extract protein, 0 is a reaction incubated with 100 μg of nuclear extract from unstimulated cells, and 4, 24, and 48 are reactions incubated with 100 μg of nuclear extract from cells stimulated with TPA 4, 24, and 48 h prior to preparation, respectively.

that bound to the NF-κB site until 24 and 48 h after TPA stimulation (compare the 0 and 4 lanes with the 24 and 48 lanes of Fig. 5).

In all DNase assays performed on the 251 LTR sequences, no protection was seen in the potential Sp-1 binding region. However, the 239 LTR sequences were protected from −52 to −38 with nuclear extracts from both HUT-78 and U937 cells. This protected region includes the 3′ half of the 3′-most Sp-1 site and is 9 bases downstream of the 11-bp insertion in the 239 LTR. This region was protected by both unstimulated and stimulated cell extracts in both cell types. In addition, a band that was hypersensitive to the DNase treatment appeared at −54, just after the protected region.

Another site, protected by nuclear extracts from HUT-78 cells, is located at −155 to −137, and a site protected by nuclear extracts from U937 cells is located at −152 to −137 in both 239 and 251. Identification of an upstream site was not unexpected, since the basal activity of 251:d2-CAT, which does not contain these sequences, was significantly lower than that of 251:d1-CAT. As with the NF-κB site, these sequences were protected by both unstimulated and stimulated HUT-78 extracts, while the protected region increased after stimulation with TPA in the assays with U937 extracts. When the 251:d2 fragment was used for DNase protection assays with HUT-78 extracts, the same sequences were protected as in the 251:d1 fragment (data not shown). A summary of the nucleotide sequences that were protected in the construct are shown in Figure 6.

**Analysis of the protein-binding region at −155 to −137.** To assess the importance of the cellular proteins binding at site −155 to −137, both 251:d1 and 239:d1 were mutated by excising the 42 bases between the two *Stu*I sites and replacement with a synthetic fragment of the same size that had random sequences from −155 to −139 (shown in Fig. 7 and described in Materials and Methods). This created 251:d1Δ and 239:d1Δ. DNase assays were done on the constructs, and the sequences at −155 to −137 were no longer protected when incubated with HUT-78 (Fig. 8) or U937 (data not shown) extracts. All other sites were protected as before.

The mutants were next tested for transcriptional activity

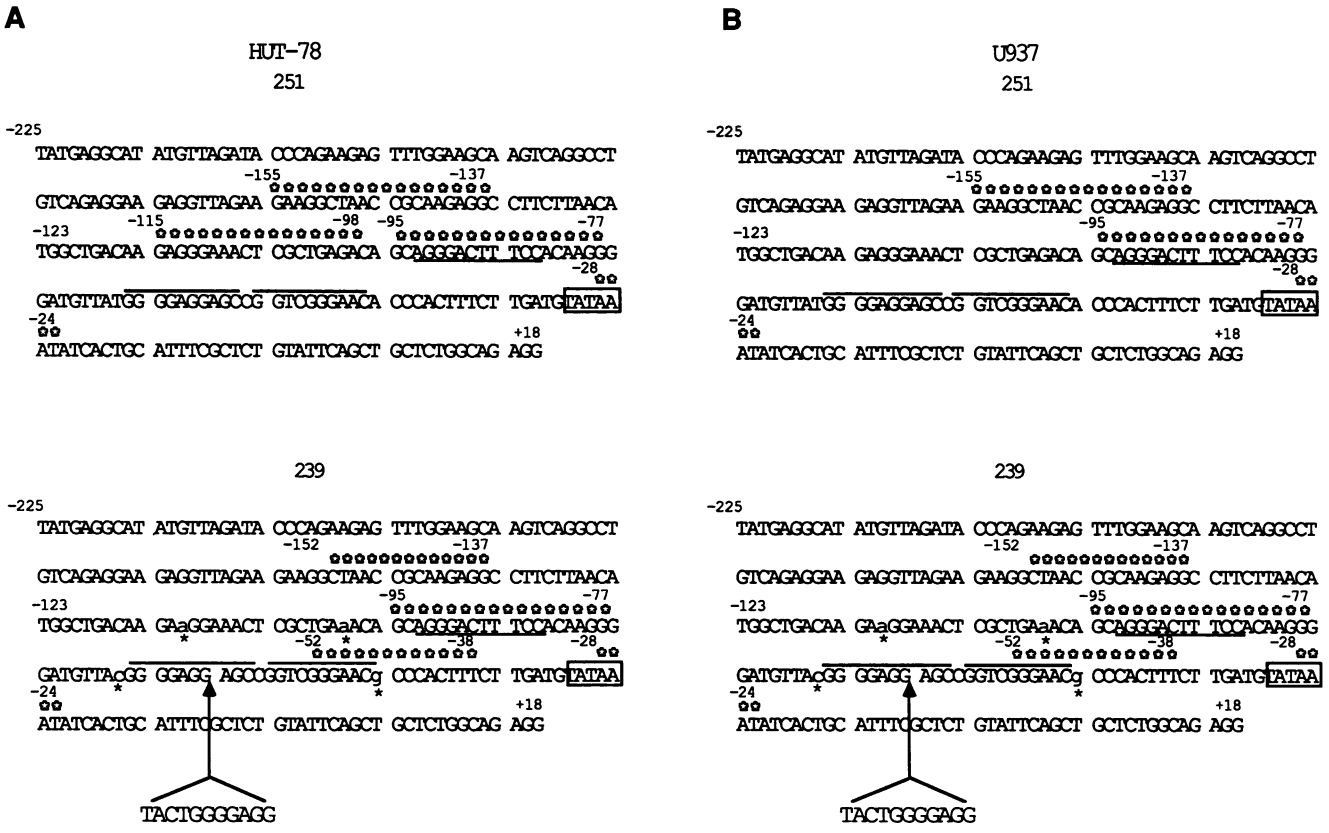


FIG. 6. Summary of DNase I protection assays. (A) Data from Fig. 4; (B) data from Fig. 5. Protected sequences are indicated by stars above the bases. Underlined bases indicate the NF-κB enhancer sequences, and overlined bases indicate the potential Sp-1 binding sites; the TATA box is boxed. Single-base differences in the 239 sequence are represented by lowercase letters with asterisks below them. The additional 11 nucleotides in 239, TACTGGGGAGG, are found where the arrow indicates.

in the CAT assay. In the HUT-78 cells, 239:d1Δ-CAT had levels of activity similar to those of 239:d1-CAT. In contrast, 251:d1Δ-CAT had fourfold less activity than did 251:d1-CAT (Table 1). In the U937 cell line, 239:d1Δ-CAT had levels of activity equivalent to those of 239:d1-CAT in unstimulated cells; however, in stimulated U937 cells, 239:d1Δ-CAT had about twofold less activity than did 239:d1-CAT (Table 2). 251:d1 had twofold less activity in unstimulated U937 cells and fourfold less activity in stimulated cells in comparison with 251:d1-CAT. These results are representative of multiple experiments.

## DISCUSSION

SIV replicates in vitro in both T4 lymphocytes and monocytes/macrophages. The regulatory sequences that control basal transcription of $SIV_{mac}251$ and $SIV_{mac}239$ in these two cell types had not been previously examined. The HIV LTR has been extensively studied, and initial experiments with the LTR of SIV suggest that it may function by using similar cellular factors. It has been shown that the SIV LTR is activated by its own *tat* gene product as well as by treatment of cells with phorbol esters (1, 2, 40, 50). In this study, it was found that the 251 and 239 full-length LTRs had basal activity considerably lower than that of the full-length HIV LTR (data not shown). This may be due to the fact that the SIV promoters contain only one NF-κB site, which is identical to the 5′ NF-κB site in HIV-1. Both NF-κB sites in the HIV LTR have been shown to be protected by cellular factors and are important for transcriptional activity (15, 37, 52). Electrophoretic mobility assays have shown that NF-κB

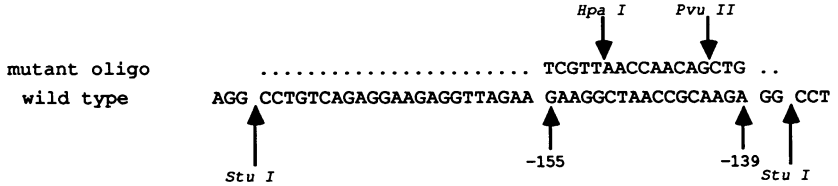


FIG. 7. Mutation of the upstream DNase-protected region. Two complementary 42-base oligonucleotides were synthesized to replace the 42 bases from −178 to −137 in both 239 and 251. The plus strand is shown. Dots indicate wild-type bases present in the mutant oligonucleotide. Both 251 and 239 wild-type sequences are identical in this region, so the same oligonucleotides were used for both. Sequences used to replace wild-type bases have the restriction sites indicated.

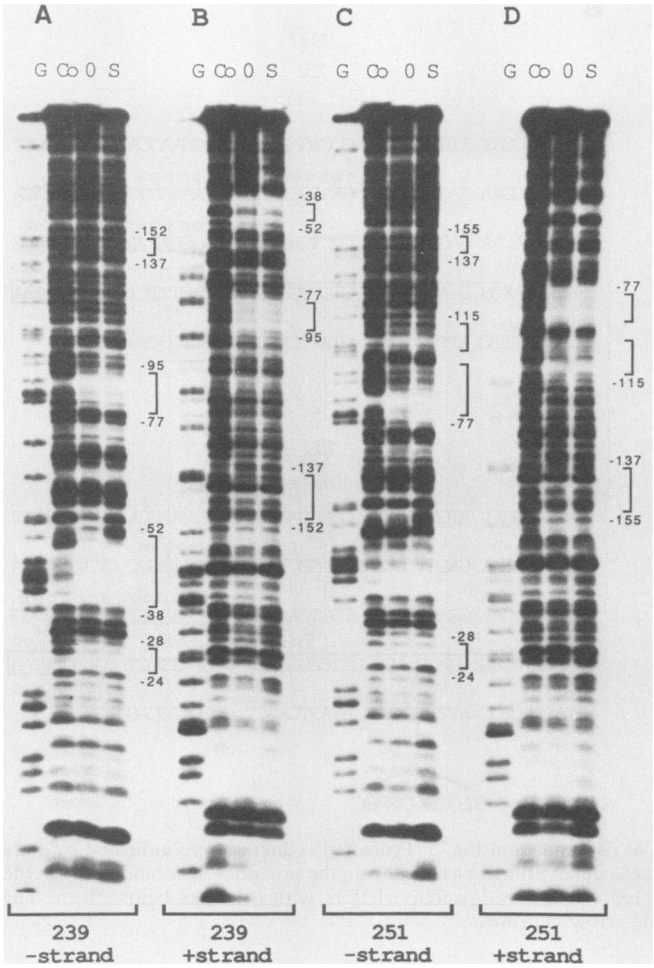


FIG. 8. DNase I protection assay using HUT-78 nuclear extracts with mutant LTR sequences (251:d2). Labeled mutant LTR DNA fragments from −225 to +18 were incubated with nuclear extracts and then treated with DNase I for 1 min. (A) $^{32}$P-labeled 239 minus strand; (B) $^{32}$P-labeled 239 plus strand; (C) $^{32}$P-labeled 251 minus strand; (D) $^{32}$P-labeled 251 plus strand. In each panel, lane G is a Maxam-Gilbert sequencing reaction, Co is a reaction incubated with BSA and no nuclear extract protein, 0 is a reaction incubated with 100 μg of nuclear extract from unstimulated cells, and S is a reaction incubated with 100 μg of nuclear extract from cells stimulated with TPA 24 h prior to preparation.

binding activity is induced in U937 cells stimulated with TPA, and enhanced expression from the HIV promoter was dependent on the presence of the NF-κB sites (17). The DNase assays in this study indicate that binding of cellular proteins to the SIV NF-κB site was also induced in U937 cells. In addition, expression of the CAT protein from the SIV promoter increased with TPA stimulation, suggesting that NF-κB binding activity may be induced in the U937 cells and play a role in activating SIV expression during differentiation from the monocyte to the macrophage (43). In the HUT-78 cells, however, the NF-κB site was protected with nuclear extracts from both unstimulated and stimulated cells. In contrast, there was an increase in transcriptional activity with TPA stimulation of HUT-78 cells. The increase in activity could be due to an increase in active NF-κB, altered binding, or interactions of NF-κB with other factors that are not revealed by the DNase protection assay.

The CAT activity of the deletion mutants, 251:d1-CAT, 239:d1-CAT, and 239:d2-CAT, was higher than that of the full-length LTR constructs, 251:fl-CAT and 239:fl-CAT, in unstimulated HUT-78 cells and U937 cells. These data indicate that the sequences necessary for basal activity are within −225 to +18. The full-length LTR constructs appear to contain a weak negative regulatory element upstream of −225. However, the effect of this element is overcome when HUT-78 and U937 cells are stimulated with TPA. Stimulation of the cells with TPA may allow the enhancer sequences to interact with cellular proteins to counteract the relatively weak negative regulation or cause the alteration of the cellular factors that mediate the negative regulation.

The upstream protein-binding region, −155 to −137, was found to be more important in the 251 LTR than in the 239 LTR, since deletion of the sequences from −225 to −124 in 251 decreased activity by 8- to 10-fold but did not significantly reduce the activity of the 239 LTR. Closer inspection of the SIV LTR revealed that this sequence contains an inverted CCAAT box at its center. The CCAAT box is an element present in many eucaryotic promoters that binds to CCAAT transcription factor, CAT-binding protein, and other cellular proteins, and it augments transcription when placed in either orientation (10, 16, 23, 35). It is, however, most frequently located close (within about 30 bp) to the start site of transcription. Mutation of this site (in the d1Δ mutants) eliminated DNase protection and resulted in lower activity. However, the activity of the delta mutants was not as low as the activity of the d2 constructs in which sequences from −225 to −124 were removed.

In the 239 LTR, the region from −155 to −137 appears to be less important than in the 251 LTR. However, this site in the 239 LTR appears to be involved in the increase in CAT activity with TPA treatment of U937 cells. Cellular factors from both HUT-78 and U937 cell extracts bind to this site in the 239 LTR. Thus, downstream sequences may compensate for the loss of this site in the 239:d2-CAT construct but not in the 251:d2-CAT construct. The region from −52 to −38, which is protected in the 239 LTR but not the 251 LTR, could be responsible for this compensation. This site is protected in both unstimulated and stimulated HUT-78 and U937 cells. In the middle of this site in 239, there is an A-to-G transition at −46 compared with the 251 sequence. This site is also 9 bp downstream of an 11-bp insertion present in the 239 LTR but not in the 251 LTR. These 11 bp are a duplication of 10 bp immediately upstream, in the potential Sp-1 binding site. This addition creates another nonconsensus Sp-1 binding site, increasing the total number of Sp-1 sites in the 239 LTR to three, the same number contained in the HIV LTR (18). It has been reported that the Sp-1 sites of HIV-1 have different affinities and that their individual importance depends on their sequence and position relative to the start site (25, 30). The inserted sequences in 239 could lead to more favorable binding conditions for cellular transcription factors at this site. Furthermore, the insertion increases the distance between the site from −52 to −38 and upstream sites, which might allow folding back of the DNA and enhanced access of cellular factors to the DNA sequences (39). In addition, a DNase-hypersensitive site was observed at −54 in the 239 footprint, which could be indicative of altered DNA structure (22).

The nucleotide sequences in the SIV LTRs that were found to be essential for basal transcription are included in the 251:d1 and 239:d2 constructs: the NF-κB binding site, the Sp-1 sites, and the TATAA box are included in these constructs. In addition, the sequences in a region containing

an inverted CCAAT box bound cellular factors in both T cells and macrophages. These sequences were important for transcriptional activity of the 251 LTR, while they were not essential for the activity of the 239 LTR. The transcriptional activity and the pattern of DNase protection were very similar for the LTRs of both 239 and 251 in T cells and monocytes/macrophages. This result was somewhat surprising, since these viruses replicate to a high level in T cells but have limited replication rates in primary macrophages in vitro.

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