JOURNAL OF VIROLOGY, Aug. 1991, p. 4350–4358
0022-538X/91/084350-09$02.00/0

Vol. 65, No. 8

Contribution of NF-κB and Sp1 Binding Motifs to the Replicative Capacity of Human Immunodeficiency Virus Type 1: Distinct Patterns of Viral Growth Are Determined by T-Cell Types

ELIZABETH K. ROSS,[1] ALICIA J. BUCKLER-WHITE,[2] ARNOLD B. RABSON,[1]† GEORGE ENGLUND,[1] AND MALCOLM A. MARTIN[1]*

Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892,[1] and Division of Molecular Virology and Immunology, Georgetown University School of Medicine, Rockville, Maryland 20854[2]

Received 13 February 1991/Accepted 9 May 1991

Starting with a replication-incompetent molecular clone of human immunodeficiency virus type 1, lacking all the NF-κB and Sp1 binding sites present in the native long terminal repeat (LTR), proviruses containing reconstructed LTRs with individual or combinations of NF-κB and Sp1 elements were generated and evaluated for their capacity to produce virus progeny following transfection-cocultivation. Virus stocks obtained from these experiments exhibited a continuum of replicative capacities in different human T-cell types depending on which element(s) was present in the LTR. For example, in experiments involving proviral clones with LTRs containing one or two NF-κB elements (and no Sp1 binding sites), a hierarchy of cellular permissivity to virus replication (peripheral blood lymphocytes = MT4 > H9 > CEM > Jurkat) was observed. Of note was the associated emergence of second-site LTR revertants which involved an alteration of the TATA box. These results suggest that the human immunodeficiency virus type 1 LTR possesses functional redundancy which ensures virus replication in different T-cell types and is capable of changing depending on the particular combination of transcriptional factors present.

The retroviral long terminal repeat (LTR), generated during the early phase of virus infection by reverse transcription, contains several *cis*-acting elements that influence viral gene expression. These include the transcriptional promoter and start site, the polyadenylation signal and addition site, and binding motifs for transcriptional activators. In some retroviral subfamilies, the LTR also contains targets for virus-encoded transactivator proteins.

During the past few years, the structure and function of the human immunodeficiency virus (HIV) LTR has been intensively investigated. Regulatory motifs (e.g., Sp1, TATA) have been identified which bind cellular proteins that are constitutively expressed in most cells (7, 11, 20); these sequences appear to be critical for basal-level HIV gene expression. Other LTR elements (e.g., NF-κB and NFAT-1) may interact with factors synthesized only in activated T lymphocytes (6, 21, 28, 37), thereby limiting vigorous virus replication to a small subset of circulating peripheral blood lymphocytes (PBLs) in vivo. Still other regions (e.g., TAR) are unique to HIV and are required for high levels of LTR-directed gene activity (4, 8, 13, 18, 36, 41). Thus, the interaction of virus-encoded (3, 40) and cellular regulatory proteins with one another and with the HIV LTR in both resting and activated human lymphocytes has the potential to effect a continuum of replicative capacities.

To date, most of our knowledge of HIV LTR structure and function comes from studies of small DNA constructs containing the LTR linked to reporter genes and tested in HeLa cells or in human T-cell lines (2, 5, 15, 28, 32, 38). Although this approach has helped to elucidate the mechanism by which the HIV LTR is activated, the interaction of virus-encoded and cellular proteins with *cis*-acting regulatory elements during a productive infection cannot be evaluated in such systems. We (23) and others (14, 24) have elected to evaluate LTR function in the context of HIV replication and have previously reported that some of the *cis*-acting elements may be dispensable, depending on the cell type infected. For example, the deletion of both NF-κB elements only modestly affects HIV replication in PBLs or in CEM and MT4 cells (23). In contrast, mutation of Sp1 motifs can drastically reduce replicative capacity in some human T-cell cultures. In particular, the deletion of all three Sp1 elements either eliminated or greatly reduced the capacity of HIV to infect CEM cells (two successes in five independent experiments); replication of this mutant in PBLs and MT4 cells was only slightly reduced (30). Finally, the simultaneous elimination of the two NF-κB and all three Sp1 elements resulted in a provirus exhibiting no detectable biological activity (23); in this report, we refer to this irreversibly replication-incompetent proviral DNA as pNL-ZERO.

To understand more fully the contribution of individual LTR components to the replicative process and to the complex interactions of cellular and viral proteins with their LTR targets, we analyzed the biological activity of pNL-ZERO into which different combinations of NF-κB and Sp1 *cis*-acting elements were added. Our results suggest a hierarchy of cellular permissivity to virus infection which very likely reflects the relative abundance of transcriptional regulatory factors and the presence of specific HIV LTR motifs.

MATERIALS AND METHODS

Construction of proviral DNAs containing LTR mutations and generation of virus stocks. pNL-ZERO is a replication-incompetent derivative of the infectious circularly permuted proviral clone pILIC (23); pNL-ZERO lacks the two NF-κB

* Corresponding author.
† Present address: Center for Advanced Biotechnology and Medicine, Piscataway, NJ 08854.

and three Sp1 sites and was previously designated pTA. pNL-ZERO DNA was digested with *Bam*HI and *Sph*I to generate a 2.1-kb fragment containing the LTR. This fragment was subcloned into M13 and subsequently used to produce the reconstructed LTRs, shown in Fig. 1, by oligonucleotide-directed mutagenesis (47) as modified by Kunkel (22). Full-length circularly permuted proviral DNAs were reconstituted by ligating the LTR-containing 1.7-kb *Xho*I-*Sph*I fragment from M13 recombinants to a similarly digested 7.4-kb cleavage product of pNL-ZERO. Following the verification of the mutations prepared in vitro by nucleotide sequence analysis (34), the replicative capacities of the different LTR mutants were evaluated by transfecting a derivative of A3.01 (9) CEM cells, designated 12D7 (generously provided by Guido Poli, Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases) with 3 μg of DNA (per 6×10^6 cells) in the presence of DEAE-dextran as previously described (23). Forty-eight hours later, the transfected cells were cocultivated with 2×10^6 MT4 (25) cells and monitored for virus production by a ^{32}P-reverse transcriptase (RT) assay (46). Virus stocks were prepared from the supernatants of transfection-cocultures when RT levels exceeded 3,500 ^{32}P cpm/μl.

Infections. PBLs, the A3.01 (9) clonal derivative of CEM cells, MT4, H9 (31), and JE6.1 (45), a clonal line of Jurkat cells (2×10^6 cells of each type), were infected with equivalent amounts of virus (300,000 cpm of ^{32}P-RT activity). Prior to infection, PBLs were stimulated with phytohemagglutinin (0.25 μg/ml) for 3 days; upon infection, the PBLs were maintained in medium containing 10% interleukin 2. Infected cells were maintained in T25 Teflon flasks (Costar) in RPMI 1640 medium (10 ml) containing 10% fetal calf serum, and progeny virion production was detected by RT activity.

PCR analysis of genomic RNA. Aliquots (100 μl) of culture supernatants from selected infections were digested with RNase-free DNase (0.05 U; Promega Biotec) for 15 min at 37°C and then incubated for 10 min at 68°C to inactivate the DNase. First-strand cDNA synthesis from viral RNA was performed as described by Morgan et al. (26). The cDNAs were then amplified in a Perkin-Elmer/Cetus DNA Thermal Cycler with the following primers: (i) a plus-sense oligonucleotide spanning positions 9073 to 9092 (HIV_{BRU} coordinates [27]) with 9 nucleotides at its 5′ end bearing an *Eco*RI site (5′-ATCGAATTCTGGAAGGGCTAATTCACTCC-3′; gift from Jonathan Silver), and (ii) a minus-sense oligonucleotide spanning positions 9504 to 9521 with 12 nucleotides at its 5′ end bearing both a *Pst*I and an *Rsa*I site (5′-AACTG CAGGTACCAGGCAAAAAGCAGCTGC-3′). The amplification protocol consisted of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 2 min. Digestion of the polymerase chain reaction (PCR) product with *Eco*RI and *Pst*I, ligation into pIBI30 (International Biotechnologies, Inc.), transformation of *Escherichia coli* DH5-αMCR (Bethesda Research Laboratories), and selection of recombinants by colony hybridization by using a 5′-end-labeled oligonucleotide probe spanning positions 143 to 182 of the HIV_{NL4-3} (1, 27) LTR were all done as previously described (33). Hybridization-positive recombinants were sequenced by the method of Sanger et al. (34).

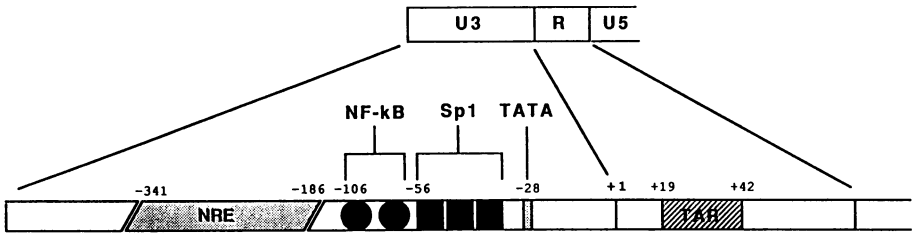
RESULTS

We previously described the construction of a circularly permuted infectious molecular clone of HIV containing a single LTR which was used to investigate the role(s) of NF-κB and Sp1 elements during virus replication in human T cells (23). Viruses in which the two NF-κB binding sites were deleted replicated efficiently in phytohemagglutinin-stimulated human PBLs, as well as in MT4 and CEM cells. HIV preparations containing one or two mutated Sp1 binding sites were also replication competent but exhibited variable growth kinetics depending on the cell type infected. Deletion of all three Sp1 sites resulted in virus which replicated efficiently in MT4 cells and in PBLs but inconsistently and with delayed kinetics in CEM T cells unless they were exposed to tumor necrosis factor alpha (30). In contrast, the deletion of 68 bp of U3 sequences encompassing the two NF-κB and the three Sp1 sites generated an HIV provirus which failed to replicate following transfection-cocultivation. This replication-defective HIV provirus, designated pNL-ZERO, was used in a series of experiments as a basal-level proviral DNA target to which NF-κB or Sp1 sites were added either individually or in combination.

Generation of mutant LTR HIV-1 stocks by transfection-cocultivation. Circularly permuted HIV proviruses containing a single wild-type or reconstructed LTR were released from plasmid vectors following digestion with *Bam*HI and ligated to regenerate "conventional" full-length proviral DNAs (i.e., containing at least two LTRs). A cloned derivative of the $CD4^+$ T-cell line A3.01, designated 12D7, was then transfected with the ligated proviral DNAs as described in Materials and Methods. Forty-eight hours following transfection, the 12D7 cells were cocultivated with MT4 cells and progeny virus production was then monitored by RT assay over a 51-day period. Supernatants were collected at the peak of RT activity and served as virus stocks for subsequent infectivity studies.

As expected, transfection-cocultivation with wild-type HIV proviral DNA resulted in rapid virus production (first detected 4 to 5 days later), whereas pNL-ZERO failed to replicate. As indicated in Fig. 1, virus production was observed following transfection-cocultivation of proviral DNAs containing one or two NF-κB elements, although replication of the former (pNL-1κB) was markedly delayed (first detected on day 27). Cloned HIV proviruses containing two or three Sp1 sites (pNL-2Sp1 and pNL-3Sp1) or a single pair of NF-κB and Sp1 sites separated by a mutated intervening NF-κB site (pNL-κB/Sp1-R) all replicated following transfection-cocultivation but with kinetics somewhat slower than that of wild type. Interestingly, the pNL-κB/Sp1-J provirus containing juxtaposed NF-κB and Sp1 single elements replicated nearly as efficiently as wild-type HIV (data not shown). Of the mutated HIV LTRs evaluated by transfection-cocultivation, only the pNL-1Sp1 proviral construction, which contains the single Sp1 element Sp1(I), failed to replicate in three independent experiments lasting 51 days, 2.5 months, and 4 months, respectively.

Infection of PBLs and human T-cell lines with HIV-1 derivatives containing NF-κB or Sp1 motifs in their reconstructed LTRs. Although the transfection-cocultivation experiments described above indicated which of the NF-κB/Sp1 insertions into the pNL-ZERO LTR restored replicative capacity, how these elements influenced virus infectivity required additional investigation. Infections were therefore performed employing virus stocks generated from the transfection-cocultures referred to in Fig. 1. The various T-cell lines were infected with equivalent amounts of wild-type or LTR mutant virus (3×10^5 ^{32}P-RT cpm, approximately equal to a multiplicity of infection of 0.001 for wild-type virus), and subsequent progeny virus production was monitored by RT activity.



	PLASMID	NF-kB(II) NF-kB(I) Sp1(III) Sp1(II) Sp1(I)	VIRUS PRODUCED
	pNL-WT	TACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGGCCTGGGCGGGACTGGGGAGTGGCGAGCCCTC	+
	pNL-ZERO	TAC CTC	-
A	pNL-1kB	TAC GCTGGGGACTTTCCAG AGCCCTC	+
A	pNL-2kB	TACAAGGGACTTTCCGCTGGGGACTTTCCAG CTC	+
	pNL-1Sp1	TAC TGGGGAGTGGCGAGCCCTC	-
B	pNL-2Sp1	TAC CTGGGCGGGACTGGGGAGTGGCGAGCCCTC	+
	pNL-3Sp1	TAC GAGGCGTGGCCTGGGCGGGACTGGGGAGTGGCGAGCCCTC	+
C	pNL-kB/Sp1-J	TAC GCTGGGGACTTTCCAGGGAGGCGTGGCC AGCCCTC	+
C	pNL-kB/Sp1-R	TACAAGGGACTTTCCGCTGCTCACTTTCCAGGGAGGCGTGGCC AGCCCTC	+

FIG. 1. Structure of reconstructed HIV LTRs associated with full-length proviral DNAs. The positions of several *cis*-acting regulatory elements within the HIV-1 LTR are indicated diagrammatically at the top. The nucleotide sequences of a 72-bp region encompassing the NF-κB and Sp1 motifs of the wild-type LTR (pNL-WT) as well as eight reconstructed LTRs are shown below. The nucleotide substitution in pNL-κB/Sp1-R, previously reported (28) to inactivate the NF-κB binding site, is underlined. Virus production following transfection-cocultivation is indicated in the column at the right.

In the first group of experiments, PBLs and different T-cell lines (MT4, CEM [A3.01 clone], H9, and Jurkat [clone JE6.1]) were infected with mutant HIV preparations containing one or two NF-κB elements (and no Sp1 motifs). Three different replicative phenotypes were observed. The first, seen with PBLs or MT4 cells, was characterized by delayed replication as the number of NF-κB sites was reduced from two to one (Fig. 2A). In H9 (Fig. 2B) and A3.01 (Fig. 2C) cells, however, HIV with the single NF-κB(I) element failed to replicate; virus containing two NF-κB sites in its reconstructed LTR replicated slowly compared with wild-type HIV, generating virus progeny 2.5 to 4 weeks postinfection. Notably, infection of A3.01 cells with HIV containing only two NF-κB elements resulted in virus production in only one of three independent experiments (the one depicted in Fig. 2C) monitored for periods of up to 42 days. The third replicative phenotype was observed in Jurkat cells and is shown in Fig. 2D. In this case, virus with LTRs containing one or two NF-κB elements failed to replicate during nearly 4 weeks of observation.

A similar approach was followed to monitor the role of Sp1 motifs on virus infectivity. Since no virus was generated following transfection-cocultivation of pNL-1Sp1, the infectivity studies were limited to virus stocks containing two [Sp1(I) plus Sp1(II)] or three [Sp1(I) plus Sp1(II) plus Sp1(III)] Sp1 motifs as shown in Fig. 1. In all cell types tested, the presence of a second Sp1 element restored infectivity. In some cells, HIVs with reconstructed LTRs containing two or three Sp1 binding sites had similar replication kinetics (PBLs in Fig. 3A), while in others, virus harboring only two Sp1 elements (H9 cells in Fig. 3B) reproducibly exhibited retarded growth properties.

Infectivity of HIV preparations with reconstructed LTRs containing combinations of NF-κB and Sp1 elements. As shown in Fig. 2 and 3, duplications of either the NF-κB or Sp1 element frequently resulted in enhanced HIV replication. Given the proximity of the NF-κB and Sp1 motifs in the wild-type LTR configuration, we wondered whether the observed augmented replicative capacity was limited to combinations of the same element. To assess the possibility that NF-κB and Sp1 elements might function in a combinatorial fashion, we generated a provirus containing LTRs with single copies of both motifs. In the five human T-cell systems evaluated, this virus ($HIV_{NL\text{-}κB/Sp1\text{-}J}$) replicated as rapidly or more rapidly than HIV preparations with LTRs harboring duplications or triplications of the NF-κB or Sp1 elements (data not shown). This phenomenon was particularly striking in Jurkat cells in which HIV with two NF-κB elements failed to replicate during a 25-day observation period (Fig. 4A). Furthermore, the NL-κB/Sp1-J virus replicated with kinetics similar to that of wild-type HIV in most of the cell lines tested (Fig. 4B).

We wondered whether the efficient viral replication directed by an LTR containing a pair of NF-κB and Sp1 elements reflected their juxtaposition or simply their presence in the reconstructed LTR. Consequently, a different LTR containing the single functional NF-κB(II) and Sp1(III) elements spatially separated from one another by a mutagenized (28) NF-κB(I) motif (see the NL-κB/Sp1-R provirus in Fig. 1) was examined for its ability to support a productive virus infection. In H9 cells, the replicative capacity of this virus was similar to that of virus containing the juxtaposed NF-κB(I) and Sp1(III) binding sites (Fig. 5). Thus, an 11-bp separation of functional NF-κB and Sp1 sites did not appreciably affect infectivity; virions with this LTR configuration replicated more rapidly than those containing only two NF-κB or two Sp1 elements.

Replicative capacity of HIV proviral DNA containing a

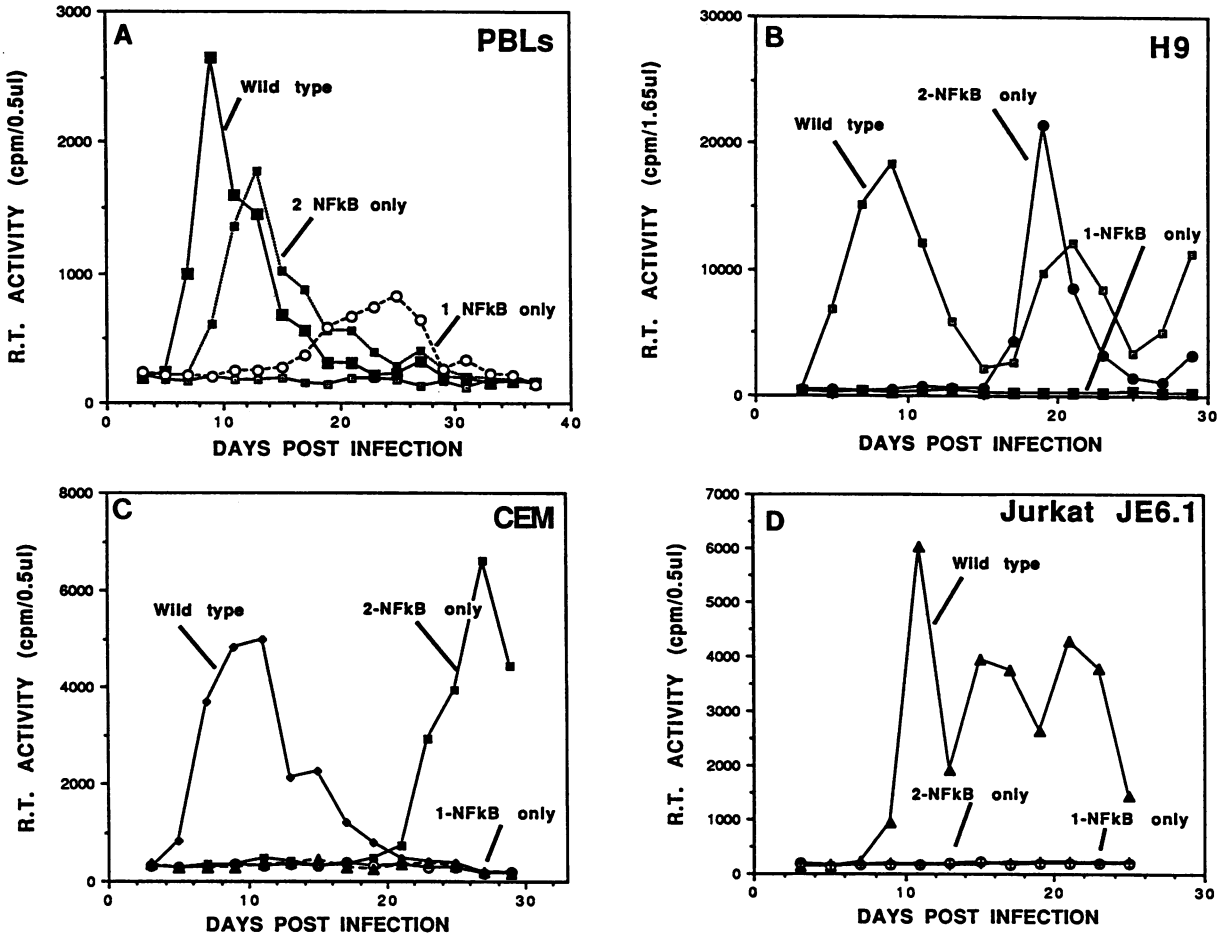


FIG. 2. Infections of human T cells with wild-type HIV or mutant HIV derivatives with LTRs containing one or two NF-κB and no Sp1 elements. Activated PBLs or H9, CEM, or Jurkat cells were infected with approximately 3×10^5 ^{32}P-RT cpm of the indicated virus preparations obtained by transfection-cocultivation. Virus production was monitored by RT assay.

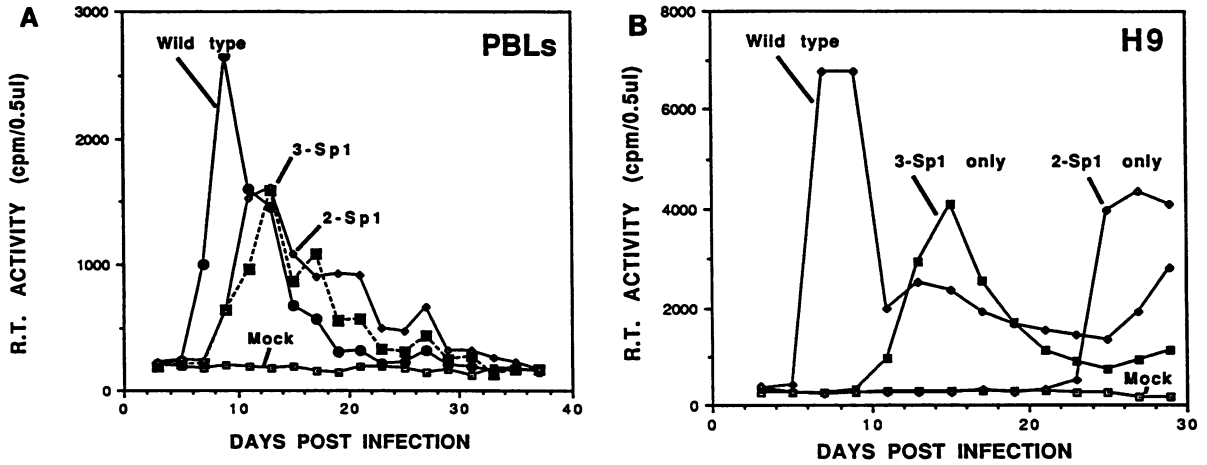


FIG. 3. Infections of human T cells with wild-type HIV or mutant HIV derivatives with LTRs containing two or three Sp1 and no NF-κB elements. Activated PBLs or H9 cells were infected with the indicated virus preparations as described in Materials and Methods. Virus production was monitored by RT assay.

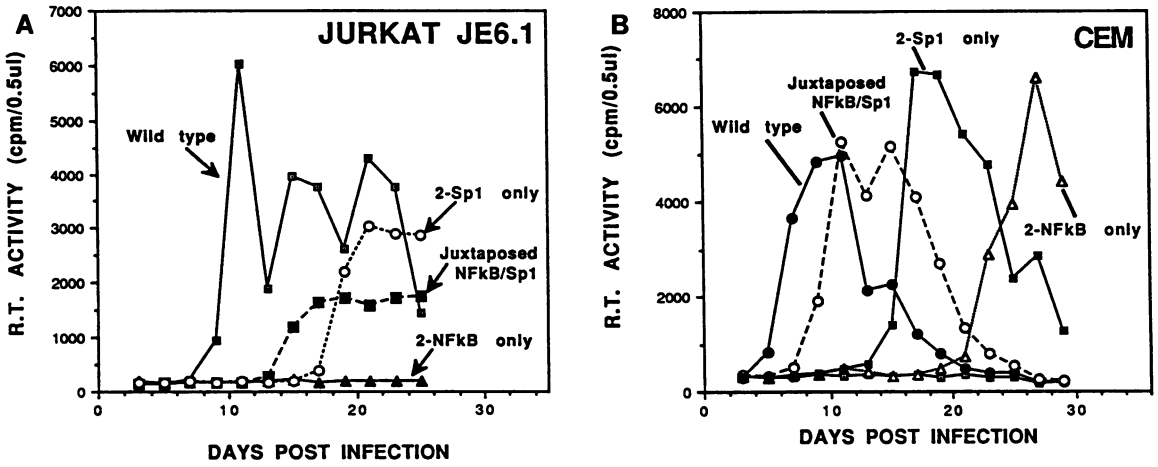


FIG. 4. Infections of human T cells with wild-type HIV or mutant HIV derivatives with LTRs containing pairs of homologous or heterologous *cis*-acting elements. Jurkat or CEM cells were infected with the indicated virus preparations as described in Materials and Methods. Virus production was monitored by RT assay.

single enhancer-activator element. The results shown in Fig. 4 and 5 suggest that paired NF-κB and Sp1 elements are acting in a combinatorial manner since HIV preparations with duplications of the same elements exhibited an impaired replication phenotype in some cell lines. Moreover, the transfection-cocultivation results presented in Fig. 1 indicated that the single Sp1 motif Sp1(I) failed to restore replication competence to pNL-ZERO, suggesting that a single Sp1 element could not direct virus production unless it was associated with either a second Sp1 element or an NF-κB element. These conclusions all assume that the biological activity of each of the single NF-κB or Sp1 elements can be predicted from the data shown in Fig. 1.

However, since the sequences of the different NF-κB or Sp1 motifs in the HIV-1 LTR are not identical, we undertook a series of experiments to evaluate more critically the functional contribution of individual enhancer-activator elements in the reconstructed LTRs. Toward this end, proviral DNAs containing only single NF-κB or Sp1 elements were assayed for virus production by transfection-cocultivation. As shown in Fig. 6, and in agreement with the data presented in Fig. 1, a proviral clone containing only the Sp1 element Sp1(I) failed to replicate in three independent experiments. In contrast, the presence of the single Sp1 motif Sp1(III) restored the capacity to produce virus. Sp1 element Sp1(II) had an intermediate effect in these assays: in one experiment, progeny virus was detected by day 25, while in a second experiment, none was produced after 77 days. A similar disparity was also observed when the two NF-κB elements were examined individually. NF-κB(I) was associated with virus production in three separate experiments, whereas NF-κB(II) restored replication competence in only one of two independent experiments. In view of the very long time (61 days) before progeny virions were detected in this latter experiment, the replication of NF-κB(II) most likely reflects a reversion event similar to that observed in the LTR sequences of other late-arising HIV variants (see next section). It is our current belief that NF-κB(II) by itself cannot restore replicative capacity to the pNL-ZERO LTR.

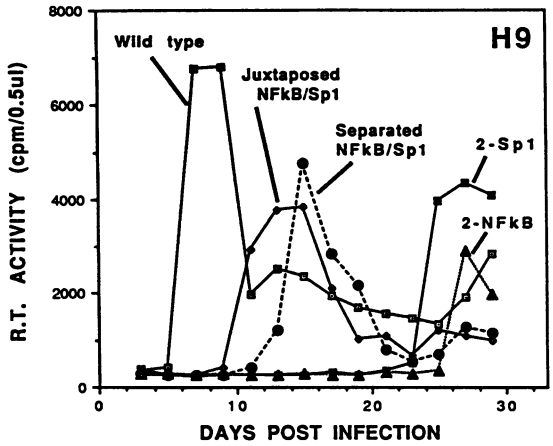


FIG. 5. Infection of H9 cells with HIV containing an LTR with single separated NF-κB and Sp1 elements. H9 cells were infected with the indicated virus preparations containing pairs of homologous or heterologous motifs. Virus production was monitored by RT assay.

Second-site reversions which appear in reconstructed LTRs following virus infection of human PBLs or T-cell lines. We previously reported that second-site revertants of replication-defective HIV mutants may emerge during long-term tissue culture infections (46). Consequently, the appearance of progeny virions at very late times following HIV infection in this study raised the possibility that a compensatory LTR alteration was responsible for the infectivity observed. We therefore focused attention on the group of proviruses containing only NF-κB elements in their reconstructed LTRs since they exhibited a spectrum of replicative capacities related to the cell type infected. As indicated in Fig. 2, a hierarchy of cellular permissiveness was observed when HIV harboring only one or two NF-κB elements was examined. PBLs and MT4 cells were the most permissive, allowing HIV containing only a single NF-κB motif to replicate, whereas constructs containing one or two NF-κB elements completely failed to replicate in Jurkat cells. H9 and CEM cells exhibited an intermediate phenotype: replication of virus with a single NF-κB sequence was restricted in these cells, but HIV containing both NF-κB elements was infectious in two of two experiments in H9 cells and one (shown in Fig. 2C) of three experiments in CEM cells.

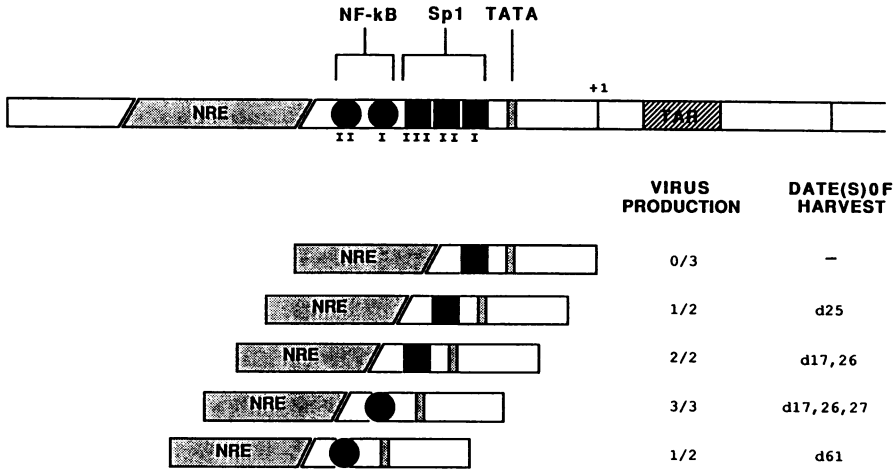


FIG. 6. Replication of HIV-1 proviruses with reconstructed LTRs containing single NF-κB or Sp1 binding motifs following transfection-cocultivation. The structure of the HIV-1 LTR is shown diagrammatically at the top. In the reconstructed LTRs, a filled square or circle indicates the presence of an individual Sp1 or NF-κB motif, respectively. NRE denotes the negative regulatory element. The frequency of virus production (number of RT-positive transfections-cocultivations per number of independent experiments) and the day of virus harvest are indicated in the columns on the right.

To assess whether the LTRs might have undergone alteration during these virus infections, we performed "RNA PCR" on the genomic RNAs isolated from particular samples of progeny virions. These samples included (i) H9 cells infected with $HIV_{NL\text{-}2\kappa B}$ (two NF-κB and no Sp1 elements), (ii) CEM cells infected with $HIV_{NL\text{-}2\kappa B}$ (two NF-κB and no Sp1 elements), and (iii) PBLs infected with $HIV_{NL\text{-}1\kappa B}$ (one NF-κB and no Sp1 elements). The resultant amplified cDNA was cloned, and the 5′ 445 bp of the U3 portion of the LTR were sequenced. In all cases, the original deletions generated in vitro remained intact. Furthermore, as shown in Fig. 7, none of the eight cDNA clones derived from H9 cells infected with pNL-2κB contained additional changes in a region of the LTR extending from the NF-κB elements to the TATAA site. In contrast, 8 of 10 PCR clones of virus recovered from CEM cells infected with $HIV_{NL\text{-}2\kappa B}$ con-

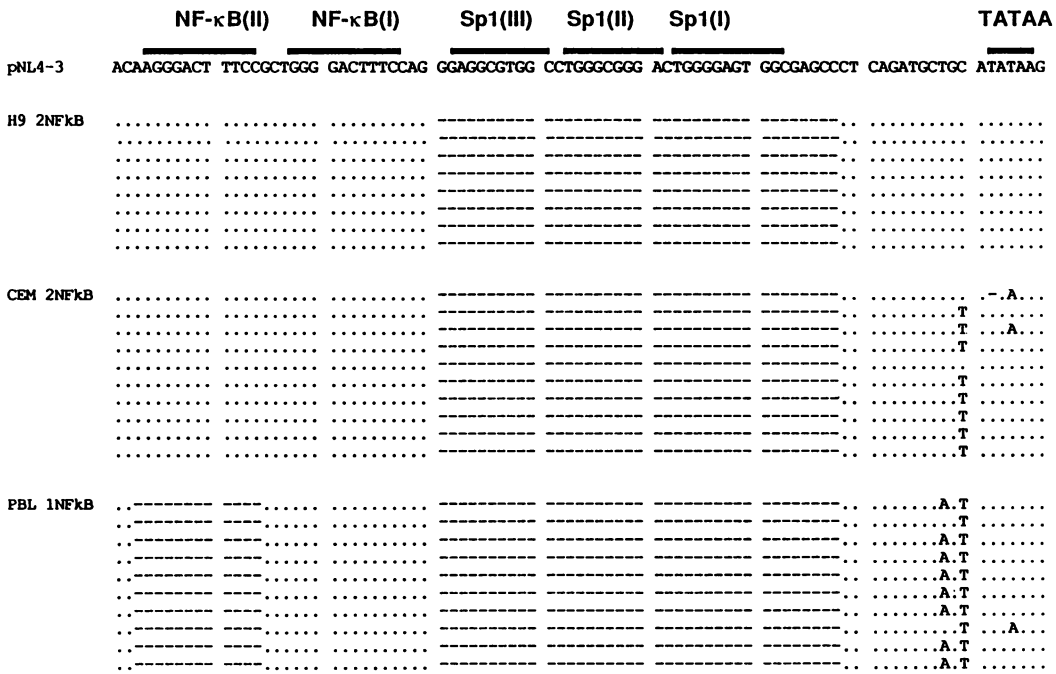


FIG. 7. PCR analysis of a portion of the U3 region of HIV LTR mutants lacking Sp1 binding motifs. The RNA associated with the cell-free virus emerging from H9 or CEM cells infected with $HIV_{NL\text{-}2\kappa B}$ (Fig. 1) or PBLs infected with $HIV_{NL\text{-}1\kappa B}$ (Fig. 1) was reverse transcribed and amplified by PCR as described in Materials and Methods. The nucleotide sequences of individual clones were determined and compared with that of the parental wild-type $HIV_{NL4\text{-}3}$ shown at the top. Identical nucleotides are indicated by the dots; dashes denote deleted nucleotides. Nucleotide substitutions are indicated.

tained a C-to-T change at nucleotide −30 (27), 2 bp upstream from the TATAA site. In addition, this same substitution was observed in all 10 clones arising from the PBL infection with $HIV_{NL\text{-}1\kappa B}$ virus and was accompanied by a second change (T to A) at nucleotide −32 in 8 of these 10 clones. Some of the amplified LTR clones had no alterations in other regions of the LTR; in the others examined, a maximum of two additional changes per LTR were noted, none of which were clustered in a specific region.

DISCUSSION

In our previous studies, a series of deletion and point mutations were introduced into the HIV LTR to study the roles of the NF-κB and Sp1 binding sites and the TAR region in virus production (23). Deletion of the two NF-κB sites did not render the resulting proviral DNA replication incompetent. The removal of the three Sp1 binding sites with preservation of the NF-κB binding sites resulted in a virus capable of replicating in human PBLs but not in cells lacking NF-κB binding activity (30). Furthermore, the simultaneous excision of both NF-κB and all three Sp1 binding sites eliminated the capacity of HIV to replicate in human T cells. These observations suggested that discrete binding sites in the LTR function as modular units, called enhansons (10, 29, 43), which can functionally substitute for one another and whose importance to viral replication varies in different cell types depending on the nature of cellular transcriptional factors present in those cells. In the present studies, we attempted to define minimal sequences required to support viral replication in a variety of human T lymphocytes and further characterized the nature of the enhansons present in the HIV promoter-enhancer sequences.

Our efforts to determine minimal promoter-enhancer sequences required for viral replication were undertaken using a reconstructive approach. The insertion of the single NF-κB element NF-κB(I) into pNL-ZERO was sufficient to restore HIV replication in CEM-MT4 cocultures; in contrast, NF-κB(II) did not restore replication competence. In analogous LTR reconstruction experiments involving individual Sp1 motifs, a continuum of replicative capacities was observed: Sp1(III) > Sp1(II) > Sp1(I). This latter result is consistent with reports showing that the Sp1(I) element has lower affinity for Sp1 protein (20) and for factors present in crude nuclear extracts of human lymphocytes (35) than does either element II or III. The ability of HIV preparations containing only Sp1(III) to infect human T cells is currently under evaluation.

As neither the NF-κB nor Sp1 binding sites are absolutely required for virus replication in PBLs (as long as the other is present in the LTR), the marked conservation of the presence and arrangement of these two types of *cis*-acting sequences in all known HIV-1 isolates is puzzling. The experiments shown in Fig. 4 and 5 suggest that the arrangement and spacing of adjacent NF-κB and Sp1 binding sites in the native configuration of the HIV-1 LTR contribute more significantly to virus replication than duplications or triplications of either element. In this regard, we previously reported that point mutations affecting the adjacent NF-κB and Sp1 elements resulted in a significant reduction of virus replicative capacity (23). Thus, two independent studies suggest the existence of a functional interaction(s) between these two different *cis*-acting elements and the proteins whose functions they mediate. Notably, NF-κB binding sites are frequently situated adjacent to binding sites for constitutive cellular transcriptional factors. One striking example is in the promoter of the cytomegalovirus immediate-early gene, in which a series of NF-κB binding sites are interspersed with the binding sites for NF-1 (12, 16). When these elements were placed adjacent to each other, 5′ to the whey acidic protein promoter, a synergistic enhancement of promoter activity was observed, suggesting that in this context, NF-κB was able to interact with factors binding to the NF-1 site to augment transcription (17). Likewise, similar studies have been performed to study the interactions between adjacent binding sites for Sp1 and OTF-1. An Sp1 element in combination with a high-affinity OTF-1 site augmented transcription 2-fold; however, when placed next to a low-affinity OTF site, the Sp1 site increased transcription 20-fold (19). Thus, as both NF-κB and Sp1 can each interact with other transcription factors to augment transcription, it is not surprising that they may interact with each other in the HIV LTR to promote viral replication.

An interesting aspect of this analysis is the apparent hierarchy of cellular permissiveness for HIV infection seen best with proviruses containing only one or two NF-κB elements. The order observed (PBLs = MT4 > H9 > CEM > Jurkat cells) is consistent with our previous findings (PBLs = MT4 > CEM) with an HIV LTR mutant containing a wild-type arrangement of NF-κB motifs but lacking all three Sp1 sites and presumably reflects various levels of cellular factors capable of binding to NF-κB (30). As shown in Fig. 2 of this study, virus containing a single NF-κB element was capable of infecting PBLs and MT4 cells (data not shown) exclusively. At the other extreme, HIV with two NF-κB elements failed to replicate in Jurkat cells. This continuum of cellular susceptibility was also reflected in the number and types of second-site revertants which appeared during infection. H9 cells consistently supported replication of HIV with two NF-κB elements; progeny virions from such an infection contained an unaltered LTR (Fig. 7). This same virus inoculum produced progeny virions in only one of three independent infections of CEM (A3.01) cells. PCR analysis revealed a consistent second-site mutation in 8 of 10 cDNA clones arising from this single positive experiment that was localized to the TATA region of the LTR. Finally, the HIV construction with a single NF-κB binding site (NL-1κB) replicated slowly in PBLs but, in the process, acquired two changes also situated immediately upstream of the TATA box. Thus, a selection for virions containing a similar TATA mutation is operative in both CEM cells and PBLs, possibly reflecting the 40-bp deletion encompassing the three Sp1 sites which effectively places the NF-κB(I) motif 13 bp upstream of the TATA box. This TATA alteration is consistent with reports describing the restricted interaction between a cell-specific enhancer and discrete

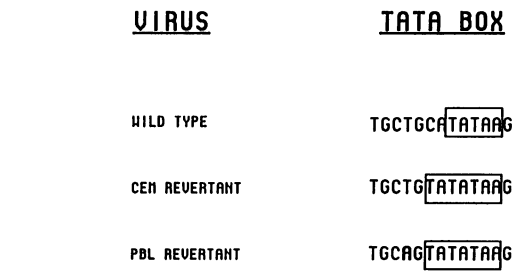


FIG. 8. TATA box revertants which appear following infection of CEM and activated PBL cells with HIV containing only an NF-κB motif(s) in the NF-κB/Sp1 domain.

TATA sequences (39, 42, 44). Perhaps the alteration of the wild-type HIV TATAA box to TATATAA is necessary for functional interaction with the dislocated NF-κB element(s).

It is worth noting that the base change common to both second-site revertant viruses extended the TATA box two nucleotides in the 5′ direction relative to that of the parental virus pNL4-3 (Fig. 8). Since the TATA element positions where RNA polymerase II initiates RNA synthesis, this change could shift the transcriptional start site from its normal location at the U3/R border. Experiments to ascertain whether the transcriptional start site has been altered in the LTR revertant viruses and to assess the effects of such TATA changes on HIV replication are presently in progress.

ACKNOWLEDGMENT

The following reagent was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases: Jurkat clone E6-1 from the American Type Culture Collection.

REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59**:284–291.
2. **Ahmad, N., and S. Venkatesan.** 1988. *Nef* protein of HIV-1 is a transcriptional repressor of HIV-1 LTR. Science **241**:1481–1485.
3. **Arya, S. K., C. Guo, S. F. Josephs, and F. Wong-Staal.** 1985. Trans-activator gene of human T-lymphotropic virus type III (HTLV-III). Science **229**:69–73.
4. **Berkhout, B., and K.-T. Jeang.** 1989. *trans* activation of human immunodeficiency virus type 1 is sequence specific for both the single-stranded bulge and loop of the *trans*-acting-responsive hairpin: a quantitative analysis. J. Virol. **63**:5501–5504.
5. **Berkhout, B., R. H. Silverman, and K.-T. Jeang.** 1989. Tat *trans*-activates the human immunodeficiency virus through a nascent RNA target. Cell **59**:273–282.
6. **Durand, D. B., J.-P. Shaw, M. R. Bush, R. E. Replogle, R. Belagaje, and G. R. Crabtree.** 1988. Characterization of antigen receptor response elements within the interleukin-2 enhancer. Mol. Cell. Biol. **8**:1715–1724.
7. **Dynan, W. S., and R. Tjian.** 1983. The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. Cell **35**:79–87.
8. **Feng, S., and E. C. Holland.** 1988. HIV-1 *tat trans*-activation requires the loop sequence within *tar*. Nature (London) **334**: 165–167.
9. **Folks, T., S. Benn, A. Rabson, T. Theodore, M. D. Hoggan, M. Martin, M. Lightfoote, and K. Sell.** 1985. Characterization of a continuous T-cell line susceptible to the cytopathic effects of the acquired immunodeficiency syndrome (AIDS)-associated retrovirus. Proc. Natl. Acad. Sci. USA **82**:4539–4543.
10. **Fromental, C., M. Kanno, H. Nomiyama, and P. Chambon.** 1988. Cooperativity and hierarchical levels of functional organization in the SV40 enhancer. Cell **54**:943–953.
11. **Garcia, J. A., D. Harrich, E. Soultanakis, F. Wu, R. Mitsuyasu, and R. B. Gaynor.** 1989. Human immunodeficiency virus type 1 LTR TATA and TAR region sequences required for transcriptional regulation. EMBO J. **8**:765–778.
12. **Ghazal, P., H. Lubon, B. Fleckenstein, and L. Hennighausen.** 1987. Binding of transcription factors and creation of a large nucleoprotein complex on the human cytomegalovirus enhancer. Proc. Natl. Acad. Sci. USA **84**:3658–3662.
13. **Guyader, M., M. Emerman, P. Sonigo, F. Clavel, L. Montagnier, and M. Alizon.** 1987. Genome organization and transactivation of the human immunodeficiency virus type 2. Nature (London) **326**:662–669.
14. **Harrich, D., J. A. Garcia, R. Mitsuyasu, and R. B. Gaynor.** 1990. TAR independent activation of the human immunodeficiency virus in phorbol ester stimulated T lymphocytes. EMBO J. **9**:4417–4423.
15. **Harrich, D., J. A. Garcia, F. K. Wu, R. Mitsuyasu, J. Gonzales, and R. B. Gaynor.** 1989. Role of Sp1-binding domains in in vivo transcriptional regulation of the human immunodeficiency virus type 1 long terminal repeat. J. Virol. **63**:2585–2591.
16. **Hennighausen, L., and B. Fleckenstein.** 1986. Nuclear factor 1 interacts with five DNA elements in the promoter region of the human cytomegalovirus major immediate early gene. EMBO J. **5**:1367–1371.
17. **Hennighausen, L., P. A. Furth, and C. W. Pittius.** 1989. κB elements strongly activate gene expression in non-lymphoid cells and function synergistically with NF1 elements. Nucleic Acids Res. **17**:8197–8206.
18. **Jakobovits, A., D. H. Smith, E. B. Jakobovits, and D. J. Capon.** 1988. A discrete element 3′ of human immunodeficiency virus 1 (HIV-1) and HIV-2 mRNA initiation sites mediates transcriptional activation by an HIV *trans* activator. Mol. Cell. Biol. **8**:2555–2561.
19. **Janson, L., and U. Pettersson.** 1990. Cooperative interactions between transcription factors Sp1 and OTF-1. Proc. Natl. Acad. Sci. USA **87**:4732–4736.
20. **Jones, J. A., J. T. Kadonga, P. A. Luciw, and R. Tijan.** 1986. Activation of the AIDS retrovirus promoter by the cellular transcription factor, Sp1. Science **232**:755–759.
21. **Kawakami, K., C. Schneidereit, and R. G. Roeder.** 1988. Identification and purification of a human immunoglobulin enhancer-binding protein (NF-κB) that activates transcription from a human immunodeficiency virus type 1 promoter *in vitro*. Proc. Natl. Acad. Sci. USA **85**:4700–4704.
22. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotype selection. Proc. Natl. Acad. Sci. USA **82**:488–492.
23. **Leonard, J., C. Parrott, A. J. Buckler-White, W. Turner, E. K. Ross, M. A. Martin, and A. B. Rabson.** 1989. The NF-κB binding site in the human immunodeficiency virus type 1 long terminal repeat are not required for virus infectivity. J. Virol. **63**:4919–4924.
24. **Lu, Y., M. Stenzel, J. G. Sodroski, and W. A. Haseltine.** 1989. Effects of long terminal repeat mutations on human immunodeficiency virus type 1 replication. J. Virol. **63**:4115–4119.
25. **Miyoshi, I., H. Taguchi, I. Kubonishi, S. Yoshimoto, Y. Ohtsuki, F. Shiraishi, and T. Akagi.** 1982. Type c virus-producing cell lines derived from adult T-cell leukemia. Gann Monogr. **28**:219–228.
26. **Morgan, R. A., K. Cornetta, and W. F. Anderson.** 1990. Applications of the polymerase chain reaction in retroviral-mediated gene transfer and the analysis of gene-marked human TIL cells. Hum. Gene Ther. **1**:135–149.
27. **Myers, G., A. B. Rabson, J. A. Berzofsky, T. F. Smith, and F. Wong-Staal (ed.).** 1990. Human retroviruses and AIDS 1990, p. I-A-64. Theoretical Biology and Biophysics, Los Alamos National Laboratory, Los Alamos, N.Mex.
28. **Nabel, G., and D. Baltimore.** 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature (London) **326**:711–713.
29. **Ondek, B., L. Gloss, and W. Herr.** 1988. The SV40 enhancer contains two distinct levels of organization. Nature (London) **333**:40–45.
30. **Parrott, C., T. Seidner, E. Duh, J. Leonard, T. S. Theodore, A. Buckler-White, M. A. Martin, and A. B. Rabson.** 1991. Variable role of the long terminal repeat Sp1-binding sites in human immunodeficiency virus replication in T lymphocytes. J. Virol. **65**:1414–1419.
31. **Popovic, M., M. G. Sarnagadharan, E. Read, and R. C. Gallo.** 1984. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science **224**:497–500.
32. **Rosen, C. A., J. G. Sodroski, and W. A. Haseltine.** 1985. The location of *cis*-acting regulatory sequences in the human T cell lymphotrophic virus type III (HTLV-III/LAV) long terminal repeat. Cell **41**:813–823.
33. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular

cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
35. **Seidner, T. J., and A. B. Rabson.** Unpublished data.
36. **Selby, M. J., E. S. Bain, P. A. Luciw, and B. M. Peterlin.** 1989. Structure, sequence, and position of the stem-loop in *tar* determine transcriptional elongation by *tat* through the HIV-1 long terminal repeat. Genes Dev. **3**:547–558.
37. **Shaw, J.-P., P. J. Utz, D. B. Durand, J. J. Toole, E. A. Emmel, and G. R. Crabtree.** 1988. Identification of a putative regulator of early T cell activation genes. Science **241**:202–205.
38. **Siekevitz, M., S. F. Josephs, M. Dukovich, N. Peffer, F. Wong-Staal, and W. C. Greene.** 1987. Activation of the HIV LTR by T cell mitogens and the *trans*-activator protein of HTLV-1. Science **238**:1575–1578.
39. **Simon, M. C., T. M. Fisch, B. J. Benecke, J. R. Nevins, and N. Heintz.** 1988. Definition of multiple, functionally distinct TATA elements, one of which is a target in the *hsp70* promoter for E1A regulation. Cell **52**:723–729.
40. **Sodroski, J., C. Rosen, F. Wong-Staal, S. Z. Salahuddin, M. Popovic, S. Arya, R. C. Gallo, and W. A. Haseltine.** 1985. Trans-acting transcriptional regulation of human T-cell leukemia virus type III long terminal repeat. Science **227**:171–173.
41. **Sodroski, J. G., C. A. Rosen, F. Wong-Staal, R. Patarca, and W. A. Haseltine.** 1985. Location of the trans-activating region on the genome of human T-cell lymphotropic virus type III. Science **229**:74–77.
42. **Struhl, K.** 1986. Constitutive and inducible *Saccharomyces cerevisiae* promoters: evidence for two distinct molecular mechanisms. Mol. Cell. Biol. **6**:3847–3853.
43. **Tanaka, M., U. Grossniklaus, W. Herr, and N. Hernandez.** 1988. Activation of the U2 snRNA promoter by the octamer motif defines a new class of RNA polymerase II enhancer elements. Genes Dev. **2**:1764–1788.
44. **Wefald, F. C., B. H. Devlin, and R. S. Williams.** 1990. Functional heterogeneity of mammalian TATA-box sequences revealed by interaction with cell-specific enhancer. Nature (London) **344**:260–262.
45. **Weiss, A. L., R. L. Wiskocil, and J. D. Stobo.** 1984. The role of T3 surface molecules in the activation of human T cells: a two-stimulus requirement for IL 2 production reflects events occurring at a pre-translational level. J. Immunol. **133**:123–128.
46. **Willey, R., D. H. Smith, L. A. Lasky, T. S. Theodore, P. L. Earl, B. Moss, D. J. Capon, and M. A. Martin.** 1988. In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. J. Virol. **62**:139–147.
47. **Zoller, M. J., and M. Smith.** 1983. Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. DNA **3**:479–488.