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Second-Site Long Terminal Repeat (LTR) Revertants of Replication-Defective Human Immunodeficiency Virus: Effects of Revertant TATA Box Motifs on Virus Infectivity, LTR-Directed Expression, In Vitro RNA Synthesis, and Binding of Basal Transcription Factors TFIID and TFIIA

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Second-site revertants from replication-incompetent molecular clones of human immunodeficiency virus (HIV) contain base substitutions adjacent to the TATA motif. The altered TATA box motifs were analyzed for their effect(s) on virus infectivity, long terminal repeat (LTR)-directed expression in transient transfection assays, in vitro RNA synthesis, and assembly of the TFIID-TFIIA preinitiation complex. The revertant TATA boxes accelerated the kinetics of HIV replication when present in the context of an LTR containing a Sp1 mutation (deletion or site specific); no effect was observed on the infectivity of wild-type HIV. In chloramphenicol acetyltransferase assays and in vitro transcription systems, the altered TATA box motifs led to elevated basal levels of RNA synthesis from NF-κB- and Sp1-mutagenized and wild-type templates, respectively, but did not increase responsiveness to Tat transactivation. The revertant TATA boxes accelerated the binding of TFIID and TFIIA to the LTR and stabilized their association with the promoter. The revertants did not assemble a more-processive elongation complex. These results suggest that in the context of an impaired enhancer/promoter (viz., three mutated Sp1 elements), a series of HIV revertants emerge which contain LTR alterations that significantly augment basal RNA synthesis. The TATA motif revertants are capable of rescuing the enhancer/promoter defect and sustain virus infectivity.

Subsequent to the integration of viral DNA, the major function of the retroviral long terminal repeat (LTR) is to regulate the production of viral mRNA. The promoter/enhancer elements needed for efficient transcriptional initiation are located within the U3 and R regions and are active in the context of the 5′ LTR. Like any other eukaryotic promoter, the human immunodeficiency virus (HIV) LTR contains its own ensemble of DNA elements to which transcriptional regulatory factors bind. These sequences were identified from mutagenesis studies of LTR-driven reporter gene constructs, from gel retardation or DNA footprinting experiments, or from searches of nucleotide sequence data bases (6, 8, 12, 13, 15, 16, 19, 20, 23, 25, 28, 32, 33, 40, 42, 50, 51). The Sp1 and TATA motifs bind cellular proteins that are constitutively expressed in most eukaryotic cells and are critical for basal levels of HIV gene expression (2, 15, 19, 20, 40). In the HIV type 1 (HIV-1) LTR, the two NF-κB motifs, together with the three Sp1 sites, function as the principal enhancers of transcription (16, 23, 33, 37, 40, 42).

Our current understanding of HIV LTR function comes primarily from analyses of transient transfections of HeLa or human T-cell lines with plasmid constructs in which the wild-type or mutagenized LTR has been fused to a reporter gene. Although this approach has been utilized for delineating important mechanisms associated with HIV LTR activation, it is unsuitable for evaluating the complex interactions of cellular proteins, *cis*-acting regulatory sequences associated with DNA and RNA forms of the viral genome, and the full ensemble of virus-encoded proteins synthesized during a productive virus infection. For example, mutations of the two NF-κB-binding sites abolish the response of the HIV LTR to T-cell activators, as measured in many reporter gene systems (9, 22, 33, 43, 45). However, when the NF-κB motifs are mutated or deleted in the context of an intact HIV genome, little if any effect was noted during subsequent virus infection of phytohemagglutinin-activated peripheral blood mononuclear cells (PBMCs) (26).

We previously reported that HIV LTR mutants, containing one or two NF-κB-binding elements and lacking Sp1 motifs (removed by deletion mutagenesis) exhibited a hierarchy of infectivity depending on the CD4-positive cell type infected (41). For example, Jurkat cells were completely refractory to infection by Sp1 mutants of HIV-1 (HIV_{Sp1del}), whereas MT-4 cells and activated PBMCs remained susceptible to HIV_{Sp1del} as well as its derivative, $HIV_{N1Sp1del}$, which, in addition to the Sp1 deletion, also lacks one of the two NF-κB elements. The CEM and H9 human T-leukemia cell lines occupied a middle ground in this continuum of susceptibility. Associated with this observed cell-specific hierarchy of susceptibility to HIVs containing mutated Sp1 sites was the emergence of revertant viruses that appeared at late times (e.g., 3 to 4 weeks) following infection. PCR analyses revealed that 20 individual revertant clones had acquired two types of second-site nucleotide

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Provirus Designation	NFkB NFkB Sp-1 Sp-1 Sp-1 TATA box	Infectivity[a]	CAT activity[c] basal	CAT activity[c] tat
$LTR_{WT}/TATA_{WT}$	TACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGGCCTGGGCGGGACTGGGGAGTGGCGAGCCCTCAGATGCTGCATATAAGC	+ (5/5)[b]	31±18	5300±1900
$LTR_{WT}/TATA_{R1}$	********** T********	+ (4/4)	41±18	5400±1800
$LTR_{WT}/TATA_{R2}$	********** A*T********	+ (3/3)	47±6.9	4300±1000
$LTR_{Sp1del}/TATA_{WT}$	**********----------**********	+ (4/4)	27±4.7	550±30
$LTR_{Sp1del}/TATA_{R1}$	**********----------**********T********	+ (2/2)	41±3.3	980±210
$LTR_{Sp1del}/TATA_{R2}$	**********----------*********A*T********	+ (2/2)	35±3.5	880±170
$LTR_{N1Sp1del}/TATA_{WT}$	***----------**********----------**********	+ (3/6)	0.52±0.12	210±100
$LTR_{N1Sp1del}/TATA_{R1}$	***----------**********----------**********T********	+ (4/4)	2.6±1.5	1100±180
$LTR_{N1Sp1del}/TATA_{R2}$	***----------**********----------**********A*T********	+ (5/5)	2.1±0.55	870±110
$LTR_{Sp1m}/TATA_{WT}$	**********TT*********TT*********TT**********	+ (3/4)	1.4±0.32	31±22
$LTR_{Sp1m}/TATA_{R1}$	**********TT*********TT*********TT**********T********	+ (2/2)	2.5±0.37	280±40

FIG. 1. Structures of mutant and revertant LTRs, infectivities of LTR mutant viruses, and CAT activities of the mutant LTR-CAT constructs. The sequences of the enhancer/promoter region are shown. The NF-κB sites, Sp1 sites, and TATA box are indicated at the top. Symbols: *, nucleotide identity with wild type; −, a nucleotide deletion. Footnotes: a, virus infectivity (+) was measured by RT activity released into the medium (>1,000 cpm/10 μl) following cocultivation of MT-4 cells with transfected 12D7 cells, and RT-negative cultures were maintained for a minimum of 3 weeks; b, values in parentheses indicate the number of experiments in which RT production was observed and the total number of experiments; c, CAT activity (basal and Tat induced) relative to the basal activity of $LTR_{WT}/TATA_{WT}$-CAT. The CAT activities represent reproducible mean values from three to five independent transfections and were normalized to the expression of cotransfected human growth hormone plasmid.

changes located immediately upstream of the TATA box; each revertant retained the original Sp1 or Sp1 plus NF-κB mutations. The revertant nucleotide substitutions effectively extended the TATA element in the 5′ direction and altered its context with respect to immediately adjacent 5′ sequences.

We have examined the functional effects of the TATA box revertant changes in two experimental systems. In the first, the biological consequences of the TATA box alterations were evaluated during productive virus infections in the context of a wild-type or Sp1-mutagenized HIV-1 LTR. The TATA revertant changes accelerated replication kinetics only when present as a component of a mutated (deleted or point) LTR; no effect was observed on virus infectivity in the context of a wild-type LTR. When evaluated in in vitro transcription systems, a 5- to 10-fold increase in LTR-directed RNA synthesis was observed in the presence of the TATA box changes. This effect appears to reflect an increase in the number of initiation events and was associated with increased stability of the basal transcription factors TFIID and TFIIA with DNA templates containing revertant TATA elements.

MATERIALS AND METHODS

Construction of proviral and LTR-CAT DNAs containing LTR mutations. pILIC (26) is an infectious, single-LTR, circularly permuted, proviral clone of HIV-1 derived from pNL4-3 (1); the proviral DNA in pILIC is inserted into the vector at the *Bam*HI site, located within the *rev/env* coding region. Mutant LTRs were constructed as previously described (41). Briefly, the *Bam*HI-*Sph*I fragment from pILIC, which encompasses *env* sequences, the LTR, and the 5′ terminus of the *gag* region, was subcloned, mutations were introduced by site-directed mutagenesis (and subsequently verified by sequencing), and an *Xho*I (422 nucleotides downstream of the *Bam*HI site)-*Sph*I subfragment was recloned into pILIC. The revertant TATA boxes $TATA_{R1}$ and $TATA_{R2}$ were originally identified following long-term passage of $LTR_{Sp1del}/TATA_{WT}$ and $LTR_{N1Sp1del}/TATA_{WT}$ HIV-1 mutant viruses (previously called Spdel/WT and N1Spdel/WT viruses, respectively) (41). The sequences of the mutated LTR and the nomenclature used to designate the various LTR mutants and revertants used in these studies are shown in Fig. 1. LTR-CAT plasmids were generated by inserting the *Xho*I-*Hin*dIII fragment (which encompasses the U3 and R regions of the LTR) from the wild type or LTR mutants of pILIC DNAs, upstream of the chloramphenicol acetyltransferase (CAT) gene containing the simian virus 40 poly(A) signal (17).

The HIV LTR mutants used in this work are shown in Fig. 1 and include the following: (i) $LTR_{Sp1del}/TATA_{WT}$, which contains a deletion of the three Sp1-binding sites (previously called pdl-Sp [36] or pNL-2kB [41]); (ii) $LTR_{N1Sp1del}/TATA_{WT}$, which contains deletions of the three Sp1 sites plus the upstream NF-κB-binding site (previously called pNL-1kB [41]); and (iii) $LTR_{Sp1m}/TATA_{WT}$, which contains point mutations in the three Sp1 motifs that abolish the binding of Sp1 (20). The revertant TATA boxes were introduced into the wild-type or enhancer mutant (41) HIV LTR to generate the proviruses listed in Fig. 1. Sequencing analysis indicated that the $LTR_{Sp1del}/TATA_{R1}$ provirus DNA clone also contained a single silent base substitution in the *gag* gene.

Cell culture and virus infection. MT-4 (18), H9 (38), 12D7 (a clonal derivative of A3.01 CEM cells) (11), and Jurkat (46) cells were maintained in RPMI 1640 medium containing 10% fetal calf serum. Human PBMCs were stimulated with 1 μg of phytohemagglutinin (Wellcome Diagnostics) per ml for 3 days prior to infection and maintained in RPMI 1640 medium containing 10% fetal calf serum and 10% interleukin 2 (Boehringer Mannheim). For preparation of virus stocks, the plasmid DNAs were digested with *Bam*HI and the released 9.2-kb proviral DNA was concatemarized in vitro with DNA ligase. The ligated DNAs (10 μg) were introduced into 10^7 12D7 cells by electroporation (Bio-Rad), followed by cocultivation with

MT-4 cells. Alternatively, transfection was performed by the DEAE-dextran method as previously described (26). Virus production was monitored by ^{32}P-labeled reverse transcriptase (RT) assay (48). Cell-free virus stocks were prepared from the coculture supernatants and used as inocula in infectivity assays. PBMC and T-cell lines were infected with equivalent amounts of wild-type or mutant virus, as determined by RT activity.

CAT assay. 12D7 cells (10^7) were coelectroporated with 5 μg (each) of the following plasmid DNAs: LTR-CAT DNA, CMV1-Tat DNA (a gift of Anne Gatinol, Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Disease) (containing Tat cDNA from HIV-1_{SF2} downstream of the simian cytomegalovirus immediate-early promoter), and human growth hormone DNA (pXGH-5; Nichols Institute Diagnostics) (used as an internal transfection control). CAT assays were performed 48 h after transfection as previously described (17). Cell lysates were diluted so that each sample converted 0.5 to 30% of the [^{14}C]chloramphenical substrate to the acetylated form during a 1-h incubation. Human growth hormone assays were performed according to the manufacturer's protocol. CAT activity was normalized for sample dilution and the amount of human growth hormone released into the culture supernatant.

In vitro transcription with unfractionated extracts. Templates for in vitro transcription were prepared by digesting 100 μg of plasmid DNA with a 5- to 10-fold unit excess of restriction enzyme for 1 to 2 h under buffer conditions suggested by the manufacturer (New England Biolabs). After termination of reaction, DNA digests were subjected to two phenol-chloroform-isoamyl alcohol (50:50:1) extractions and a subsequent enthanol precipitation. For most transcription experiments, preincubation of protein extracts (40 μg) and DNA template in the absence of nucleoside triphosphates was followed by the addition of nucleoside triphosphates and a second incubation. All incubations were at 30°C. The in vitro transcription buffer contained 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.9), 50 mM KCl, 0.5 mM EDTA, 1.5 mM dithiothreitol (DTT), 6.25 mM $MgCl_2$, and 8.5% glycerol. The DNA templates were linearized with *Eco*RI and added to a concentration of 3.3 to 10 μg/ml (50 to 300 ng per reaction mixture). HeLa whole-cell extracts, prepared as described previously (29), were added to a final concentration of 2.4 mg/ml (40 μg per reaction mixture). Purified Tat [in Tat storage buffer, which consists of phosphate-buffered saline (without Ca^{2+} and Mg^{2+}), 0.1% bovine serum albumin (BSA) (RNase and DNase free), and 0.1 mM DTT] was added to a final concentration of 0.4 μM. Nucleoside triphosphates in water were added to a final concentration of 500 μM. After a 30-min preincubation period, 20 μCi (2 μl) of [α-^{32}P]UTP (400 Ci/mmol) was added. Transcription reactions were terminated by the addition of 20 mM Tris-HCl (pH 7.8), 150 mM NaCl, and 0.2% sodium dodecyl sulfate. RNA was purified and analyzed on a denaturing 4% polyacrylamide–urea gel as described previously (3).

Band shift analysis. Partially purified TFIIA, TFIIB, and TFIID were prepared by sequential phosphocellulose, DEAE, and Mono S column chromatography by the method of Meisterernst et al. (31). The oligonucleotide probe was labeled with [γ-^{32}P]ATP by using T4 kinase enzyme. The labeled oligonucleotide (6.25 ng) (approximately 10^5 cpm) was incubated with partially purified TFIID (1 μl; DEAE column fraction) and TFIIA (2 μl; Mono S column fraction) for 30 min at room temperature. The sense-strand wild-type oligonucleotide probe contained upstream HIV promoter sequences from −38 to −8 (5′-GATGCTGCATATAAGCAGCTGCTTTTTGCC-3′). The final volume of the reaction mixture was 15 μl, and the binding reaction buffer contained 12 mM HEPES (pH 7.9), 10% glycerol, 5 mM $MgCl_2$, 60 mM KCl, 1 mM DTT, 20 μg of BSA per ml, 0.5 mM EDTA, 0.01% Nonidet P-40, and 3 μg of poly(dG-dC) · poly(dG-dC) (Pharmacia). For competition studies, 100 ng of unlabeled oligonucleotide was added to the incubation mixture. After incubation, DNA-protein complexes were analyzed on a 4% native polyacrylamide gel with 0.25× TBE (Tris-borate-EDTA) as the running buffer. The polyacrylamide gel was preelectrophoresed for 1 h at 150 V.

RESULTS

Effects of second-site TATA region changes on HIV replication. To verify that the TATA box alterations observed were indeed responsible for restoring the infectivity of the family of HIV LTR mutants with Sp1 motifs deleted, the two types of revertant changes (TGCATATAA to TGTATATAA [TA TA_{R1}] or AGTATATAA [$TATA_{R2}$]) were introduced into the wild-type or mutagenized LTR associated with the circularly permuted full-length proviral DNA (designated pILIC [26]), previously used for the analysis of HIV LTR mutants. The nomenclature used to designate the structures of the HIV LTRs present in different HIV molecular clones and virus derivatives and the presence or absence of the wild-type or revertant TATA box ($LTR_{XX}/TATA_{YY}$) is indicated in Fig. 1.

Virus stocks were generated by transfecting the clonal 12D7 derivative of CEM cells with wild-type or HIV LTR mutant proviruses followed by cocultivation with MT-4 cells, as described in Materials and Methods. Human T-cell leukemia virus type I-transformed MT-4 cells are exquisitely sensitive to infection by HIV-1, presumably because they constitutively synthesize the Tax_1 protein and contain constitutively activated NF-κB (36). The MT-4 cells serve two functions in the transfection-cocultivation system: (i) they rapidly amplify viral progeny released following a single cycle of replication in transfected 12D7 cells, and (ii) since MT-4 cells are susceptible to infection by all members of the family of HIV LTR mutants with Sp1 deletion (41), they are the host cell of choice for the preparation of virus stocks for subsequent infectivity studies.

As shown in Fig. 2A, the Sp1-deleted provirus $LTR_{Sp1del}/TATA_{WT}$ exhibited slightly delayed replication kinetics compared with that of the parental wild type ($LTR_{WT}/TATA_{WT}$) in the transfection-cocultivation assay. This result is consistent with our previously reported study of HIV_{Sp1del} in infectivity assays (41) which showed that peak production of HIV_{Sp1del} progeny virions was delayed 2 to 3 days compared with that of wild-type HIV-1 in highly susceptible MT-4 cells (the T-cell type used in the transfection-cocultivation system). The replication properties of the $LTR_{Sp1del}/TATA_{R1}$ and $LTR_{Sp1del}/TATA_{R2}$ proviruses were also very similar to those of $LTR_{WT}/TATA_{WT}$ in the transfection-cocultivation system (Fig. 2A). Consequently, to ascertain whether the revertant TATA boxes have any effect on the replication properties of the Sp1-deleted HIV-1 proviruses, direct infections (rather than transfection and cocultivation) were performed on CEM cells, previously shown to be less susceptible to this family of HIV LTR mutants than MT-4 cells (41). The 12D7 subclone of CEM cells was infected with cell-free $LTR_{Sp1del}/TATA_{WT}$, LTR_{Sp1del}/TA TA_{R1}, and $LTR_{Sp1del}/TATA_{R2}$ virus stocks, and particle production was measured by RT assay. As shown in Fig. 3, very low levels of progeny $LTR_{Sp1del}/TATA_{WT}$ virions were detected 3 to 4 weeks following infection, whereas the revertant TATA box motifs augmented replication of the $LTR_{Sp1del}/TATA_{R1}$ and $LTR_{Sp1del}/TATA_{R2}$ HIV derivatives significantly. It should be noted, however, that the revertant TATA box changes did not restore virus infectivity to wild-type levels.

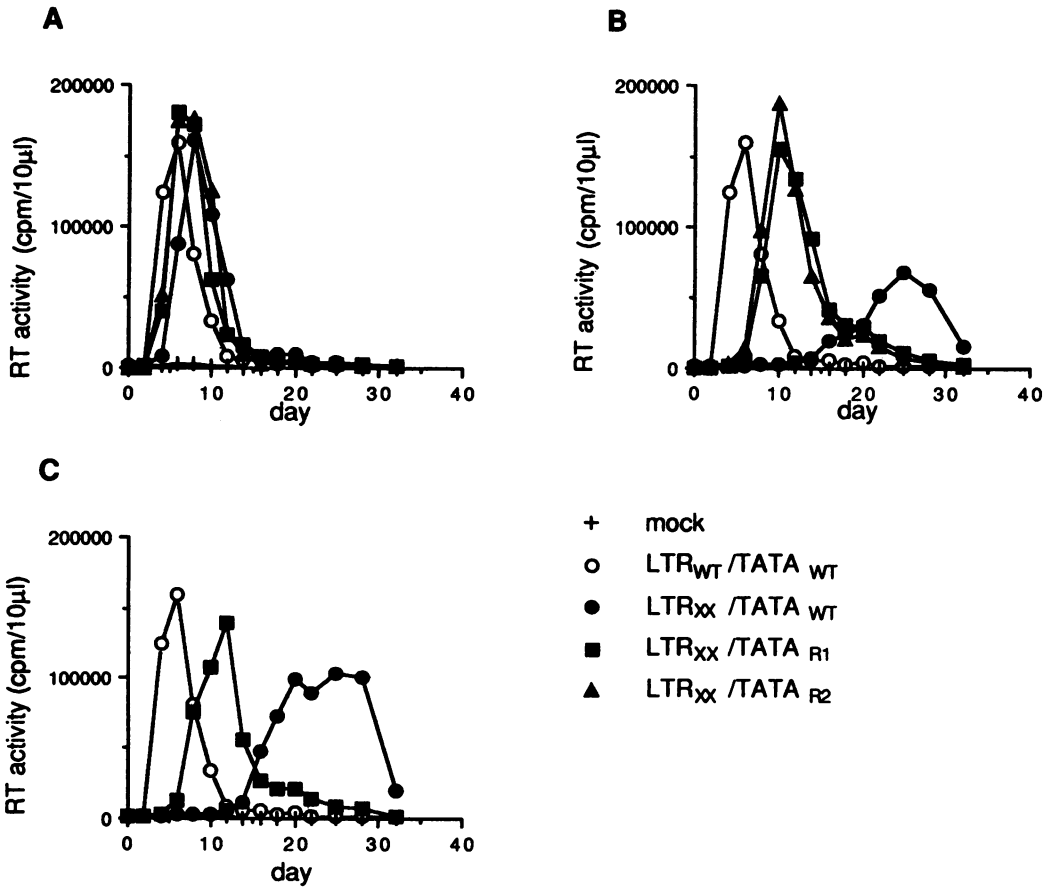

FIG. 2. Replication kinetics of $LTR_{Sp1del}/TATA_{WT}$ (A), $LTR_{N1Sp1del}/TATA_{WT}$ (B), and $LTR_{Sp1m}/TATA_{WT}$ (C) proviral DNAs in transfection-cocultivation assays. Replication kinetics of $LTR_{WT}/TATA_{WT}$ proviruses is also shown. Ten micrograms of circularly permuted proviral plasmid DNA was digested with *Bam*HI, and the released proviral DNA was concatemerized by ligation in vitro. DNAs were introduced into 1×10^7 12D7 cells by electroporation followed by cocultivation with 5×10^6 MT-4 cells. Virus production was monitored by RT assay.

Since the infection kinetics of HIV containing the more-extensive $LTR_{N1Sp1del}/TATA_{WT}$ mutation had been previously shown to be markedly delayed in all CD4-positive cells including MT-4, we elected to return to the transfection-cocultivation system and monitor the effects of the revertant TATA boxes on the replication of $LTR_{N1Sp1del}/TATA_{WT}$ proviral DNA. As is shown in Fig. 2B, the replication of the $LTR_{N1Sp1del}/TATA_{WT}$ mutant was delayed by nearly 3 weeks compared with that of $LTR_{WT}/TATA_{WT}$; in fact, no progeny virus production was detected in three of six independent experiments with this LTR mutant (see tabulated infectivity data in Fig. 1). However, the markedly impaired replication capacity of $LTR_{N1Sp1del}/TATA_{WT}$ was restored following the introduction of the revertant TATA boxes: both $LTR_{N1Sp1del}/TATA_{R1}$ and $LTR_{N1Sp1del}/TATA_{R2}$ proviruses exhibited a striking acceleration of replication kinetics compared with that of $LTR_{N1Sp1del}/TATA_{WT}$ in the transfection-cocultivation assays (Fig. 2B).

It was also important to examine the biological properties of HIVs containing a revertant TATA box ($TATA_{R1}$) in the context of Sp1 mutations in which the spatial organization of the LTR had been preserved (i.e., site-specific Sp1 mutations). This was accomplished by substituting a TT dinucleotide for the GG dinucleotide in each of the three Sp1 motifs, which eliminates Sp1 binding (20) (Fig. 1). The replicative properties of such a mutant provirus ($LTR_{Sp1m}/TATA_{WT}$) as well as proviral DNA containing the Sp1m mutation plus the revertant TATA box ($LTR_{Sp1m}/TATA_{R1}$) were evaluated by transfection and cocultivation. Two interesting results were obtained. First, in contrast to the original Sp1-deleted LTR mutant, $LTR_{Sp1del}/TATA_{WT}$, the replication of $LTR_{Sp1m}/TATA_{WT}$ was markedly delayed compared with replication of the wild-type virus in the highly sensitive transfection-cocultivation assay (contrast Fig. 2C with A). In fact, no replication of $LTR_{Sp1m}/TATA_{WT}$ was detected in one of four independent experiments (see tabulated infectivity data in Fig. 1), and in the other three experiments, progeny virus production was delayed significantly. Second, the presence of the revertant TATA box in $LTR_{Sp1m}/TATA_{R1}$ greatly accelerated the kinetics of infection (Fig. 2C). Thus, the TATA box alteration conferred augmented biological activity to a HIV provirus with mutagenized Sp1 motif in the context of an HIV LTR with a wild-type spatial organization.

Finally, we were curious whether the revertant TATA boxes might accelerate wild-type HIV infections. Accordingly, in vitro mutagenesis was used to introduce the $TATA_{R1}$ and $TATA_{R2}$ changes into pILIC DNA, and virus stocks were generated by transfection and cocultivation, as described in Materials and Methods. Six independent infections of activated human PBMCs and 12D7 cells (carried out over a 3-log-unit range of multiplicities of infection) failed to reveal

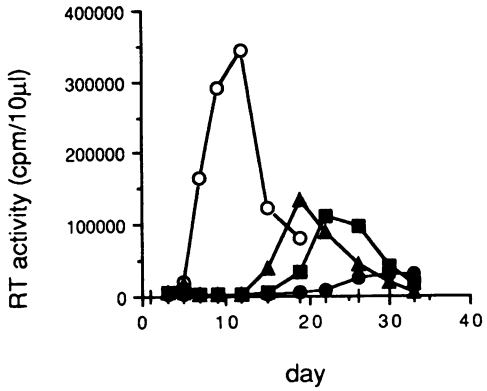

FIG. 3. Infection kinetics of the HIV_{Sp1del} family of viruses in 12D7 cells. 12D7 cells (2×10^6 cells) were inoculated with equal amounts (5×10^6 cpm of ^{32}P-labeled RT activity) of wild-type or $HIV_{Sp1del}/TATA_{WT}$, $HIV_{Sp1del}/TATA_{R1}$, or $HIV_{Sp1del}/TATA_{R2}$ produced by MT-4 and 12D7 cocultivation; progeny virion production was monitored by RT assay. Symbols: +, mock infected; ○, $LTR_{WT}/TATA_{WT}$; ●, $LTR_{Sp1del}/TATA_{WT}$; ■, $LTR_{Sp1del}/TATA_{R1}$; ▲, $LTR_{Sp1del}/TATA_{R2}$.

measurable differences between $LTR_{WT}/TATA_{WT}$, $LTR_{WT}/TATA_{R1}$, and $LTR_{WT}/TATA_{R2}$ HIVs (data not shown).

Role of TATA region changes on HIV LTR-directed expression in transient transfection assays. It seemed appropriate, from a mechanistic standpoint, to evaluate the possible functional role(s) of revertant TATA boxes on HIV LTR-directed RNA synthesis, because they had emerged during protracted infections with viruses containing mutations affecting the binding sites for transcription regulatory factors (Sp1 and NF-κB). As a first step in this process, the effects of the TATA motif changes on LTR-directed CAT expression in electroporated CEM cells were examined. As indicated from the data presented in the right-hand columns of Fig. 1, the revertant TATA boxes had little if any effect on the basal or Tat-induced expression directed by wild-type or Sp1-deleted LTRs and conferred a consistent two- to fivefold increase of basal and Tat-induced levels of CAT directed by $LTR_{N1Sp1del}$ and LTR_{Sp1m} mutant HIV promoters. Thus, similar to the virus replication and infectivity studies presented in Fig. 2 and 3, the revertant TATA box alterations appear to function only in the context of a mutagenized LTR in CAT assays where a modest increase in expression was observed.

Functional activity of revertant TATA boxes during in vitro transcription. We next decided to monitor the effect(s) of the revertant TATA boxes in an in vitro transcription system so that individual steps associated with LTR-directed RNA synthesis could be analyzed. CAT reporter plasmid DNAs containing HIV $LTR_{WT}/TATA_{WT}$, $LTR_{WT}/TATA_{R1}$, or $LTR_{WT}/TATA_{R2}$ promoters were added (50 to 150 ng per reaction mixture) to the HeLa cell in vitro transcription system described in Materials and Methods. A small amount of RNA transcript (330 bases long) was synthesized following incubation with 150 ng of HIV $LTR_{WT}/TATA_{WT}$ template DNA; no RNA was detected when smaller amounts of this construct were used (Fig. 4A, lanes 1 to 3). When the HIV $LTR_{WT}/TATA_{R1}$ or $LTR_{WT}/TATA_{R2}$ promoters were assayed in this system, considerably larger amounts of the 330-base RNA transcript was produced (Fig. 4A, lanes 4 to 9). PhosphorImaging analysis revealed that HIV $LTR_{WT}/TATA_{R2}$ directed the synthesis of 5- to 10-fold-more RNA than the HIV $LTR_{WT}/TATA_{WT}$ promoter did. In several independent experiments, the level of HIV $LTR_{WT}/TATA_{R1}$-directed transcription was two- to threefold higher than that observed with HIV $LTR_{WT}/TATA_{WT}$. Because no exogenously supplied Tat was present in these assays, the increased RNA synthesis directed by the HIV $LTR_{WT}/TATA_{R1}$ and $LTR_{WT}/TATA_{R2}$ promoters clearly reflected elevated basal levels of transcription in vitro.

To determine whether the TATA box revertants affected transcription initiation, the synthesis of promoter-proximal RNA was examined in a shorter (15-min) in vitro transcription assay that permitted, on the basis of previous time course studies, analysis of transcripts generated during the initial round of RNA synthesis. The ^{32}P-labeled RNA produced was purified, hybridized to a promoter-proximal probe consisting of the 5′-terminal 80 nucleotides of the LTR-directed transcript, and digested with S1 nuclease, and the protected RNA products were analyzed on a polyacrylamide-urea gel. As shown in Fig. 4B, approximately fivefold-more promoter-proximal transcripts were present in the reaction mixture containing the $LTR_{WT}/TATA_{R2}$ template than in that containing the $LTR_{WT}/TATA_{WT}$ promoter (lanes 2 and 4). This result is consistent with the run-off transcription assays presented in Fig. 4A and suggests that the revertant TATA box increases LTR-directed expression by stimulating transcription initiation.

Similar results were obtained using the HIV $LTR_{Sp1del}/TATA_{WT}$, $LTR_{Sp1del}/TATA_{R1}$, and $LTR_{Sp1del}/TATA_{R2}$ promoters in coupled in vitro transcription-S1 nuclease assays. Transcription driven by the HIV $LTR_{Sp1del}/TATA_{WT}$ template in vitro was lower than transcription driven by the $LTR_{WT}/TATA_{WT}$ promoter as a result of the absence of the three Sp1-binding sites. Nonetheless, the $LTR_{Sp1del}/TATA_{R1}$ and $LTR_{Sp1del}/TATA_{R2}$ promoters directed 2- to 5-fold-higher basal levels of RNA than $LTR_{Sp1del}/TATA_{WT}$ did (data not shown).

The Tat responsiveness of HIV LTRs containing revertant TATA boxes was next investigated in the in vitro transcription system by performing reactions (reaction mixtures contained 75 ng of CAT reporter plasmid DNA) in the presence or absence of 0.4 μM Tat protein (3, 30). In the presence of Tat, transcription directed by the $LTR_{WT}/TATA_{WT}$ promoter increased 13-fold (Fig. 5, lanes 1 and 2); with the $LTR_{WT}/TATA_{R1}$ promoter, a 16-fold stimulation was observed (Fig. 5, lanes 3 and 4). The effect of Tat on a promoter containing the revertant $TATA_{R2}$ box was more difficult to quantitate because of very high levels of basal transcription. In a reaction mixture similar to that used for $LTR_{WT}/TATA_{WT}$ and $LTR_{WT}/TATA_{R1}$, the $LTR_{WT}/TATA_{R2}$ promoter directed the synthesis of 20-fold-more RNA than the $LTR_{WT}/TATA_{WT}$ promoter in the absence of Tat did (compare Figure 5, lanes 1 and 5); the addition of Tat resulted in a further 2-fold elevation of expression (Fig. 5, lanes 5 and 6). Because the in vitro transcription reactions driven by the $LTR_{WT}/TATA_{R2}$ promoter were no longer in the linear range, a separate experiment that included less (35-ng) template DNA was performed; under these conditions, Tat effected a sevenfold increase in transcriptional activity (Fig. 5, lanes 7 and 8). Taken together, the results presented in Fig. 5 show that the TATA box revertants elevate basal transcription levels but do not increase responsiveness to Tat. These results are consistent with the effects of TATA box revertants in the LTR-directed CAT assays described earlier (summarized in the right-hand columns of Fig. 1).

The revertant TATA boxes accelerate the assembly of basal transcription factors TFIID and TFIIA. The binding of TFIID to the TATA element is one of the initial steps that occurs

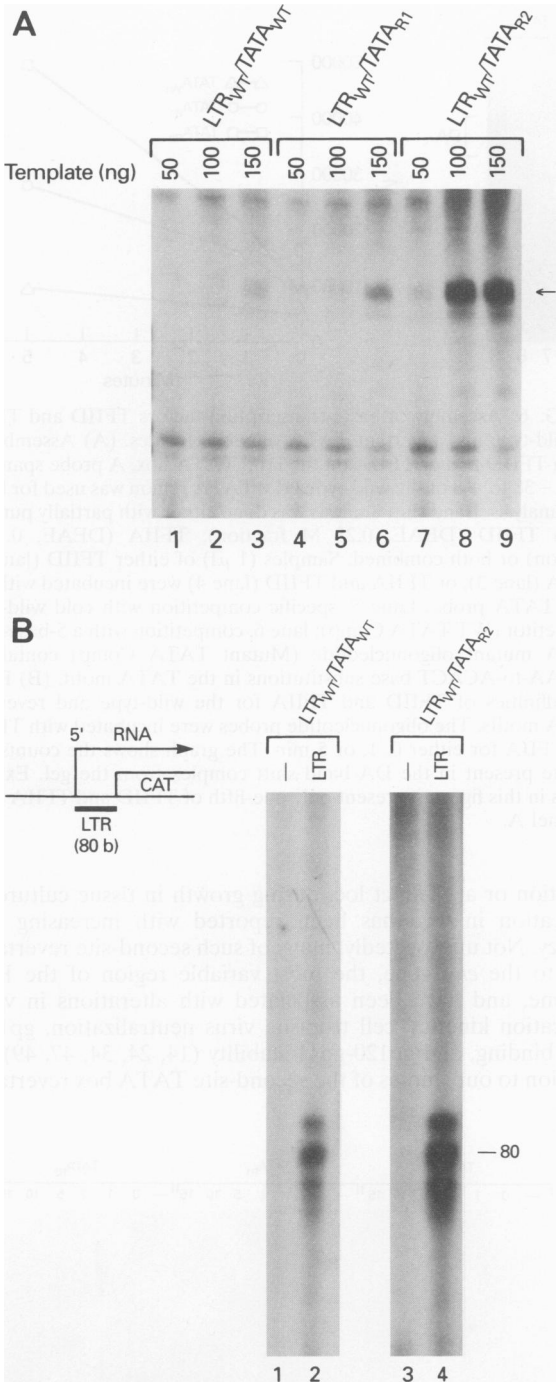

FIG. 4. In vitro transcription of HIV wild-type and TATA revertant templates. (A) Titration of input DNA templates in an in vitro transcription reaction. Hela whole-cell extract was incubated with various concentrations of DNA template, as described in Materials and Methods. After a 1-h incubation, RNA products were purified and analyzed on a 4% denaturing polyacrylamide–urea gel. LTR$_{WT}$/TATA$_{WT}$, LTR$_{WT}$/TATA$_{R1}$, and LTR$_{WT}$/TATA$_{R2}$ promoters were used. The arrow indicates 330-base HIV transcript. (B) S1 nuclease analysis of RNA transcribed from wild-type or revertant HIV promoters. In vitro transcription reactions were performed as described in Materials and Methods. [α-^{32}P]UTP-labeled RNA, synthesized from either LTR$_{WT}$/TATA$_{WT}$ or LTR$_{WT}$/TATA$_{R2}$ template, was hybridized with the cold oligonucleotide probe (+1 to +80; lanes 2 and 4) followed by S1 nuclease digestion. Protected RNA was analyzed on a 6% denaturing polyacrylamide–urea gel. b, bases. The presence (LTR) or absence (−) of the 80-b LTR probe is indicated over the lanes.

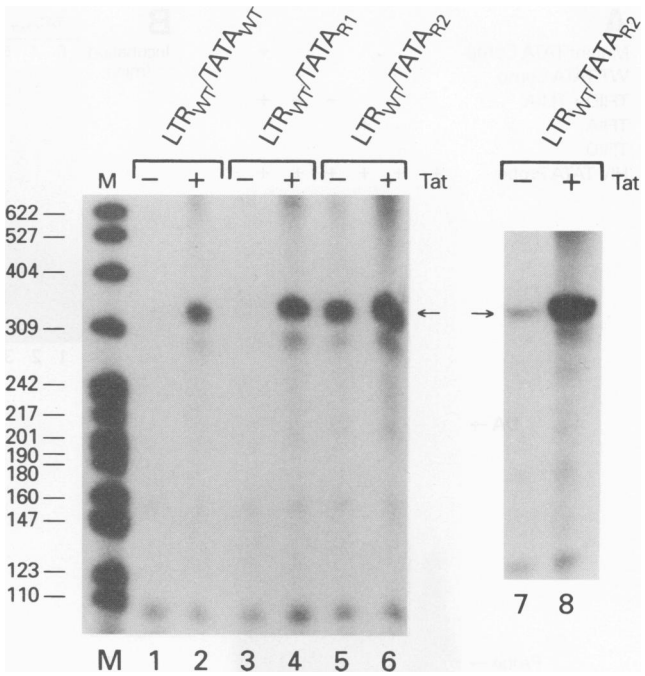

FIG. 5. In vitro Tat transactivation of HIV promoters containing wild-type and revertant TATA boxes. In vitro transcription reactions were carried out as described in Materials and Methods. Lanes 1, 3, and 5 reflect basal promoter activity (75 ng of template DNA); lanes 2, 4, and 6 contain the indicated template plus Tat (0.4 μM). The LTR$_{WT}$/TATA$_{R2}$ template (35 ng) was added to the transcription reaction mixtures presented in lanes 7 and 8. The presence (+) or absence (−) of the Tat protein is indicated over the lanes.

during the assembly of the *pol*II transcription complex. To assess whether the augmentation of RNA synthesis associated with the revertant TATA boxes might be due to increased binding of TFIID (and TFIIA) to the promoter, band shift analyses were initially performed with an oligonucleotide from the wild-type HIV LTR which contained sequences mapping −8 to −38 nucleotides upstream of the transcription initiation site. As shown in Fig. 6A (lanes 2 and 3), the TATA$_{WT}$ oligonucleotide did not form a band shift complex following incubation with either TFIID or TFIIA alone. In contrast, a single, prominent, slowly migrating complex (designated DA) was observed when the TATA$_{WT}$ oligonucleotide was incubated with both TFIID and TFIIA (Fig. 6A, lane 4). As expected, when unlabeled TATA$_{WT}$ oligonucleotide was included in the reaction mixture, the DA band shift complex failed to form (Fig. 6A, lane 5), whereas a mutated oligonucleotide, unable to bind TFIID, did not prevent the formation of DA (Fig. 6A, lane 6).

The affinities of TFIID plus TFIIA for the revertant TATA boxes, TATA$_{R1}$ and TATA$_{R2}$, were compared with that for the TATA$_{WT}$ oligonucleotide in band shift assays similar to that shown in Fig. 6A, except that the TFIID and TFIIA concentrations were reduced fivefold to limit the formation of complexes with the TATA$_{WT}$ oligonucleotide. As shown in Fig. 6B, three- and sixfold increases in TFIID-TFIIA binding to the labeled TATA$_{R1}$ and TATA$_{R2}$ oligonucleotides, respectively,

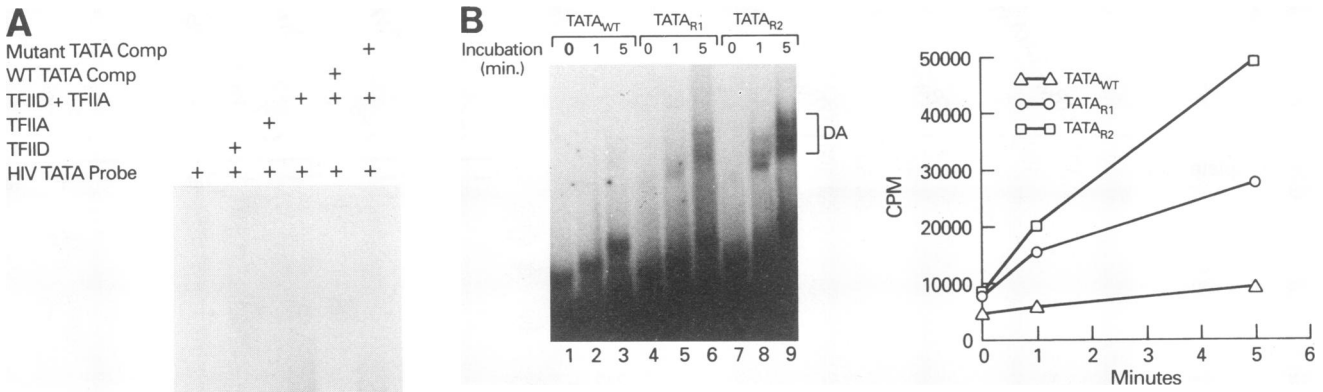

FIG. 6. Assembly of basal transcription factors TFIID and TFIIA on wild-type and revertant TATA oligonucleotides. (A) Assembly of HeLa TFIID and/or TFIIA on the HIV TATA box. A probe spanning from −38 to −8 of the wild-type HIV TATA region was used for band shift analysis. Band shift analysis was done either with partially purified HeLa TFIID (DEAE, 0.25 M fraction), TFIIA (DEAE, 0.1 M fraction) or both combined. Samples (1 μl) of either TFIID (lane 2), TFIIA (lane 3), or TFIIA and TFIID (lane 4) were incubated with the HIV TATA probe. Lane 5, specific competition with cold wild-type competitor (WT TATA Comp); lane 6, competition with a 5-base-long TATA mutant oligonucleotide (Mutant TATA Comp) containing TATAA-to-ACACT base substitutions in the TATA motif. (B) Relative affinities of TFIID and TFIIA for the wild-type and revertant TATA motifs. The oligonucleotide probes were incubated with TFIID and TFIIA for either 0, 1, or 5 min. The graph shows the counts per minute present in the DA band shift complex from the gel. Experiments in this figure represent only one-fifth of TFIID and TFIIA used in panel A.

were observed compared with that of $TATA_{WT}$. Because only limiting amounts of TFIID and TFIIA were used in this experiment, only 1 to 2% of the labeled probe formed a complex following 5 min of incubation. Similar results were obtained in several independent experiments (data not shown).

TFIIA and TFIID form a more-stable complex with the revertant TATA boxes than with the wild-type TATA motif. The relative stabilities of the TFIID-TFIIA complex bound to wild-type and revertant TATA boxes was evaluated in a two-step experiment in which purified TFIIA and TFIID were first incubated with the three different TATA-containing oligonucleotides for 10 min to allow the DA complex to form. A 50-fold excess of unlabeled $TATA_{WT}$ was then added to each reaction mixture, and aliquots were removed at various times for analysis on a neutral polyacrylamide gel. More than 90% of the DA complex could be displaced from the $TATA_{WT}$ template in less than 1 min (Fig. 7, lanes 2 to 7), whereas greater stability was observed when TFIIA and TFIID were initially bound to either $TATA_{R1}$ or $TATA_{R2}$ (Fig. 7, lanes 9 to 21). PhosphorImaging analysis indicated that approximately 49% of the initial $TATA_{R2}$-DA complex was still present following 15 min of incubation with the unlabeled $TATA_{WT}$ oligonucleotide. In this experiment, we estimate that the half-life of the DA complex bound to the $TATA_{WT}$ template was approximately 20 s, whereas it was considerably longer (>5 min) when a promoter-containing $TATA_{R2}$ element was included in the reaction mixture.

DISCUSSION

The emergence of HIV-1 revertants bearing nucleotide changes at a site(s) different from the position of an initial mutation or at distinct loci during growth in tissue culture or replication in vivo has been reported with increasing frequency. Not unexpectedly, many of such second-site revertants map to the *env* gene, the most variable region of the HIV genome, and have been associated with alterations in virus replication kinetics, cell tropism, virus neutralization, gp120-CD4 binding, and gp120-gp41 stability (14, 24, 34, 47, 49). In addition to our studies of the second-site TATA box revertants

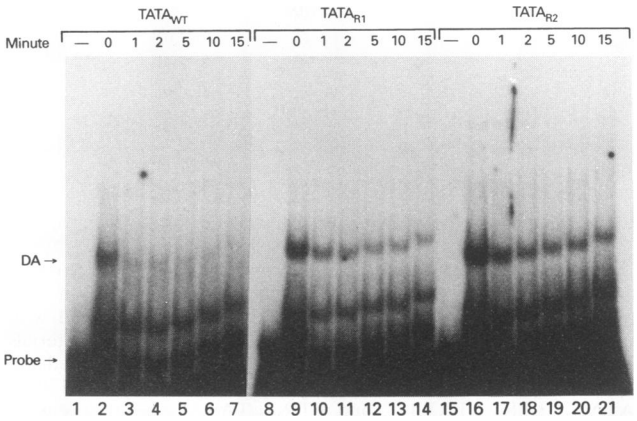

FIG. 7. Stability of TFIID and TFIIA complex on the wild-type and revertant TATA sequences. The wild-type or revertant TATA probes were incubated with TFIID and TFIIA for 10 min after which a 50-fold excess unlabeled wild-type TATA oligonucleotide ($TATA_{WT}$) was added to the incubation mixture. At various time points, an aliquot of the reaction mixture was removed and loaded on a running neutral gel. Quantitations (in counts per minute) were done with a PhosphorImager.

(41), nucleotide changes involving other *cis*-acting HIV regulatory sequences, which appeared following the tissue culture propagation of viral mutants with deletions of portions of the tRNA primer-binding site (pbs), have been reported (39). In the latter work, fully infectious virus was regenerated from an initial mutant provirus in which the 3′-terminal 12 nucleotides of the 18-nucleotide pbs had been deleted. Despite the restoration of a complete pbs, a new deletion located adjacent to and immediately 3′ to the pbs (in the Gag leader sequence) was invariably present.

The mechanism responsible for reverting a disabling mutation affecting virus replicative capacity or conferring a novel biological phenotype is most likely the obligate reverse transcription reaction that occurs during infections induced by replication-competent retroviruses. The lack of proofreading activity to correct errors in RNA-dependent DNA polymerization generates viral genomes containing a multitude of point and deletion mutations, many of which render the resultant viruses noninfectious. Given sufficient selective pressure (e.g., rapid growth in tissue culture, antiviral therapy, or immunological surveillance in vivo) and an intrinsically error-prone process that generates genetically heterogeneous genomic intermediates, retroviruses such as HIV continuously produce progeny with enormous adaptive potential. In those cases where second-site revertants emerge following the introduction of the initial disabling point or deletion mutation, virus infectivity was clearly never lost. Low but undetectable levels of virus must have been continually produced, including revertant progeny that eventually outgrew other members of the virus quasi-species, and eventually dominated the pool of released virus particles. In the mutants under study here, the point when progeny virions again became detectable could reflect the transition in HIV LTR-directed RNA synthesis when the extremely low basal levels of viral RNA directed by an Sp1-deleted LTR were suddenly boosted as a consequence of the acquisition of a revertant TATA box. The increased level of HIV RNA produced might exceed a hypothetical threshold and generate levels of Tat protein required to sustain a productive virus infection and release sufficient amounts of virion-associated RT activity to be detected in our assays. No augmentation of virus infectivity or CAT expression was observed when the TATA revertant motifs were inserted into an otherwise wild-type LTR. This latter finding suggests that when basal levels of HIV transcription are sufficiently high, the further increase due to the presence of the revertant TATA boxes is neither needed nor measurable. Thus, in the context of HIV mutants containing known biochemical defects or virus isolates with defined biological phenotypes, second-site revertants become immensely useful tools for elucidating structure-function relationships. In the context of the present study, the observed emergence of second-site revertants in viral mutants may be an example of the mechanism by which HIV might evade therapies aimed at disrupting enhancer function.

It is interesting to note that the HIV variant in which all three Sp1 sites were mutated displayed markedly slower kinetics of replication than a virus in which the Sp1 sites were deleted. This is of interest not only as it pertains to the ability of NF-κB to function at a distance from the TATA box, but also in light of the reported interaction between NF-κB and Sp1 (37). In the case of the Sp1 deletion mutant, the NF-κB site is positioned 34 bp closer to the TATA sequence. We suggest that the role of the TATA revertant changes may be to compensate for the lost NF-κB or Sp1 effect by facilitating an interaction between NF-κB and the basal transcription factors. In the Sp1 point mutant, the effect of the TATA revertant may be less efficient as a result of the increased distance between NF-κB and the basal transcription factors.

This analysis of the mutant Sp1 HIVs and their LTRs suggests that the revertant TATA boxes serve primarily to increase basal levels of transcription; no increased responsiveness to Tat could be demonstrated in transfected tissue culture or in vitro systems. The augmented promoter activity observed was dependent not only on the structure of LTR in which the revertant TATA box resided but also on the type of assay performed. For example, the revertant TATA motifs accelerated HIV infection kinetics only when present within an LTR containing a Sp1 mutation. No effect was observed when the TATA box reversion was associated with a wild-type LTR. Similar results were obtained in CAT assays. In contrast, less-stringent constraints were operative in in vitro systems. There, the stimulatory effects of the revertant TATA boxes on basal transcription were readily observed even in the context of the wild-type LTR. Previous investigations of the HIV TATA motif have suggested that the substitution of other eukaryotic TATA boxes or non-TATA initiator sequences minimally affected basal transcription but, in most cases, markedly reduced responsiveness to Tat (2, 27, 35). Mutations corresponding to the $TATA_{R1}$ and $TATA_{R2}$ motifs were not included in the promoters tested. It would appear therefore, that a distinct class of TATA revertants has been selected for in which basal but not Tat-transactivated transcription has been affected. These results also imply that HIV basal transcription must play a critical role in virus replication at some point in the infection cycle.

One interpretation of our findings, which ties together the results of virus infections, CAT assays, and the in vitro transcription experiments, is that the original Sp1 mutation so severely compromised basal activity of the HIV promoter that no detectable progeny could be detected. The revertant TATA boxes emerged as a result of long-term selective pressure (for enhanced growth in infected tissue cultures), because they provided a more-optimal template for assemblying *pol*II transcription complexes and directing larger amounts of viral RNA. This could reflect the increased binding of TFIID or, alternatively, the selective binding of a unique species of TFIID containing a specific set of TAFs (4, 5, 7, 10, 44, 52) to the revertant TATA motifs. Such a hypothesis suggests that the intrinsic interaction of TFIID with the HIV basal promoter is weak or generates full-length viral transcripts very inefficiently. In this regard, we have recently demonstrated that Tat binds to TFIID, facilitating binding of the basal transcription factor to the promoter, thereby increasing HIV transcription (21). Thus, increased levels of transcription can be obtained either by altering the primary sequence with which TFIID interacts, as has occurred in the revertants, or by modifying the interaction of TFIID through protein-protein interactions, as in Tat transactivation. The revertants analyzed in this study do not selectively assemble a more-processive initiation/elongation complex (data not shown). Experiments to analyze the interaction of and the TAFs associated with the TFIID that binds to the wild type, TATA revertant, and Tat-transactivated HIV promoter are presently in progress. Such studies should help to define the complex mechanisms of HIV transcription regulation.

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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. **Berkhout, B., and K.-T. Jeang.** 1992. Functional roles for the TATA promoter and enhancers in basal and Tat-induced expression of the human immunodeficiency virus type 1 long terminal repeat. J. Virol. **66:**139–149.
3. **Bohan, C. A., F. Kashanchi, B. Ensoli, L. Buonaguro, K. Boris-Lawrie, and J. N. Brady.** 1992. Analysis of Tat transactivation of human immunodeficiency virus transcription in vitro. Gene Express. **2:**391–407.
4. **Brou, C., S. Chaudhary, I. Davidson, Y. Lutz, J. Wu, J.-M. Egly, L. Tora, and P. Chambon.** 1993. Distinct TFIID complexes mediate the effect of different transcriptional activators. EMBO J. **12:**489–499.
5. **Brou, C., J. Wu, S. Ali, E. Scheer, C. Lang, I. Davidson, P. Chambon, and L. Tora.** 1993. Different TBP-associated factors are required for mediating the stimulation of transcription *in vitro* by the acidic transactivator GAS-VP16 and the two nonacidic activation functions of the estrogen receptor. Nucleic Acids Res. **21:**5–12.
6. **Bucher, P., and E. N. Trifonov.** 1986. Compilation and analysis of eukaryotic POL II promoter sequences. Nucleic Acids Res. **14:** 10009–10026.
7. **Chiang, C. M., H. Ge, Z. Wang, A. Hoffman, and R. G. Roeder.** 1993. Unique TATA-binding protein-containing complexes and cofactors involved in transcription by RNA polymerases II and III. EMBO J. **12:**2749–2762.
8. **Cooney, A. J., S. Y. Tsai, B. W. O'Malley, and M. J. Tsai.** 1991. Chicken ovalbumin upstream promoter transcription factor binds to a negative regulatory region in the human immunodeficiency virus type 1 long terminal repeat. J. Virol. **65:**2853–2860.
9. **Dinter, H., R. Chiu, M. Imagawa, M. Karin, and K. A. Jones.** 1987. In vitro activation of the HIV-1 enhancer in extracts from cells treated with a phorbol ester tumor promoter. EMBO J. **6:**4067–4071.
10. **Dynlacht, B. D., T. Hoey, and R. Tjian.** 1991. Isolation of coactivators associated with the TATA-binding protein that mediate transcriptional activation. Cell **66:**563–576.
11. **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.
12. **Franza, B. R., Jr., S. F. Josephs, M. Z. Gilman, W. Ryan, and B. Clarkson.** 1987. Characterization of cellular proteins recognizing the HIV enhancer using a microscale DNA-affinity precipitation assay. Nature (London) **330:**391–395.
13. **Franza, B. R., Jr., F. J. Rauscher III, S. F. Josephs, and T. Curran.** 1988. The Fos complex and Fos-related antigens recognize sequence elements that contain AP-1 binding sites. Science **239:** 1150–1153.
14. **Fujita, K., J. Silver, and K. Peden.** 1992. Changes in both gp120 and gp41 can account for increased growth potential and expanded host range of human immunodeficiency virus type 1. J. Virol. **66:**4445–4451.
15. **Garcia, J. A., D. Harrich, E. Soultanaki, 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.
16. **Garcia, J. A., F. K. Wu, R. Mitsuyasu, and R. B. Gaynor.** 1987. Interactions of cellular proteins involved in the transcriptional regulation of the human immunodeficiency virus. EMBO J. **6:**3761–3770.
17. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
18. **Harada, S., Y. Koyanagi, and N. Yamamoto.** 1985. Infection of HTLV-III/LAV in HTLV-I-carrying cells MT-2 and MT-4 and application in a plaque assay. Science **229:**563–566.
19. **Harrich, D., J. Garcia, F. Wu, R. Mitsuyasu, J. Gonzalez, and R. 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.
20. **Jones, K. A., J. T. Kadonaga, P. A. Luciw, and R. Tjian.** 1986. Activation of the AIDS retrovirus promoter by the cellular transcription factor, SP1. Science **232:**755–759.
21. **Kashanchi, F., G. Piras, M. F. Radonovich, J. F. Duvall, A. Fattaey, and J. N. Brady.** 1994. Direct interaction of human TFIID with the HIV-1 transactivator Tat. Nature (London) **367:**295–299.
22. **Kaufman, J. D., G. Valandra, G. Roderiquez, G. Bushar, C. Giri, and M. A. Norcross.** 1987. Phorbol ester enhances human immunodeficiency virus-promoted gene expression and acts on a repeated 10-base-pair functional enhancer element. Mol. Cell. Biol. **7:**3759–3766.
23. **Kawakami, K., C. Scheidereit, 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.
24. **Keller, R., K. Peden, S. Paulous, L. Montagnier, and A. Cordonnier.** 1993. Amino acid changes in the fourth conserved region of human immunodeficiency virus type 2 strain HIV-2_{ROD} envelope glycoprotein modulate fusion. J. Virol. **67:**6253–6258.
25. **Kim, J. Y., F. Gonzalez-Scarano, S. L. Zeichner, and J. C. Alwine.** 1993. Replication of type 1 human immunodeficiency viruses containing linker substitution mutations in the −201 to −130 region of the long terminal repeat. J. Virol. **67:**1658–1662.
26. **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 sites in the human immunodeficiency virus type 1 long terminal repeat are not required for virus infectivity. J. Virol. **63:**4919–4924.
27. **Lu, X., T. M. Welsh, and B. M. Peterlin.** 1993. The human immunodeficiency virus type 1 long terminal repeat specifies two different transcription complexes, only one of which is regulated by Tat. J. Virol. **67:**1752–1760.
28. **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.
29. **Manley, J. L., A. Fire, A. Cano, P. A. Sharp, and M. L. Gefter.** 1980. DNA-dependent transcription of adenovirus genes in a soluble whole-cell extract. Proc. Natl. Acad. Sci. USA **77:**3855–3859.
30. **Marciniak, R. A., B. J. Calnan, A. D. Frankel, and P. A. Sharp.** 1990. HIV-1 Tat protein trans-activates transcription *in vitro*. Cell **63:**791–802.
31. **Meisterernst, M., A. L. Roy, H. M. Lieu, and R. G. Roeder.** 1991. Activation of class II gene transcription by regulatory factors is potentiated by a novel activity. Cell **66:**981–993.
32. **Meyers, G., J. A. Berzofsky, B. Korber, R. F. Smith, and G. N. Pavlakis.** 1992. Human retroviruses and AIDS. A compilation and analysis of nucleic acid and amino acid sequences. Theoretical Biology and Biophysics Group T-10, Los Alamos National Laboratory, Los Alamos, N.Mex.
33. **Nabel, G., and D. Baltimore.** 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature (London) **326:**711–713.
34. **Nara, P. L., L. Smit, N. Dunlop, W. Hatch, M. Merges, D. Waters, J. Kelliher, R. C. Gallo, P. J. Fischinger, and J. Goudsmit.** 1990. Emergence of viruses resistant to neutralization by V3-specific antibodies in experimental human immunodeficiency virus type 1 IIIB infection of chimpanzees. J. Virol. **64:**3779–3791.
35. **Olsen, H. S., and C. A. Rosen.** 1992. Contribution of the TATA motif to Tat-mediated transcriptional activation of human immunodeficiency virus gene expression. J. Virol. **66:**5594–5597.
36. **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.
37. **Perkins, N. D., N. L. Edwards, C. S. Duckett, A. B. Agranoff, R. M. Schmid, and G. J. Nabel.** 1993. A cooperative interaction between

NF-κB and Sp1 is required for HIV-1 enhancer activation. EMBO J. **12**:3551–3558.

38. **Popovic, M., M. G. Sarngadharan, 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.
39. **Rhim, H., J. Park, and C. D. Morrow.** 1991. Deletions in the $tRNA^{Lys}$ primer-binding site of human immunodeficiency virus type 1 identify essential regions for reverse transcription. J. Virol. **65**:4555–4564.
40. **Rosen, C. A., J. G. Sodroski, and W. A. Haseltine.** 1985. The location of cis-acting regulatory sequences in the human T cell lymphotropic virus type III (HTLV-III/LAV) long terminal repeat. Cell **41**:813–823.
41. **Ross, E. K., A. J. Buckler-White, A. B. Rabson, G. Englund, and M. A. Martin.** 1991. 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. J. Virol. **65**:4350–4358.
42. **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.
43. **Siekevitz, M., S. F. Josephs, M. Dukovich, N. Peffer, F. Wong-Staal, and W. C. Greene.** 1987. Activation of the HIV-1 LTR by T cell mitogens and the trans-activator protein of HTLV-I. Science **238**:1575–1578.
44. **Tanese, N., B. F. Pugh, and R. Tjian.** 1991. Coactivators for a proline-rich activator purified from the multisubunit human TFIID complex. Genes Dev. **5**:2212–2224.
45. **Tong-Starksen, S. E., P. A. Luciw, and B. M. Peterlin.** 1987. Human immunodeficiency virus long terminal repeat responds to T-cell activation signals. Proc. Natl. Acad. Sci. USA **84**:6845–6849.
46. **Weiss, A., 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.
47. **Willey, R. L., and M. A. Martin.** 1993. Association of human immunodeficiency virus type 1 envelope glycoprotein with particles depends on interactions between the third variable and conserved regions of gp120. J. Virol. **67**:3639–3643.
48. **Willey, R. L., E. K. Ross, A. J. Buckler-White, T. S. Theodore, and M. A. Martin.** 1989. Functional interaction of constant and variable domains of human immunodeficiency virus type 1 gp120. J. Virol. **63**:3595–3600.
49. **Willey, R. L., 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.
50. **Wright, C. M., B. K. Felber, H. Pavlakis, and G. N. Pavlakis.** 1986. Expression and characterization of the transactivator of HTLVIII/LAV virus. Science **234**:988–992.
51. **Wu, F. K., J. A. Garcia, D. Harrich, and R. B. Gaynor.** 1988. Purification of the human immunodeficiency virus type 1 enhancer and TAR binding proteins EBP-1 and UBP-1. EMBO J. **7**:2117–2130.
52. **Zhou, Q., P. M. Liberman, T. G. Boyer, and A. J. Berk.** 1992. Holo-TFIID supports transcriptional stimulation by diverse activators and from a TATA-less promoter. Genes Dev. **6**:1964–1974.