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Evolution of the Human Immunodeficiency Virus Type 1 Long Terminal Repeat Promoter by Conversion of an NF-κB Enhancer Element into a GABP Binding Site

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Human immunodeficiency virus type 1 (HIV-1) transcription is regulated by the viral Tat protein and cellular factors, of which the concentration and activity may depend on the cell type. Viral long terminal repeat (LTR) promoter sequences are therefore optimized to suit the specific nuclear environment of the target host cell. In long-term cultures of a Tat-defective, poorly replicating HIV-1 mutant, we selected for a faster-replicating virus with a 1-nucleotide deletion in the upstream copy of two highly conserved NF-κB binding sites. The variant enhancer sequence demonstrated a severe loss of NF-κB binding in protein binding assays. Interestingly, we observed a new binding activity that is specific for the variant NF-κB sequence and is present in the nuclear extract of unstimulated cells that lack NF-κB. These results suggest that inactivation of the NF-κB site coincides with binding of another transcription factor. Fine mapping of the sequence requirements for binding of this factor revealed a core sequence similar to that of Ets binding sites, and supershift assays with antibodies demonstrated the involvement of the GABP transcription factor. Transient transfection experiments with LTR-chloramphenicol acetyltransferase constructs indicated that the variant LTR promoter is specifically inhibited by GABP in the absence of Tat, but this promoter was dramatically more responsive to Tat than the wild-type LTR. Introduction of this GABP site into the LAI virus yielded a specific gain of fitness in SupT1 cells, which contain little NF-κB protein. These results suggest that GABP potentiates Tat-mediated activation of LTR transcription and viral replication in some cell types. Conversion of an NF-κB into a GABP binding site is likely to have occurred also during the worldwide spread of HIV-1, as we noticed the same LTR modification in subtype E isolates from Thailand. This typical LTR promoter configuration may provide these viruses with unique biological properties.

Human immunodeficiency virus type 1 (HIV-1) transcription is directed by the promoter located in the 5′ long terminal repeat (LTR) of the integrated provirus. Transcription is controlled both by cellular factors that bind to enhancer elements in the U3 region of the LTR and by the virally encoded Tat protein (reviewed in reference 29). The Tat protein transactivates the HIV-1 promoter several hundred-fold and is essential for virus replication. Tat binds an RNA hairpin, termed TAR, that is present at the 5′ end of all viral transcripts (8, 15). This unique Tat protein-TAR RNA complex stimulates transcription by recruiting a cyclin–cyclin-dependent kinase complex to the promoter that phosphorylates the C-terminal domain of RNA polymerase II (25, 27, 29, 60).

The U3 enhancer region of the LTR promoter contains binding sites for the Sp1 and NF-κB transcription factors. Both Sp1 and NF-κB are constitutively expressed, but the latter factor is present as an inactive complex with IκB protein in the cytoplasm of unstimulated cells. Dissociation of this complex and migration of active NF-κB into the nucleus can be induced by a large number of extracellular stimuli (52, 56). Such stimuli include infection by some viruses, phorbol esters, and multiple cytokines. NF-κB is a heterodimer composed of two proteins of the Rel/κB family of transcription factors, p50 and RelA (52). NF-κB recognizes a 10-bp stretch of DNA with the consensus sequence 5′-GGGPuNNPyPyCC-3′ (52). Both the p50 and RelA subunits are required for DNA binding, but the transactivation domain is provided by RelA (33).

The two tandem NF-κB binding sites in the HIV-1 LTR are highly conserved among different viral isolates, suggesting an essential role in virus replication. Consistent with this idea, mutation of either NF-κB site resulted in a dramatic loss of LTR promoter activity in transient transfection studies with LTR-CAT reporter constructs (7, 42). Mutations of the NF-κB binding sites in infectious HIV-1 clones yielded conflicting results regarding their contribution to virus replication. Initial studies indicated that the NF-κB enhancer elements are dispensable for virus growth (36, 45), but more recent analyses demonstrated the importance of these elements for optimal HIV-1 replication (1, 11). Interestingly, a direct correlation was observed between the severity of the replication defect of NF-κB site-mutated viruses and the NF-κB protein level of the cell type used for infection (11, 36).

As part of an analysis of Tat protein structure and function, we constructed a set of HIV-1 molecular clones with a mutant Tat protein. Several replication-impaired HIV-1 mutants with a defective Tat function were described previously (58). To select for revertant viruses with improved replication capacity, we maintained the transfected cell cultures for a prolonged period. The *tat* gene of several, but not all, revertant viruses demonstrated first- or second-site amino acid changes that explain the reversion event (unpublished data). The LTR pro-

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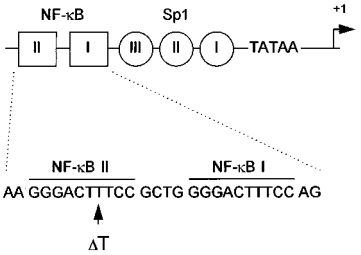

FIG. 1. Scheme of the HIV-1 LTR promoter-enhancer. The NF-κB and Sp1 binding sites are indicated by boxes and circles, respectively. The TATAA motif and the start site of transcription (+1) are indicated. The nucleotide sequences of the two NF-κB enhancers are shown. In a revertant of a Tat-mutated virus, a single T deletion (ΔT) was observed in the upstream NF-κB site II.

moter region was also analyzed for mutations that may improve transcription of a Tat-defective virus through modulation of the promoter-enhancer elements. In this study, we describe one revertant virus with a single-nucleotide deletion in the upstream NF-κB enhancer (Fig. 1) that is predicted to abolish NF-κB binding (33, 52). Because a virus with a loss of enhancer function is unlikely to repair a Tat defect, it was anticipated that a binding site for another transcription factor could have been generated. Indeed, such a gain of function was apparent in protein binding studies with the variant NF-κB site. It is demonstrated that the GABP protein of the Ets family of transcription factors binds to the variant LTR sequence. This tissue culture evolution experiment not only underscores the enormous genetic flexibility of HIV-1 but also indicates that virus variants with modified LTR promoter-enhancer motifs that may respond differently to cellular activation signals can evolve. Interestingly, we observed the same enhancer switch in HIV-1 isolates that belong to subtype E.

MATERIALS AND METHODS

Cloning and sequencing of proviral 3′ LTR segments. Chromosomal DNA from HIV-infected cells was isolated as described elsewhere (12). Proviral 3′ LTR sequences were amplified in a standard 35-cycle PCR using the 5′ primer Nefseq1 (5′ ATTCGCCACATACCTAGAAG 3′, proviral positions 8789 to 8808) and the 3′ U5 primer C(N1) (5′ GGTCTGAGGGATCTCTAGTTACC AGAGTC 3′, proviral positions 9737 to 9709). The 948-bp PCR product was gel purified and digested with *Xho*I (position 8944) and *Hin*dIII (position 9663), and the resulting 719-bp fragment was cloned into pBlue 3′ LTR-CAT (30) to replace the wild-type LTR sequences. These constructs were used as LTR-chloramphenical acetyltransferase (CAT) reporter plasmids in transient transfection assays. Clones were sequenced by using ET Dyeprimer technology (Amersham) on an Applied Biosystems 373 DNA sequencer.

Transient transfections and CAT assays. The T-cell lines SupT1 and Jurkat were cultured in RPMI medium supplemented with 10% fetal calf serum and penicillin (100 U/ml)-streptomycin (100 μg/ml). Transient transfection of these cells was carried out by means of electroporation as described previously (31). The cell lines were transfected with the indicated amounts of LTR-CAT reporter plasmid to determine basal promoter activity. The activated promoter activity was measured in transfections with 1 μg of LTR-CAT and 2.5 μg of pcDNA3-Tat (58), unless indicated otherwise. GABPα and -β1 expression plasmids (44) were used at 1.8 μg per transfection. In some experiments, cells were treated with phorbol myristate acetate (PMA; 25 ng/ml)-phytohemagglutinin (PHA; 1 μg/ml) or tumor necrosis factor alpha (TNF-α; 30 ng/ml) at 24 h posttransfection. Cell lysates for CAT assays were prepared at 3 days posttransfection as described (58) and CAT assays were performed in the linear range of the assay by the phase extraction method (49). The Tat response was calculated as the ratio of activated over basal LTR activity, with a correction for the different amounts of LTR-CAT plasmid used in the transfections with and without Tat. This correction is valid, because the basal transcription level depends on the quantity of LTR-CAT plasmid in a linear manner up to 40 μg in this transfection system (results not shown).

EMSA. Nuclear extracts from HeLa and SupT1 cells were prepared essentially as described previously (14). For some extracts, the cells were treated prior to extraction with TNF-α (30 ng/ml) for 15 min at 37°C to induce NF-κB binding activity in the nucleus. The oligonucleotides used in the electrophoretic mobility shift assays (EMSAs) are listed in Table 1. We hybridized 250 pmol of sense oligonucleotide to 250 pmol of the corresponding antisense oligonucleotide in 100 μl of buffer consisting of 10 mM Tris (pH 7.9), 50 mM NaCl, 0.5 mM EDTA, and 10% glycerol. The samples were heated at 90°C for 4 min and slowly cooled to room temperature. End labeling was performed for 30 min at 37°C with 2.5 pmol of double-stranded DNA, [γ-^{32}P]ATP, and T4 polynucleotide kinase according to the protocol of the manufacturer (Boehringer Mannheim GmbH, Mannheim, Germany). The reaction was stopped with 1 μl of 0.5 M EDTA, and free label was removed on a Sephadex G50 column. EMSAs were performed with 5 μg of HeLa or 2 μg of SupT1 nuclear extract–0.5 μg of poly(dI-dC)–10 mg of bovine serum albumin in a 20-μl reaction mixture containing 10 mM Tris (pH 7.9), 50 mM NaCl, 0.5 mM EDTA, 10% glycerol, and 1 mM dithiothreitol. The labeled probe (30,000 to 50,000 cpm) was added to this mixture, and the sample was incubated for 25 min at room temperature. In competition assays, we added 1 pmol of unlabeled double-stranded oligonucleotide to the reaction before addition of the labeled probe (approximately 20 fmol). In supershift EMSAs, we preincubated the EMSA sample for 5 min, after which 1 μl of antiserum was added and incubation was continued for an additional 20 min at room temper-

TABLE 1. EMSA oligonucleotides

Name	Sequence (5′ to 3′)	
	Sense	Antisense
wt	AAGGGACTTTCCGC	GCGGAAAGTCCCTT
mut	AAGGGACTTCCGC	GCGGAAGTCCCTT
wt-wt	AAGGGACTTTCCGCTGGGGACTTTCCAG	CTGGAAAGTCCCCAGCGGAAAGTCCCTT
mut-wt	AAGGGACTTCCGCTGGGGACTTTCCAG	CTGGAAAGTCCCCAGCGGAAGTCCCTT
d3-11	AAGGGACTTCCGCTGG	CCAGCGGAAGTCCCTT
d3-8	AAGGGACTTCCGCTGGGGA	TCCCCAGCGGAAGTCCCTT
d3-5	AAGGGACTTCCGCTGGGGACTT	AAGTCCCCAGCGGAAGTCCCTT
d3-2	AAGGGACTTCCGCTGGGGACTTTCC	GGAAAGTCCCCAGCGGAAGTCCCTT
d5-3	GGACTTCCGCTGGGGACTTTCCAG	CTGGAAAGTCCCCAGCGGAAGTCC
d5-6	CTTCCGCTGGGGACTTTCCAG	CTGGAAAGTCCCCAGCGGAAG
d5-9	CCGCTGGGGACTTTCCAG	CTGGAAAGTCCCCAGCGG
MD-wt	GGACTTTCCGCTGGGGA	TCCCCAGCGGAAAGTCC
MD-mut	GGACTTCCGCTGGGGA	TCCCCAGCGGAAGTCC
MD-A	AAACTTCCGCTGGGGA	TCCCCAGCGGAAGTTT
MD-B	GGGTTTCCGCTGGGGA	TCCCCAGCGGAAACCC
MD-C	GGACCCCCGCTGGGGA	TCCCCAGCGGGGGTCC
MD-D	GGACTTTTGCTGGGGA	TCCCCAGCAAAAGTCC
MD-E	GGACTTCCATTGGGGA	TCCCCAATGGAAGTCC
MD-F	GGACTTCCGCCAGGGA	TCCCTGGCGGAAGTCC
MD-G	GGACTTCCGCTGAAGA	TCTTCAGCGGAAGTCC
MD-H	GGACTTCCGCTGGGAG	CTCCCAGCGGAAGTCC

ature. The complexes were resolved on nondenaturing 4% polyacrylamide gels in 0.25× Tris-borate-EDTA at 200 V for 2 h. Gels were dried and exposed to X-ray film at −70°C. Quantitation of EMSA signals was performed with a Molecular Dynamics PhosphorImager and ImageQuant software.

Protein and antibody reagents. Purified recombinant NF-κB p50 and p52 (p49) were obtained from Promega, Madison, Wis. Monoclonal antibodies against the RelA and p50 NF-κB subunits were purchased from Rockland, Gilbertsville, Pa. The preimmune sera and antisera against the GABP α and β1 subunits were described previously (28).

Construction of the variant LAI virus and replication studies. The GABP promoter region was cut from the pBlue 3′ LTR-CAT vector with *Bsp*EI and *Bfr*I and cloned into plasmid pBlue 3′ LTR (30), thus replacing the wild-type sequence. This plasmid was used to replace the wild-type 3′ LTR in pLAI by exchange of the *Xho*I-*Bgl*I fragment as described previously (30). All constructs were verified by sequence analysis.

C33A cells were cultured and transfected as described previously (13). Viral stocks were prepared by transfection of C33A cells with 1 μg of the wild-type plasmid pLAI and the GABP variant in 24-well multidish plates. The supernatant was taken at 2 days posttransfection and frozen at −70°C in aliquots. CA-p24 levels were determined by antigen capture enzyme-linked immunosorbent assay as described previously (2), and these values were used to normalize the amount of virus in subsequent infection assays on susceptible cells.

MT-2 and C8166 cells were cultured as described above for the SupT1 cell line. Infections were performed with 10^6 cells in 1 ml of RPMI medium with Polybrene (5 μg/ml) for 1 h at 37°C. The culture volume was subsequently increased to 5 ml with RPMI medium. Peripheral blood mononuclear cells (PBMC) were cultured as described elsewhere (2), and 5×10^6 cells were used for infection as described above. Cells were infected with an amount of virus indicated in the figure legends. All cultures were monitored for CA-p24 production following infection.

Mixed infections of SupT1 cells with an equal amount of the wild-type and GABP virus (8 ng of CA-p24 each) were performed to determine the relative fitness of the two viruses. At the peak of infection, as indicated by the presence of massive syncytia, virus was passaged onto fresh SupT1 cells and virus replication was continued for 40 days. Samples of infected cells were taken at different days, and chromosomal DNA was isolated as described previously (12). Proviral LTR sequences were PCR amplified as described above, using primers XhoU3 (5′ CCGCTCGAGTGGAAGGGCTAATTCACT 3′, proviral positions 9133 to 9150) and C(N1). Population-based sequencing was performed with the 3′ TATA primer (5′ GCAAAAAGCAGCTGCTTATATGCA 3′, proviral positions 9578 to 9555) and DyeTerminator sequencing technology (Amersham) on an ABI automated sequencer. The sequence of both HIV-1 isolates is identical up to the ΔT mutation in the NF-κB site but will be read in different reading frames downstream of this position. Overlapping sequence signals representing the two viruses were quantitated at several positions to determine the ratio of wild-type to variant virus.

RESULTS

Selection of an HIV-1 variant with an unusual LTR mutation. We previously described several replication-impaired HIV-1 mutants with a single amino acid substitution in the cysteine-rich domain of the Tat protein (58). Two Tat-mutated viruses (Tyr26Ala and Phe32Ala) were chosen for the selection of revertant viruses in several SupT1 T-cell cultures that were maintained for over 4 months. The rationale behind this forced evolution approach is to identify second-site mutations within the Tat protein that are able to restore the function of this small transactivator protein. Although rapidly replicating viruses appeared in most of the cultures, only some of these revertant viruses contained either first-site or second-site Tat mutations (unpublished data). To identify putative adaptive changes in the HIV-1 promoter-enhancer motifs and the TAR element that constitutes the Tat binding site, we analyzed the sequence of the LTR region of each of these revertant viruses. One culture that was infected with the Tyr26Ala mutant yielded a fast-replicating virus that maintained the original Tat mutation but acquired a remarkable sequence variation in the upstream NF-κB motif that is predicted to abolish NF-κB binding (Fig. 1, site II). Because it is difficult to understand how the loss of NF-κB binding can assist a Tat-defective virus, we reasoned that perhaps a binding site for another transcription factor was generated by this mutation.

The LTR sequence variation results in a loss of NF-κB binding. First, we analyzed the effect of the 1-nucleotide (nt) deletion in the NF-κB site II on protein binding in EMSA with double-stranded DNA oligonucleotides and nuclear extract from HeLa cells. Since NF-κB is an inducible transcription factor, we also prepared extracts from HeLa cells that were stimulated with TNF-α. The oligonucleotides used for EMSA represent the NF-κB site II, either the wild-type or mutant sequence, with two flanking base pairs on each side (Fig. 2A, wt and mut). As shown in Fig. 2B, stimulation of HeLa cells with TNF-α resulted in the induction of NF-κB binding activity in the nucleus (compare lanes 1 and 2), and this binding was largely abolished for the mutant NF-κB site (lane 4). This result indicated a loss of NF-κB binding function for the mutant HIV-1 promoter. Quantitation of the signals in lanes 2 and 4 demonstrated an eightfold decrease in NF-κB binding for the mutant LTR motif. When larger oligonucleotides including the downstream NF-κB site I were used (Fig. 2A, wt-wt and mut-wt), a somewhat different binding pattern was observed. The binding properties of the wt-wt probe (lanes 5 and 6) is identical to that of the shorter wt probe (lanes 1 and 2). However, a new binding activity was detected with the mut-wt probe in unstimulated HeLa nuclear extract (lane 7). This activity is specific for the mutant sequence, as it is not observed with the wt-wt probe (lane 5). Interestingly, this new protein-DNA complex was not observed in EMSA with the shorter mut probe (lane 3), indicating that sequences downstream of NF-κB site II are required for binding of this new factor that is present in nuclear extract of unstimulated cells. The mut-wt probe demonstrated increased protein binding in stimulated nuclear extract (lane 8). This most likely represents NF-κB binding to the intact site I. However, because the new protein-DNA complex comigrated with the regular NF-κB complex, it is not possible to distinguish between these two complexes in

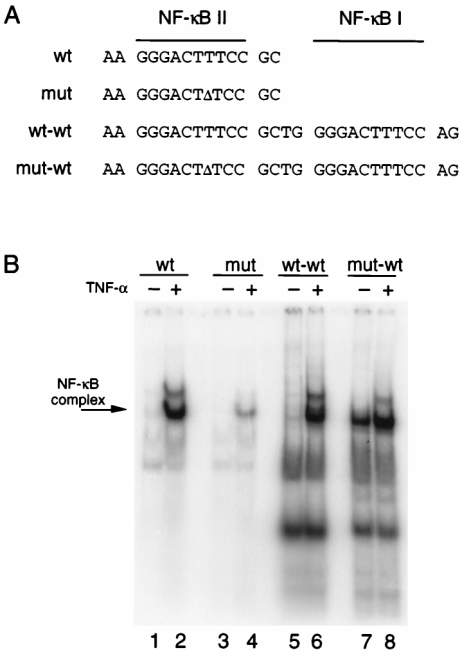

FIG. 2. The variant HIV-1 enhancer does not bind NF-κB protein. (A) Sequences of the probes used in the EMSA (plus strand only is shown). Probes wt and mut represent the wild-type and variant (ΔT) NF-κB site II, flanked by two nucleotides on each side. Probes wt-wt and mut-wt also contain the downstream NF-κB site I. (B) EMSA with nuclear extract from HeLa cells treated with (+) or without (−) TNF-α to induce NF-κB binding activity. The probes used in EMSA are indicated above the lanes. The signal corresponding to the NF-κB complex is indicated by an arrow.

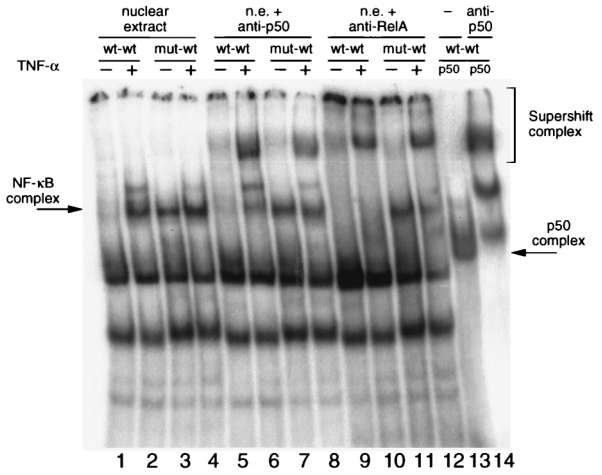

FIG. 3. The variant HIV-1 enhancer binds a new nuclear protein. EMSA was performed with HeLa nuclear extract (n.e.) stimulated (+) or unstimulated (−) with TNF-α (lanes 1 to 12) and probes wt-wt and mut-wt as indicated above the lanes. In lanes 13 and 14, purified recombinant NF-κB p50 was used for EMSA. Monoclonal antibodies specific for the NF-κB subunits RelA (lanes 9 to 12) and p50 (lanes 5 to 8) were used to supershift the NF-κB complex. Positions of the NF-κB, p50, and supershift complexes are indicated.

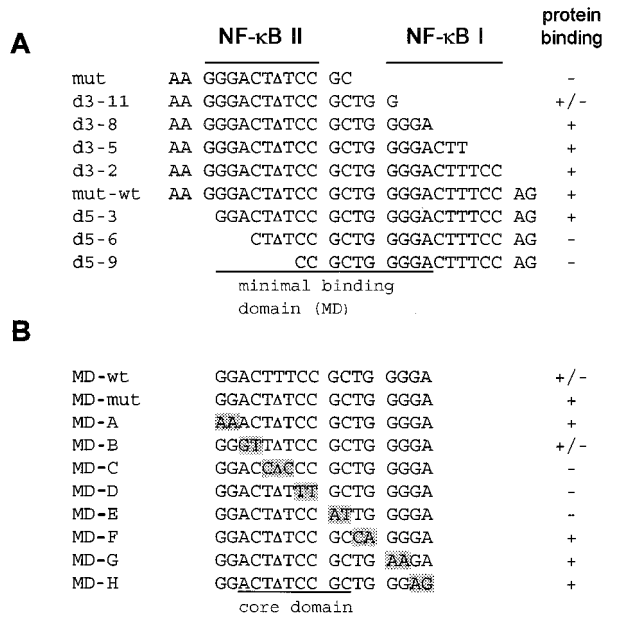

FIG. 4. Mutational analysis of the new protein binding site. (A) A set of 5′- and 3′-deleted probes was tested in EMSA for the ability to compete with probe mut-wt for protein binding in unstimulated nuclear extracts. Efficient competition (and thus protein binding) is scored as +; no binding is scored as −. The MD, which overlaps both NF-κB sites, is underlined. (B) Determination of the sequence requirements for GABP binding. Mutant probes (MD-A to -H; substituted nucleotides are shaded) were tested for the ability to compete for protein binding competition to the MD-mut probe in EMSA. The sequence-specific core of 8 nt is underlined.

this experiment. The faster-migrating bands observed with the extended wt-wt and mut-wt probes (lanes 5 to 8) represent aspecific protein-DNA complexes that are frequently observed in NF-κB EMSA (48, 61).

The NF-κB protein is a p50-RelA (p65) heterodimeric complex, but numerous alternative homo- or heterodimers can be formed by different members of the Rel family of transcription factors. Each complex displays a different DNA binding specificity and transactivation potential (52, 56), and it is therefore feasible that the mutant NF-κB site has been optimized to accommodate the binding of an alternative NF-κB complex that is distinct from p50-RelA. For instance, the p52-RelA heterodimer has been suggested to be involved in specific transactivation of the HIV-1 promoter (47, 48). To investigate this possibility, we performed EMSA in the presence of antibodies directed against the NF-κB subunits (Fig. 3). The new binding activity, which is present in unstimulated nuclear extract and specific for probe mut-wt, is shown in lane 3. Lanes 1, 2, and 4 represent the appropriate control experiments; the regular NF-κB complex is, for instance, detected with probe wt-wt and stimulated nuclear extract (lane 2). The same four EMSA samples were incubated with an anti-p50 antibody (lanes 5 to 8) and a RelA-specific antibody (lanes 9 to 12). Incubation with anti-p50 supershifted the NF-κB complex (lane 6), but the new protein-DNA complex is not recognized by this antiserum (lane 7). As a control for the specificity of this antiserum, it is demonstrated that a complex made with probe wt-wt and purified recombinant p50 protein can be supershifted with this monoclonal antibody (lanes 13 and 14). Similar results were obtained with the RelA-specific antibody: an NF-κB supershift (lane 10) but no effect on the new DNA-protein complex (lane 11). Interestingly, the protein complex observed with probe mut-wt in stimulated nuclear extract is only partially supershifted by both NF-κB antisera (lanes 8 and 12). Since the amount of antibody used is sufficient to efficiently supershift the NF-κB signal obtained with probe wt-wt (lanes 6 and 10), we propose that the unreactive signal with probe mut-wt represents the new protein-DNA complex. In fact, this result may suggest that the new factor and NF-κB are not simultaneously bound to probe mut-wt.

We also analyzed the binding of the wt-wt and mut-wt DNA probes to recombinant NF-κB homodimer forms. The p50-p50 homodimer bound both probes (not shown), in line with the observation that binding of p50 requires the 5′ half of the NF-κB recognition sequence (33), which is not affected by the T deletion. The p52-p52 homodimer did not bind to any of the probes, consistent with the low binding affinity of p52 for the HIV-1 NF-κB sites (47). These combined results indicate that the factor that binds the variant NF-κB II site is not related to the Rel family of transcription factors.

Fine mapping of the binding site for the new protein factor. To further analyze the factor that binds specifically to the modified LTR sequence, we first determined the minimal DNA sequence requirements. Because binding was observed with probe mut-wt but not with probe mut (Fig. 2B), it is possible that sequences downstream of the NF-κB site II contribute to protein binding. To test this, a nested set of 5′/3′-truncated probes was synthesized and analyzed for the ability to compete with probe mut-wt for binding of the new protein factor in EMSA with unstimulated cell extracts (Fig. 4A). Deletion of 2, 5, and 8 nt from the 3′ end of probe mut-wt did not influence protein binding (d3-2, d3-5, and d3-8). Deletion of 11 nt partially affected binding (d3-11), and deletion of 14 nt abrogated binding (d3-14, the original mut probe). Removal of 3 nt from the 5′ end of probe mut-wt showed no effect (d5-3), but deletion of 6 and 9 nt abolished protein binding (d5-6 and d5-9). These results suggest that the minimal DNA binding domain (MD) constitutes the sequence GGACTTCCGCTGG GGA, which overlaps both NF-κB sites (Fig. 4A).

We next synthesized this MD and demonstrated protein binding for the mutant LTR sequence. Scanning mutagenesis was used to replace all nucleotides of this MD-mut probe by

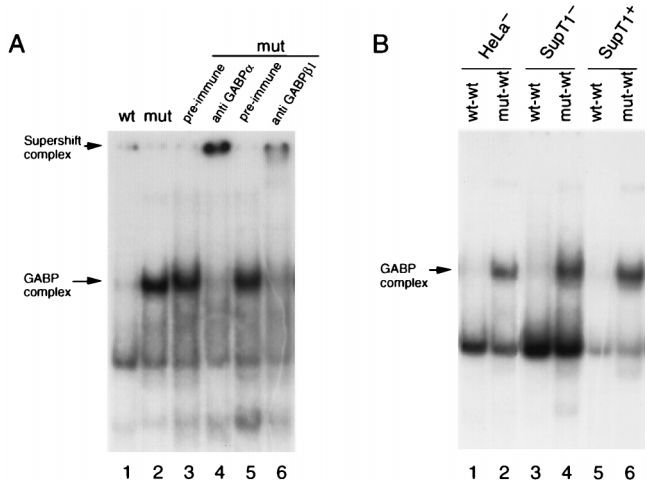

FIG. 5. The variant enhancer binds the GABP transcription factor. (A) EMSA was performed with probes wt-wt (lane 1) and mut-wt (lanes 2 to 6). The latter sample was supershifted with rabbit polyclonal antiserum specific for the α and β1 subunits of GABP or the control preimmune sera. Positions of the GABP and supershift complexes are indicated. (B) EMSA was performed with probes wt-wt and mut-wt in nuclear extracts of unstimulated HeLa cells (HeLa$^-$) and unstimulated and stimulated SupT1 cells (SupT1$^-$ and SupT1$^+$). The position of the GABP complex is indicated.

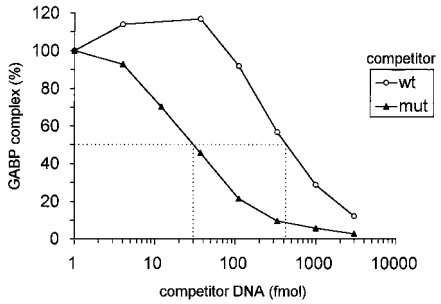

FIG. 6. Mutation of the NF-κB enhancer greatly stimulates GABP binding. The relative affinity of GABP for the MD-mut and MD-wt probes was measured as follows. EMSA was performed with unstimulated HeLa nuclear extract and the ^{32}P-labeled MD-mut probe, and unlabeled MD-mut and MD-wt probes were added as competitor in the range of 4 to 3,000 fmol. The GABP complex was quantitated with a PhosphorImager and plotted (signal in the absence of competitor set at 100%). The dotted lines mark the position at which 50% competition was observed.

transitional mutation in a pairwise manner (Fig. 4B, MD-A to -H). This set of MD probes was tested for the ability to compete with the MD-mut probe for protein binding. Mutation of two 5′-terminal G residues in probe MD-A did not affect protein binding. Binding was partially lost upon mutation of the AC dinucleotide in probe MD-B and was abrogated for mutants MD-C, MD-D, and MD-E. The 3′-terminal sequence of the MD-mut probe does not contribute to protein binding in a sequence-specific manner, as efficient competition was measured for the mutants MD-F, MD-G, and MD-H. These results are summarized in Fig. 4B, where the core domain that provides important sequence information is underlined.

The variant LTR gains GABP binding activity. The core domain ACTTCCGC is reminiscent of the binding site for Ets transcription factors because the noncoding strand contains the GGA motif that is essential for Ets factor binding (24). Unlike most Ets binding sites, the mutant HIV-1 LTR has the GGA motif in the noncoding strand. The thrombopoietin gene is one example of a cellular gene with the HIV-like orientation of an Ets binding site in its promoter (28). This Ets site binds GABP, and the core of this GABP binding site (ACTTCCG) is identical to that of the variant HIV-1 sequence. These combined observations raised the possibility that GABP is the factor that binds the modified HIV-1 LTR. GABP is a heterodimer consisting of an α and a β subunit of 60 and 53 kDa, respectively. GABPα is an Ets family transcription factor with a DNA binding Ets domain; GABPβ1 is a heterotypic protein related to *Drosophila melanogaster* Notch and contains a series of ankyrin repeats that interact with GABPα (34). Formation of the heterodimer enhances DNA binding and specificity (5).

To test whether the T deletion in the NF-κB site II facilitates GABP binding, we performed EMSA with rabbit polyclonal antibodies directed against the α and β subunits of GABP. Figure 5A shows the protein-DNA complex that is specific for probe mut-wt (lane 2). Incubation of this EMSA sample with preimmune sera demonstrated no effect (lanes 3 and 5), but the DNA-protein complex was supershifted with both anti-GABP antibodies (lanes 4 and 6). This experiment identified the GABP transcription factor as the protein that binds to the variant HIV-1 enhancer element. This idea is supported by two additional pieces of evidence. First, GABP is constitutively expressed (34) and therefore present in unstimulated nuclear extract. Second, the GABP heterodimer is similar in size to the NF-κB dimer, which is consistent with the similar migration of the corresponding DNA complexes on EMSA gels.

GABP is expressed in many cell types, including the HeLa cells that were used for preparation of the nuclear extract (34). Since the HIV-1 revertant with the variant LTR was selected upon long-term replication in the SupT1 T-cell line, we expected GABP to be present in the nuclear extract of these cells as well. To test this, we performed EMSA with nuclear extract from unstimulated and TNF-α-stimulated SupT1 cells (Fig. 5B, SupT1$^-$ and SupT1$^+$, respectively). For comparison, the unstimulated HeLa nuclear extract (HeLa$^-$) was included to demonstrate specific GABP binding to probe mut-wt (Fig. 5B, lane 2). Indeed, a complex with the gel mobility of the DNA-GABP complex was detected both in unstimulated and stimulated SupT1 nuclear extracts (lanes 4 and 6). This binding was specific for the variant LTR probe. Note the absence of NF-κB binding activity in the stimulated SupT1 nuclear extract, which should have reacted with probe wt-wt (lane 5). This is consistent with the observation that stimulated SupT1 cells contain extremely low levels of NF-κB (11).

The results so far indicate a gain of GABP binding upon mutation of the HIV-1 LTR sequence, but there is some evidence that the GABP transcription factor can also bind with a low affinity to the wild-type HIV-1 promoter. First, such an effect was described by Flory and coworkers (17). Second, we measured some GABP binding with the MD-wt probe, which has the wild-type NF-κB sequence (Fig. 4B). To analyze the relative binding affinity of GABP for the MD-wt and MD-mut probes, we performed a competition experiment. EMSA was performed with the labeled MD-mut probe and unstimulated HeLa nuclear extract. Either the MD-mut or MD-wt probe was added in increasing amounts as competitor. The GABP bandshift signals were quantitated on a PhosphorImager, and the results are presented in Fig. 6. The EMSA signal in the absence of competitor was set at 100%, and the relative EMSA signals were plotted as a function of the amount of competitor DNA. It is obvious that the MD-mut probe is a much more efficient competitor than the MD-wt probe. A 14-fold increase in relative binding affinity was calculated by comparing the

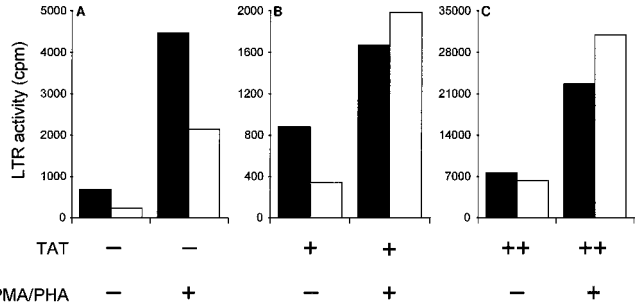

FIG. 7. The variant LTR promoter combines low basal activity with high Tat inducibility. SupT1 T cells were electroporated with 40 (A) or 1 (B and C) μg of wild-type (■) or mutant (□) LTR-CAT reporter plasmid. Some samples were cotransfected with 1 (+) or 3 (++) μg of the Tat expression plasmid and treated with PMA-PHA 24 h posttransfection as indicated. Transfections were performed simultaneously, and results of a representative experiment are shown. Similar results were obtained in five independent transfection experiments.

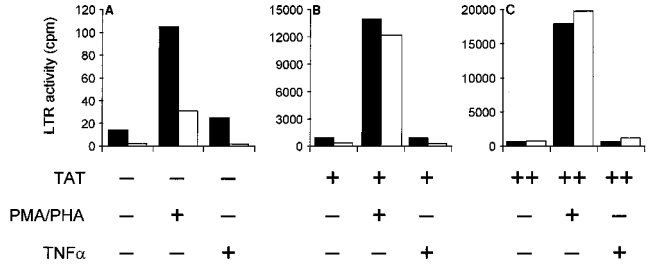

FIG. 8. The variant LTR promoter has the same activity profile in NF-κB-containing cells. Jurkat T cells were electroporated with 1 μg of the wild-type (■) or mutant (□) LTR-CAT reporter construct in the absence or presence of 1 (+) or 2 (++) μg of the pcDNA3-Tat expression vector. The cells were either untreated or stimulated by PMA-PHA or TNF-α at 24 h posttransfection as indicated.

amounts of competitor that reduce the EMSA signal by 50%: 30 fmol for MD-mut and more than 400 fmol for MD-wt.

The GABP binding site changes the activity of the HIV-1 LTR promoter. To perform transcription studies, we inserted the wild-type and variant LTR promoters upstream of the CAT reporter gene. To accurately measure the extremely low basal promoter activity, 40 μg of LTR-CAT plasmid was transfected into SupT1 cells. Gene expression with the variant LTR promoter was less than half of that with the wild-type LTR promoter (Fig. 7A). The mitogens PMA and PHA stimulate signal transduction pathways and can activate NF-κB expression in the nucleus (52). In addition, PMA activates the tyrosine kinase/Ras/Raf signaling pathway, leading to enhanced expression from promoters that are regulated by Ets transcription factors (6). Specifically, it has been demonstrated that GABP subunits are phosphorylated by the mitogen-activated protein kinase/ERK kinase pathway upon treatment of cells by phorbol ester, and such a posttranslational modification may regulate the transcriptional activity of GABP (17). We therefore determined the effects of these stimuli on the wild-type and mutant LTRs. The addition of PMA and PHA greatly increased basal activities of both the wild-type and mutant promoters, but the latter remained twofold less active than the former.

Transactivation of the LTR promoter by the viral Tat protein was assayed in transfections with 1 μg of LTR-CAT and 1 or 3 μg of Tat plasmid (Fig. 7B and C). Both situations were tested in the absence and presence of PMA-PHA. Both promoters demonstrate an approximately 50-fold Tat induction compared with an LTR-CAT transfection in the absence of Tat (not shown). The variant LTR remained less active than the wild-type LTR at low Tat levels, but this difference was less prominent at high Tat levels, and the mutant promoter activity exceeded that of the wild-type LTR in the presence of Tat and PMA-PHA. These results show that replacement of the upstream NF-κB site by a GABP element can have a positive effect on the HIV-1 gene expression level under certain conditions. This result is striking compared with the behavior of other NF-κB knockout mutations, which show a severe reduction in LTR promoter activity (reference 7 and data not shown).

The loss of the NF-κB enhancer may be less detrimental in the SupT1 T-cell line that was used in the virus selection experiment because this cell type contains very low levels of this transcription factor (11). To determine whether the NF-κB-to-GABP switch is more detrimental to LTR promoter activity in cells that express higher levels of NF-κB, we also analyzed the promoter constructs in the Jurkat T-cell line. The cells were treated with PMA-PHA at 24 h posttransfection or were left untreated. Compared with the SupT1 cell line, a more dramatic loss of the basal promoter activity was measured in Jurkat cells for the mutant LTR (Fig. 8A), which correlates with the higher NF-κB levels in the latter cell type (11). However, in the presence of either suboptimal amounts of Tat in combination with PMA-PHA (Fig. 8B) or high Tat levels (Fig. 8C), the variant LTR was able to fully overcome this defect. Thus, the variant LTR promoter with a GABP site can outperform the wild-type LTR even in cell types that contain abundant levels of the NF-κB protein. We also analyzed the effect of TNF-α treatment on promoter activity. This proinflammatory cytokine stimulates NF-κB binding activity in the nucleus, but presumably via a signaling cascade other than that induced by PMA (52). For a mouse major histocompatibility complex class I gene promoter, it has been reported that tandem NF-κB sites are required for optimal TNF-α induction (23). We therefore compared the wild-type and variant LTR promoters with two (wild type) and one (variant) NF-κB site for their TNF responsiveness in the Jurkat cell line. The results indicate that both types of HIV-1 LTR promoter do not respond to TNF-α treatment (Fig. 8).

We next tested the effects of overexpression of the two GABP subunits on transcription from the wild-type and mutant LTR promoter. SupT1 cells were cotransfected with combinations of LTR-CAT plasmid and Tat, GABPα, and GABPβ1 expression vectors. We plotted the basal LTR activities measured in the absence of Tat (Fig. 9A) and the Tat-activated LTR activities (Fig. 9B). These two values were used to calculate the relative Tat response of the two LTR promoters (Fig. 9C). Cotransfection of the GABP vectors inhibited basal activity of the mutant LTR promoter, whereas the wild-type LTR was relatively unaffected (Fig. 9A). Cotransfection of the mutant LTR-CAT vector with an individual GABP plasmid inhibited transcription approximately twofold, and an eightfold reduction was measured when both GABP plasmids were cotransfected. In contrast, cotransfection of the GABP plasmids in the presence of Tat yielded similar transcriptional activities for the wild-type and variant LTR promoters (Fig. 9B). Thus, overexpression of GABP results in a selective inhibition of the variant LTR promoter, but this GABP-mediated inhibition is fully overcome in the presence of Tat. In other words, the variant promoter is more Tat responsive than the wild-type LTR upon overexpression of GABP (Fig. 9C).

The GABP site improves virus replication in SupT1 cells. Interestingly, we observed that the 1-nt deletion in the NF-κB

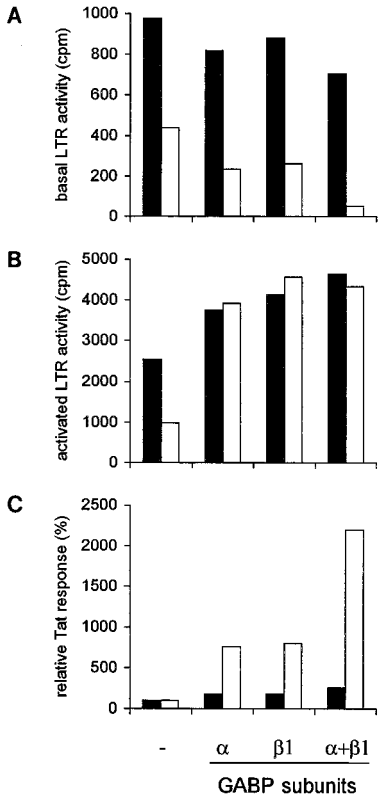

FIG. 9. Overexpression of GABP specifically affects the variant LTR promoter. SupT1 T cells were electroporated with 20 (A) or 1 (B) μg of the wild-type (■) or mutant (□) LTR-CAT in the presence or absence of 2.5 μg of the pcDNA3-Tat expression vector. Vectors encoding the α and β1 subunits of GABP were included individually or in combination as indicated. The relative Tat response (C) was calculated from the data in panels A and B (see Materials and Methods). In this calculation, we corrected for the different amounts of transfected LTR-CAT plasmid. The Tat response of each promoter in the absence of GABP was set at 100% (in this experiment, an approximately 50-fold induction was measured for both promoters). Similar results were obtained in three independent transfection experiments.

enhancer is also present in the LTR promoter of subtype E viruses (see Discussion). Thus, the NF-κB-to-GABP site modification represents natural variation in the LTR promoter that could provide the different subtype viruses with unique transcriptional properties. To test this, we introduced the 1-nt mutation in the HIV-1 LAI isolate and compared the replication of the wild-type and GABP variant in different cell types, including the SupT1 cell line in which the GABP site was selected. A small but significant increase in replication capacity was observed in these cells (Fig. 10A and B). No difference in replication was measured in other T-cell lines (Fig. 10C and D) and in primary cells (PBMC; Fig. 10E). This selective gain of fitness may correlate with the extremely low amount of NF-κB in SupT1 cells. We also performed a coculture of the two viruses to allow more accurate calculation of the fitness gain. Samples of infected cells were taken over time, and the proviral LTR was analyzed by population-based sequencing to determine the relative concentrations of the wild-type virus and GABP variant (Fig. 11). This experiment demonstrated the rapid outgrowth of the GABP variant. Fitness calculations indicated an approximately 37% increase in replication capacity for the GABP variant compared with wild-type LAI.

DISCUSSION

We observed a single-nucleotide deletion in the upstream NF-κB site of the tandem enhancer motif in the HIV-1 LTR promoter. This mutation was selected in a long-term culture of a Tat-defective HIV-1 mutant. EMSAs demonstrated loss of NF-κB binding, and a concomitant loss of basal promoter activity was measured. However, outgrowth of this particular LTR variant suggested that the mutation does contribute to improved virus replication. A new binding activity that is largely specific for the mutant LTR was identified in EMSAs with nuclear extracts of unstimulated cells. This DNA-protein complex did not react with NF-κB-specific antibodies, indicating that the factor is not an alternative NF-κB complex. Deletion and mutation analyses delineated the minimal DNA binding site and the sequence-specific core that is recognized by the new protein factor. This DNA sequence is homologous to the binding site recognized by the GABP transcription factor (59), and antibody-mediated supershifts demonstrated that it is indeed GABP that binds to the mutated NF-κB site. The 1-nt deletion decreased binding of NF-κB by a factor 8 and, at the same time, increased the affinity for GABP approximately 14-fold.

The mutant LTR promoter displayed a partial loss of activity at low transcription levels, but this promoter outperformed the wild-type LTR at high transcription levels in the presence of the Tat transactivator protein and PMA-PHA stimulation. A simple interpretation of these results is that NF-κB contributes primarily to basal LTR activity, which is consistent with previous studies (7), whereas the major contribution of the new GABP motif is to improve the level of activated LTR transcription. However, the situation may be more complex. For instance, cotransfection of GABP expression vectors reduced the basal activity of the mutant HIV-1 LTR in a specific manner, suggesting that the GABP site is responsible in part for both the reduced basal promoter activity and increased level of activated promoter activity. It is obvious that such altered promoter characteristics may provide HIV-1 with unique biological properties.

Because the HIV-1 promoter variant was selected in the SupT1 T-cell line, which is known to have an extremely low level of NF-κB protein, we reasoned that the conversion of an NF-κB site into a GABP binding site could represent a SupT1-specific adaptation. In other words, it is possible that this enhancer switch is more detrimental in a cell line with higher NF-κB levels. This was tested in the Jurkat cell line, which expresses high NF-κB levels (11). However, a very similar pattern of reduced basal transcription and improved activated transcription was measured for the mutant LTR promoter in the Jurkat cell line. Obviously, the new LTR enhancer configuration may have been altered to repair, at least partially, the Tat defect of the HIV-1 mutant that was used to initiate the long-term infection experiment. Indeed, the transient transfection data indicate that the mutant LTR with the GABP site is more Tat responsive. In this way, the virus could amplify the residual Tat activity encoded by the mutant Tyr26Ala virus. We measured poor transcriptional activity with the variant LTR promoter in combination with the Tyr26Ala Tat mutant (results not shown). Thus, other changes elsewhere in the HIV-1 genome are likely to have contributed to the reversion event. In fact, a second-site mutation within the *tat* gene was observed for this revertant virus, and this putative Tat revertant protein is currently being analyzed.

It has been reported that other Ets transcription factors also bind the HIV-1 LTR. Multiple weak Ets binding sites were detected in the promoter-distal LTR region, around position

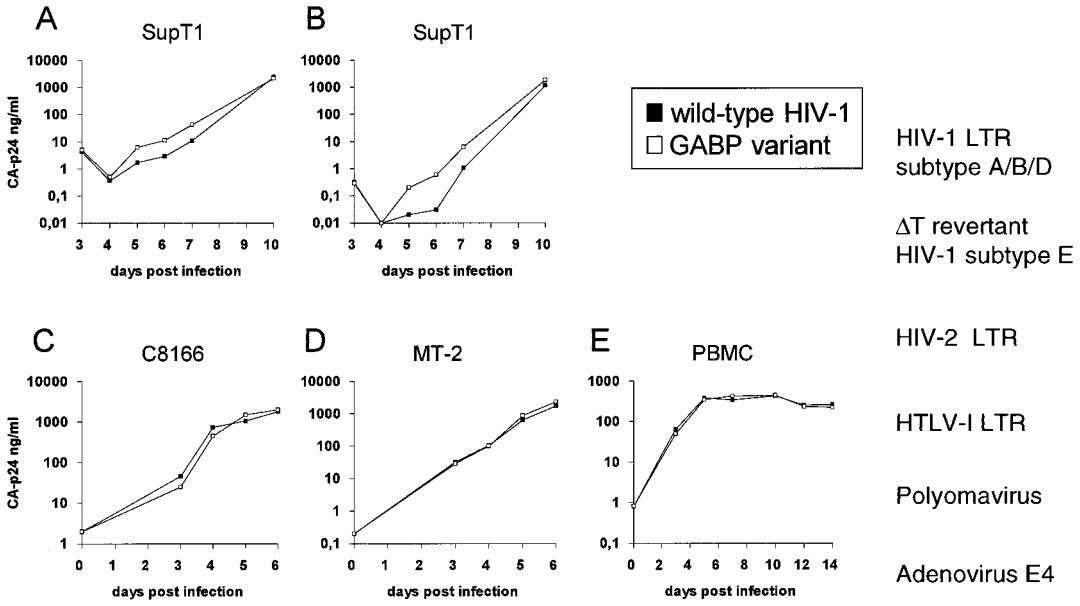

FIG. 10. Replication of wild-type LAI virus and the GABP variant in different cell types. Different T-cell lines (A to D) and primary cells (PBMC; E) were infected with the wild-type and GABP virus (20 [A], 2 [B], 10 [C], 1 [D], and 4 [E] ng of CA-p24). Virus production was measured at several days post infection.

−145 relative to the transcription start site (4, 22, 53). The Ras/Raf activation pathway, which leads to activation of many transcription factors including Ets proteins, triggers expression from the HIV-1 promoter, and Ras/Raf-responsive elements that overlap the NF-κB elements were identified (6, 9). It has been demonstrated that NF-κB is not responsible for this stimulation, and subsequent analyses revealed the involvement of several Ets transcription factors, including Ets-1, Ets-2, ERGB/Fli-1, and GABP (17, 21, 50). In addition, the HIV-1 sequence overlapping the NF-κB binding sites has been shown to bind other transcription factors such as the zinc finger-containing protein PRDII-BF1 (3) and the cell cycle regulator E2F-1 (32). Except for GABP, we did not test how the single T-deletion within the upstream NF-κB site affects the binding of these factors.

Ets family transcription factors are involved in the transcriptional regulation of a variety of viruses (Fig. 12). For instance, the HIV-2 LTR contains a single NF-κB site with an upstream Elf-1 binding site (35, 37, 41). Human T-cell leukemia virus type 1 (HTLV-1) and polyomavirus transcription is, among other factors, regulated by the Ets-1 factor (24). The GABP transcription factor was first identified in studies on transcriptional regulation of the adenovirus E4 promoter (59). A well-known nonviral promoter regulated by GABP is the retino-

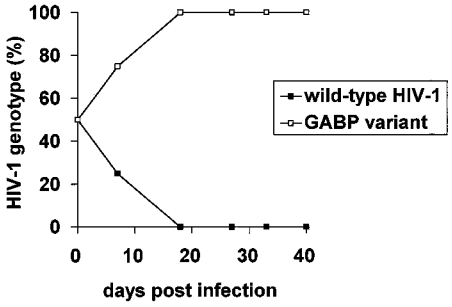

FIG. 11. The GABP variant outgrows the wild-type LAI virus in a mixed infection of SupT1 cells. An equimolar mixture of the wild-type virus and GABP variant (8 ng of CA-p24 each) was used to infect SupT1 cells at day 0. Virus was passaged at the peak of infection onto fresh SupT1 cells. Infected cell samples were used for PCR amplification of a proviral LTR fragment, and population-based sequencing was performed to determine the composition of the virus mixture. Fitness calculations were performed as described previously (20), with the viral generation time set at 2.0 days.

FIG. 12. The GABP site is also present in HIV-1 subtype E. Promoter organizations of several viral and cellular genes with binding sites for Ets factors are shown. The GABP site identified in this study is identical in sequence to the motif present in HIV-1 subtype E. Arrows over the GABP, Ets-1, and Elf-1 motifs indicate the 5′-to-3′ direction of the GGA core recognition sequence. See text for further details.

blastoma (Rb) tumor suppressor gene promoter (46, 51, 55). The role of GABP in regulating Rb transcription may be crucial, since it is speculated that GABP gene inactivation is associated with the occurrence of some malignancies (55). The orientation of most Ets binding sites is such that the core binding sequence GGA is in the coding strand (Fig. 12; arrows over Ets sites indicate the 5′-to-3′ direction of the GGA sequence). The GABP element in the HIV-1 revertant is in the opposite orientation, similar to the GABP site in the thrombopoietin gene promoter (28). The latter GABP element was shown to be the most important transcription factor for the regulation of this cellular promoter.

A characteristic of Ets factors is that they synergize with other transcription factors in the activation of transcription. For instance, interaction of Ets-1 with Sp1 has been proposed to be required for full transcriptional activity of the HTLV-1 promoter (Fig. 12) (19). For the HIV-1 LTR promoter, interactions between the Ets-1 factor and either USF-1 (53) or NF-κB and NFAT (4) have been reported. The Elf-1 factor can interact directly with NF-κB (26), and this may explain the synergistic activation of the HIV-2 LTR by NF-κB and Elf-1 (37). Experiments are under way to analyze in further detail the role of GABP in transcription of the variant HIV-1 LTR, e.g., whether its contribution is orientation specific and whether there is a functional interaction with NF-κB bound at site I or other transcription factors. Although our preliminary protein binding experiments indicate that these factors cannot bind simultaneously to the LTR, this may merely represent a limitation of the EMSA.

There is evidence that the conversion of an NF-κB into a GABP site is not a unique tissue culture adaptation phenomenon. Alignment of the new LTR promoter configuration with published HIV-1 sequences (39–41) revealed identity with LTR sequences of subtype E isolates. Whereas HIV-1 subtypes A, B, and D contain tandem NF-κB sites, subtype E viruses have the typical GABP/NF-κB enhancer configuration as described in this study (Fig. 12). The LTR sequences of a total of 18 subtype E isolates have been determined, and all isolates have the exact GABP site as described and tested in this study (references 39 and 40 and unpublished results from our laboratory). It has been reported that NF-κB binding to the upstream NF-κB site of subtype E viruses is abolished, without an apparent loss of promoter function (40). We can now explain this result: the T-deletion represents a modified rather than a defective enhancer element.

Because HIV-1 subtype E is thought to represent a relatively recent branch of the HIV-1 phylogeny of the current epidemic, it is likely that the T deletion in the upstream NF-κB site occurred at least once during the worldwide spread of HIV-1. We demonstrate that the LAI virus with the GABP site can outgrow the wild-type virus in certain cell types. For animal retroviruses, there is ample evidence for a role of LTR enhancer switches as an important regulator of pathogenicity and oncogenicity (57). The alternative enhancer configuration of the subtype E viruses may also have implications for cell tropism. For instance, it has been reported that HIV-1 subtype E viruses replicate more efficiently than other subtypes in Langerhans cells, the possible target in heterosexual transmission (54), although follow-up studies could not confirm these results (16, 43). The lentivirus equine infectious anemia virus provides an interesting example where the presence of an Ets-1 site in the LTR promoter was found to be essential for productive virus replication in macrophages (10, 38). Despite accumulating sequence data on the genomes of HIV-1 strains belonging to the different subtypes (18, 41), no subtype-specific differences in virus biology have been described. Because it has been suggested that subtype E is a more pathogenic and virulent virus, it will be important to test the biological function of the GABP site-containing LTR promoter in more detail.

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