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# Central Nervous System-Derived Cells Express a κB-Binding Activity That Enhances Human Immunodeficiency Virus Type 1 Transcription In Vitro and Facilitates TAR-Independent Transactivation by Tat

J. PAUL TAYLOR,[1] ROGER J. POMERANTZ,[2] GANESH V. RAJ,[1] FATAH KASHANCHI,[3] JOHN N. BRADY,[3] SHOHREH AMINI,[1] AND KAMEL KHALILI[1]*

*Molecular Neurovirology Section, Jefferson Institute of Molecular Medicine, Department of Biochemistry and Molecular Biology, Thomas Jefferson University,[1] and Division of Infectious Disease, Department of Medicine, Jefferson Medical College,[2] Philadelphia, Pennsylvania 19107, and Laboratory of Molecular Virology, National Cancer Institute, Bethesda, Maryland 20892[3]*

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**The Tat protein of human immunodeficiency virus type 1 (HIV-1) is a potent activator of long terminal repeat-directed transcription. While in most cell types, activation requires interaction of Tat with the unusual transcription element TAR, astrocytic glial cells support TAR-independent transactivation of HIV-1 transcription by Tat. This alternative pathway of Tat activation is mediated by the viral enhancer, a κB domain capable of binding the prototypical form of the transcription factor nuclear factor kappa B (NF-κB) present in many cell types, including T lymphocytes. Tat transactivation mediated by the κB domain is sufficient to allow replication of TAR-deleted mutant HIV-1 in astrocytes. The present study demonstrates the existence of κB-specific binding factors present in human glial astrocytes that differ from prototypical NF-κB. The novel astrocyte-derived κB-binding activity is retained on an HIV-1 Tat affinity column, while prototypical NF-κB from Jurkat T cells is not. In vitro transcription studies demonstrate that astrocyte-derived κB-binding factors activate transcription of the HIV-1 long terminal repeat and that this activation is dependent on the κB domain. Moreover, TAR-independent transactivation of HIV-1 transcription is reproduced in vitro in an astrocyte factor-dependent manner which correlates with κB-binding activity. The importance of the central nervous system-enriched κB transcription factor in the regulation of HIV-1 expression is discussed.**

The *tat* gene of human immunodeficiency virus type 1 (HIV-1) is essential for viral replication (16, 20). Tat is expressed early in the viral life cycle and serves as a potent activator of transcription directed by the HIV-1 long terminal repeat (LTR), leading to the increased expression of all viral genes (1, 54). Tat binds to a specific RNA structure, termed the TAR element, found at the 5′ end of all HIV-1 mRNAs (12, 13, 39, 49). Experiments carried out with a limited number of cell types, primarily HeLa cells and T-lymphocytic cell lines, indicate that the interaction of Tat with this specific RNA target is an important component of the activation mechanism (8, 12, 13). It has been suggested that binding to TAR serves to localize Tat to the vicinity of the promoter, where it interacts with unspecified targets, resulting in enhanced transcriptional initiation (53). This model of Tat function is consistent with results implicating the participation of specific upstream regulatory elements of the viral LTR, such as the κB domain and the GC-rich Sp1-binding sites, in Tat transactivation (7, 30, 55), as well as with kinetic studies which suggest that a transient interaction between Tat and nascent TAR RNA is sufficient for achieving wild-type levels of transactivation (7, 8, 28, 30, 53, 55). Recently, a number of reports have suggested that the primary function of Tat is to increase the efficiency of transcriptional elongation (19, 36). The ability of Tat to influence elongation seems surprising in light of evidence that the target of Tat function is at the promoter (7, 30, 52, 55) and that the kinetics of Tat function indicate an effect at the time of, or immediately after, initiation of transcription (28). In an attempt to resolve this apparent paradox, it has been proposed that Tat functions at the promoter of HIV-1, requiring the participation of upstream regulatory elements, and biases initiation towards the formation of "more-processive" transcription complexes (22).

The clinical course of HIV disease is frequently complicated by multiple disorders of the central nervous system (CNS) (47, 48). At least 60% of AIDS patients exhibit clinical symptoms of neurologic disease, and 90% demonstrate neuropathologic abnormalities at autopsy (32, 42). While HIV-1 predominantly infects microglial cells in the CNS, viral antigens have also been detected in astrocytes in vivo (25, 57), and it has been demonstrated that HIV-1 infects glial astrocytes in vitro (14, 15, 17). Studies examining the regulation of HIV-1 gene expression in cells derived from the CNS revealed that glial astrocytes support TAR-independent activation of HIV-1 transcription by Tat (60). The alternative pathway of Tat utilization in glial astrocytes was found to allow HIV-1 replication to circumvent the requirement for a functional TAR element (6, 60). We further determined that the RNA-binding domain of Tat protein was dispensable for TAR-independent transactivation but that the activation domain of the protein remained necessary (59). The κB domain within the HIV-1 LTR, also referred to as the viral enhancer, was determined to be Tat responsive within astrocytes, conferring Tat responsiveness to heterologous promoters and permitting TAR-independent replication of HIV-1 (6, 60). These observations clearly differed from many previous reports concerning lymphocytes and monocytes, where TAR is reported to be absolutely essential for Tat transactivation and viral replication, and suggested the potential for differential utilization of κB elements within astrocytes. Further, these observations may provide insight

* Corresponding author. Phone: (215) 955-5534. Fax: (215) 923-8021.

into the mechanism by which Tat influences cellular gene expression.

The HIV-1 enhancer found between nucleotides −80 and −105 of the viral LTR contains two tandem repeats of the decanucleotide sequence 5′-GGGACTTTCC-3′ which have been found to bind two copies of the transcription factor NF-κB. Induction of NF-κB binding is responsible for the increased HIV-1 transcription observed upon activation of T lymphocytes by specific antigen or stimulation with mitogens such as phorbol esters and lectins (40). This activation works in concert with Tat in stimulating HIV-1 gene expression (34, 40). In addition, the κB domain mediates activation of HIV-1 by the regulatory proteins of a number of heterologous viruses, including the ICP0 protein of herpes simplex virus type 1, the E1a protein of adenovirus, and the Tax protein of human T-lymphotropic virus type 1 (41, 64). The induction of HIV-1 replication caused by the cytokines tumor necrosis factor alpha and interleukin 1, specifically implicated in reactivation of HIV-1 from latency in astrocytes (61), is also mediated by the κB domain (43, 45).

The importance of the κB domain to the regulation of HIV-1 gene expression globally, and, specifically, the involvement of the κB domain in mediating transactivation by Tat in astrocytes, led us to study the status of κB transcription factors in astrocytes. The results demonstrate the presence of κB-specific binding factors in human glial astrocytes that differ from prototypical NF-κB of lymphocytes. Unlike prototypical NF-κB, the form of κB present in the CNS is specifically retained on an HIV-1 Tat affinity column, indicating either a direct or an indirect interaction between astrocyte-derived κB and Tat. Astrocyte-derived κB-binding factors activate transcription of HIV-1 in vitro in a promoter-specific fashion that is dependent on the presence of κB-binding sites. Moreover, TAR-independent transactivation of HIV-1 transcription is reproduced in vitro and is found to be dependent on an astrocyte factor which correlates with κB-binding activity.

## MATERIALS AND METHODS

**Oligonucleotides and in vitro transcription templates.** Templates for in vitro runoff transcription were prepared by restriction enzyme digestion of expression plasmids p117/80, p117/3, and 80/3 (containing HIV-1 LTR nucleotides −117 to +80, −117 to +3, and −80 to +3, respectively [60]) with *Eco*RI or of pAdMLP (containing a 430-bp *Alu*I fragment of pAd6 [9]) with *Bam*HI. Full-length runoff transcription from template −117/+80 produces a transcript of 356 nucleotides. Templates −117/+3 and −80/+3 produce transcripts of 279 nucleotides. Template AdMLP produces a transcript of 375 nucleotides. Oligonucleotides used in electrophoretic mobility shift assays (EMSAs) and in vitro transcription-competition analysis are as follows (single strand shown only): 5′-TCGACAGAG**GGGACTTTCC**GAGAGGC-3′ (wild-type κB site) and 5′-TCGACAGAG**CTCACTTTCC**GAGAGGC-3′ (mutant κB site).

**Cells and tissue culture.** U-87MG cells are human astrocytic glioblastoma cells and were grown in Dulbecco's modified Eagle media (Gibco) supplemented with 10% (vol/vol) fetal bovine serum (Gibco). U-87MG cells, originally cloned by Ponten et al. (46) in 1966, show characteristic markers of astrocytic differentiation and are available from the American Type Culture Collection. The biology of HIV-1 in U-87MG cells has been studied extensively and is representative of behavior in a wide variety of astrocytes (6, 14, 15, 46, 58–60). Jurkat T cells were grown in RPMI 1640 (Gibco) supplemented with 10% (vol/vol) fetal bovine serum. For induced cultures, 50 ng of phorbol 12-myristate 13-acetate (PMA) (Sigma) per ml was added to the growth media 1, 3, 6, 12, or 20 h prior to harvesting the cells.

**EMSA.** Nuclear extracts from U-87MG glial astrocytes and Jurkat T cells for use in EMSA and in vitro transcription were prepared as described previously (2). 40,000 counts of $^{32}$P-end-labeled wild-type κB oligonucleotide was incubated with 8 μg of U-87MG nuclear extract in a binding buffer consisting of 10 mM Tris (pH 7.4), 50 mM NaCl, 1 mM Na-EDTA, 5% glycerol, 1 mM dithiothreitol (DTT), 4 μg of poly(dI-dC), 0.5 mg of bovine serum albumin (BSA) per ml, and 3 mM GTP. Following a 30-min incubation on ice, the resulting complexes were resolved on a low-ionic-strength 6% native polyacrylamide gel containing 6.7 mM Tris (pH 7.4), 3.3 mM Na-acetate (NaOAc), and 1 mM Na-EDTA. Electrophoresis was carried out at 4°C at a constant 140 V with a recirculating buffer. The gel was then dried and autoradiographed at −70°C with an instrument fitted with an intensifying screen. For competition analysis, a 50-fold excess of unlabeled wild-type or mutant oligonucleotide was added to the binding reaction mixture.

**Affinity column chromatography.** A 400-μg amount of purified protein (Tat or BSA) was dialyzed against coupling buffer containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.9), 20 mM NaCl, 5 mM β-mercaptoethanol, and 10% glycerol and then coupled to 200 μl of washed, equilibrated Affi-Gel 10 matrix (Bio-Rad) in a final volume of 2.5 ml overnight at 4°C. A 400-μl volume of U-87MG or Jurkat T whole-cell extract (10 mg/ml), dialyzed against the column buffer (20 mM HEPES [pH 7.9], 50 mM KCl, 10 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 20% glycerol [21]), was subjected to chromatography at 4°C at a flow rate of 1 ml/12.5 min. The flowthrough was recycled four times, and the column was subsequently washed with 5 ml of column buffer. Bound proteins were step eluted with KCl concentrations ranging from 0.3 to 2.0 M. Samples of the flowthrough, the first 400 μl of wash buffer, the final 400 μl of wash buffer, and each of the salt elution fractions were dialyzed against dialysis buffer containing 50 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 40 mM $(NH_4)_2SO_4$, 0.2 mM EDTA, 1 mM DTT, and 15% glycerol and analyzed for κB-binding activity by EMSA. Samples of all fractions were examined by silver staining of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis gels, which demonstrated that all fractions retained full integrity (no degradation detectable). No gross differences were observed between Tat and BSA fractions by silver stain.

**In vitro transcription analysis.** To analyze basal transcription, 50 to 100 ng of each template was incubated with 1.3 mM ATP, 1.3 mM GTP, 1.3 mM CTP, 20 μCi of [α-$^{32}$P]UTP (400 Ci/mmol), and 7 μl of HeLa whole-cell extract in a 15-μl reaction volume at 30°C for 1 h. HeLa whole-cell extract was prepared as described previously (35). The order of addition of the components of the transcription reaction mixture was consistently as follows: (i) template DNA [and poly(dI-dC) when appropriate], (ii) nucleoside triphosphates, (iii) transcription extract, and (iv) [α-$^{32}$P]UTP. Transcription reactions were stopped by addition of 200 μl of stop solution containing 10 mM Tris-HCl (pH 7.9), 1% SDS, 0.1 mM EDTA, and 50 mg of tRNA per ml and 200 μl of phenol. This mixture was gently rocked for 5 min at room temperature, extracted three to five times with phenol-chloroform and once with chloroform, and ethanol precipitated with 0.3 M NaOAc on dry ice. After centrifugation, the RNA was resuspended in gel loading buffer (90% deionized formamide, 5× Tris-borate-EDTA, and 0.5% bromophenol blue) and analyzed by electrophoresis on a denaturing acrylamide-urea gel. To analyze the influence of PMA-stimulated U-87MG nuclear extracts on transcription,

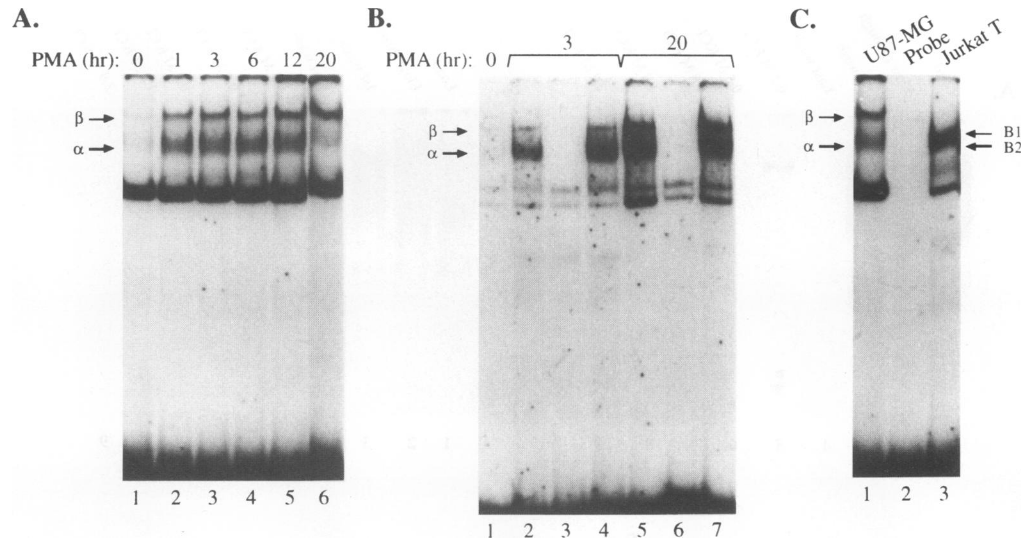



FIG. 1. Astrocytes express κB-binding activity that is induced in a bimodal fashion and differs from the NF-κB of Jurkat T cells. (A) EMSA demonstrating κB-binding activity (designated α and β) of unstimulated U-87MG cells and cells stimulated with PMA for 1, 3, 6, 12, or 20 h as indicated. (B) Specificity of astrocytic κB-binding activity. Competition analysis was performed by including a 50-fold excess of unlabeled wild-type κB oligonucleotide (lanes 3 and 6) or mutant κB oligonucleotide (5′-TCGACAGAGCTCACTTTCCGAGAGGC-3′) (lanes 4 and 7) in the binding reaction mixture. Lanes: 1, unstimulated U-87MG nuclear extract; 2 to 4, 3-h PMA-stimulated U-87MG nuclear extract; 5 to 7, 20-h PMA-stimulated U-87MG nuclear extract. (C) Astrocytic κB-binding activity differs from prototypical NF-κB of Jurkat T cells. A comparison of the migration of κB complexes formed in Jurkat T cells with that of those formed in U-87MG cells is shown. Lanes: 1, Jurkat T cells; 2, probe; 3, U-87MG cells. To reveal the B1 and B2 NF-κB complexes, Jurkat T cells were stimulated for 20 h with PMA before nuclear extract preparation.

the reaction mixtures, containing suboptimum concentrations of the template DNAs (20 ng) and 4 μl of HeLa whole-cell extract, were supplemented with 2 μl of the appropriate astrocytic nuclear extract. Titration of the template indicated that 25 ng provided an optimal basal activity for analysis of transcriptional activation (data not shown). For the in vitro transcription competition experiments, the glial nuclear extracts were preincubated with 1 μg of either wild-type or mutant κB oligonucleotide for 15 min on ice before being added to the transcription reaction mixture. In addition, the in vitro transcription competition reaction mixtures contained only 15 μg of template DNA and were supplemented with 0.5 μg of poly(dI-dC). For the in vitro transcription transactivation experiments, the transcription extracts were preincubated with 1 μM synthetic Tat protein for 15 min on ice. Synthetic Tat protein (amino acids 1 to 86), a generous gift of S. A. Khan, Wistar Institute, Philadelphia, Pa., was stored lyophilized at −70°C. For use, Tat was dissolved at a concentration of 1 mg/ml in 20 mM HEPES-KOH (pH 7.9)–20% glycerol (vol/vol)–100 mM KCl–0.2 mM EDTA–1 mM DTT and degassed with $N_2$. In quantitation, densitometry results were normalized for RNA recovery and gel loading by comparison with a reference band labeled R. This product is transcription independent and probably represents tRNA labeled with [$^{32}$P] UTP.

## RESULTS

**Human astrocytes contain κB-binding activity.** Observation that the HIV-1 κB domain mediated cell-specific Tat transactivation prompted an analysis of κB-binding factors from astrocytes (U-87MG, an astrocytic glioblastoma cell line, is described in Materials and Methods). EMSA using nuclear extracts prepared from U-87MG astrocytes reveals two complexes, designated α and β, which form with an oligonucleotide probe containing the HIV-1 κB sequence (GGGACTTTCC). A biphasic pattern of induction in κB complex formation is observed upon treatment with the mitogen PMA. In the absence of PMA treatment, both the α and β complexes are present at a low, basal level with a comparable ratio of α and β (Fig. 1A, lane 1). Appearance of the α complex is substantially induced at 1 to 3 h after PMA stimulation, while the β complex is slightly induced, resulting in a dramatic increase in α complex relative to β complex (Fig. 1A, lanes 2 and 3). After 6 to 12 h of continuous stimulation by PMA, the α/β ratio returns to the levels observed in nontreated cells, although the quantity of both complexes remains elevated above basal levels (Fig. 1A, lanes 4 and 5). After 20 h of PMA stimulation, the β complex is greatly induced, while the α complex remains unaltered, resulting in a dramatic increase in the ratio of β complex relative to α, an inversion of the previous ratio (Fig. 1A, lane 6). Both the α and β complexes were determined to represent a specific interaction with the κB sequence by competition analysis. Formation of the α complex, induced upon stimulation of U-87MG cells with PMA for 3 h (Fig. 1B, lane 2), was blocked in the presence of 50-fold excess of unlabeled wild-type oligonucleotide (Fig. 1B, lane 3) but not in the presence of an oligonucleotide containing a mutant κB-binding site (lane 4). Similarly, formation of the β complex, induced upon stimulation of U-87MG cells with PMA for 20 h (Fig. 1B, lane 5), was blocked by a 50-fold excess of unlabeled, wild-type oligonucleotide (Fig. 1B, lane 6) but not by oligonucleotide containing a mutant κB-binding site (lane 7).

**Astrocyte-derived κB-binding factors differ from those observed in T lymphocytes.** The prototypical NF-κB induced by PMA in Jurkat T cells appears as two complexes that have been previously designated B1 and B2 (10). The α complex of U-87MG astrocytes comigrates with the B1 complex of Jurkat T cells (Fig. 1C, lanes 1 and 3) and is induced with kinetics similar to those reported for B1 following stimulation with PMA (38). The B1 complex of Jurkat T cells consists of

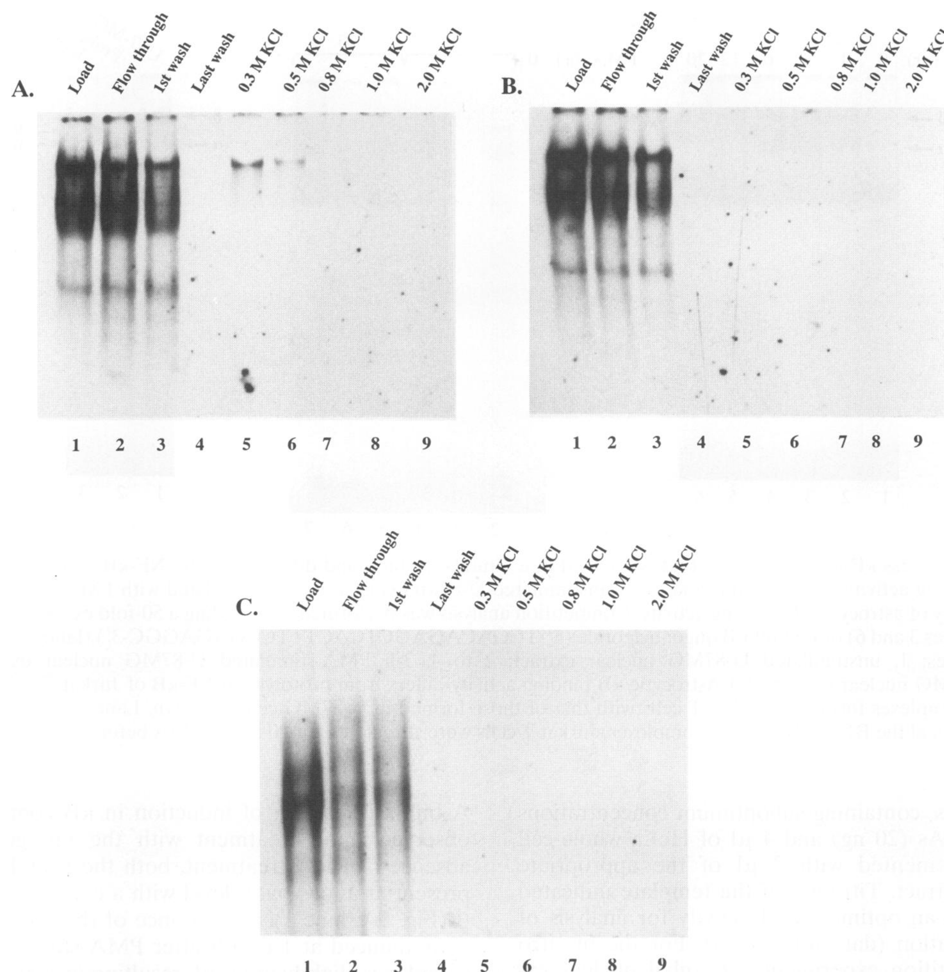
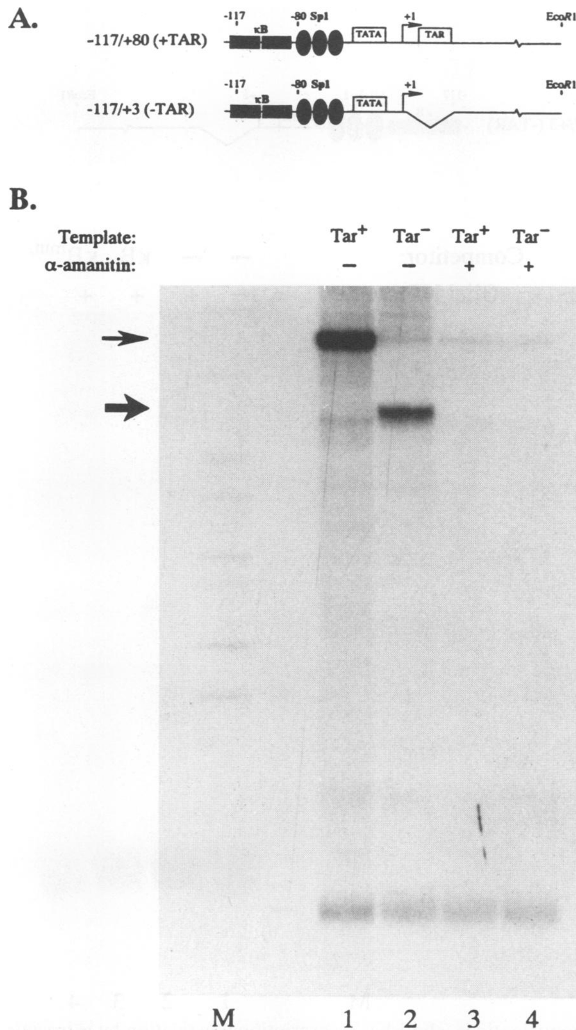



FIG. 2. Retention of astrocyte-derived κB activity on a Tat affinity column. (A) EMSA analysis of PMA-stimulated U-87MG whole-cell extract following chromatography on a Tat affinity column. First wash, recycled flowthrough diluted with column buffer; Last wash, the final 400 μl of a 5-ml wash with column buffer. Further fractions were step eluted from the column with the indicated concentrations of KCl. (B) EMSA analysis of PMA-stimulated U-87MG whole-cell extract following chromatography on a BSA affinity column. (C) EMSA analysis of PMA-stimulated Jurkat-T whole-cell extract following chromatography on a Tat affinity column.

proteins of 75, 86, and 50 to 55 kDa, believed to represent p65, c-Rel, and p50, respectively (38). The β complex of U-87MG astrocytes migrates very slowly in the native gel, suggestive of a very large complex (Fig. 1C, lane 1). A similarly large complex which migrates more slowly than NF-κB, coincidentally termed BETA, was previously observed in astrocytes and neurons (31) and has also been observed in mouse and human fetal brain nuclear extracts (unpublished observation). The induction kinetics of β complex suggest that de novo protein synthesis may precede the formation of this complex. No complex of comparable migration is observed in Jurkat T cells, suggesting that astrocytes contain additional, or perhaps novel, κB-binding factors (Fig. 1C, lane 1). The B2 complex of Jurkat T cells, which consists solely of 50- to 55-kDa proteins and probably represents a homodimer of p50 (38), does not appear in astrocytic extracts, demonstrating further heterogeneity between κB-binding factors in U-87MG astrocytes and Jurkat T cells (Fig. 1C, lanes 1 and 2). In addition, astrocytic κB-binding factors differ from prototypical NF-κB in susceptibility to dissociation by oxidation, salts, and treatment with detergents (unpublished data).

**Astrocyte-derived κB is specifically retained on an HIV-1 Tat affinity column, while prototypical NF-κB is not.** To investigate the potential interaction between κB-binding factors and Tat, whole-cell extract prepared from PMA-stimulated U-87MG and Jurkat T cells was subjected to chromatography over an HIV-1 Tat affinity column. The presence of both α and β complexes from U-87MG cells can be observed in the loaded extract, as well as in the flowthrough and the first washing fraction (Fig. 2A, lanes 1 to 3). No κB-binding activity is observed in the last washing fraction, which contains very little protein as determined by silver staining (unpublished observation). Band shift analysis of salt elution fractions demonstrates that κB-binding activity, in the form of a β complex, retained on the Tat column is released in the presence of 0.3 to 0.5 M KCl (Fig. 2A, lanes 5 and 6). The α complex, in contrast, is not retained by the Tat column, suggesting a structural difference between these two astrocyte-derived κB-binding activities. To examine binding specificity, U-87MG whole-cell extract was subjected to chromatography over a control affinity column containing BSA. As can be seen in Fig. 2B, κB-binding activity is not retained on a BSA affinity column. In a parallel experiment, whole-cell extract from PMA-stimulated Jurkat T cells was subjected to chromatogra-



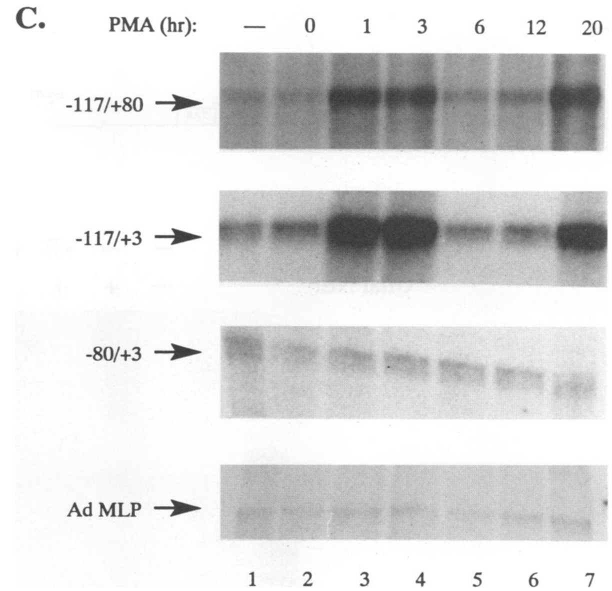




FIG. 3. (A) HIV-1 LTR templates used for in vitro transcription. (B) In vitro transcription of templates −117/+80 (Tar$^+$) and −117/+3 (Tar$^-$). Lanes: 1, template −117/+80 generates a runoff transcript of 356 nucleotides (thin arrow); 2, template −117/+3 generates a runoff transcript of 279 nucleotides (thick arrow). The reaction mixtures in lanes 3 and 4 contained α-amanitin (0.5 μg/ml) and template −117/+80 (lane 3) and template −117/+3 (lane 4). M, marker. − and + indicate absence and presence, respectively, of α-amanitin. (C) Nuclear factors from U-87MG astrocytes stimulate HIV-1 transcription. Shown is in vitro transcription of the appropriate template (−117/+80, −117/+3, −80/+3, or AdMLP) in HeLa transcription extract alone (lane 1) (−), HeLa transcription extract supplemented with unstimulated U-87MG nuclear extract (0) (lane 2), or HeLa transcription extract supplemented with nuclear extract from U-87MG cells stimulated with PMA for the indicated times (in hours) (lanes 3 to 7). The intensity of the bands was determined by densitometry of multiple experiments. Representative gels are shown.

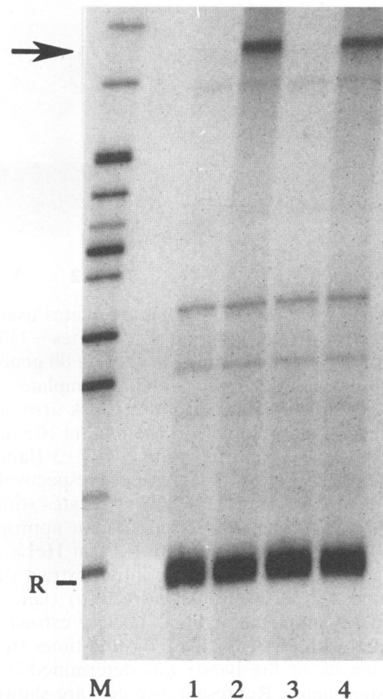
phy over the Tat affinity column and the control BSA affinity column. All of the prototypical NF-κB from these cells was found in the flowthrough and early wash steps, as was the α complex from U-87MG cells, suggesting that these factors are unable to bind to Tat and be retained on the Tat affinity column (Fig. 2C). Band shift analysis of the Jurkat extract on the control BSA showed no κB-binding activity in the salt-eluted fractions (data not shown). The ability of the β complex derived from astrocytes to bind Tat further distinguishes this κB activity from prototypical NF-κB and promotes this activity as a candidate for mediating TAR-independent transactivation.

**Transcriptional transactivation correlates with κB-binding activity.** To examine the influence of astrocytic nuclear factors on HIV-1 transcription, a cell-free in vitro transcription system was established by using HeLa whole-cell extract. Templates for runoff transcription were generated by *Eco*RI digests of LTR-chloramphenicol acetyltransferase constructs (Fig. 3A). Template −117/+80 contains the HIV-1 LTR sequences from −117 to +80 relative to the transcription start site, which includes the κB domain (−105 to −80), a GC-rich Sp1-binding domain (−47 to −79), a canonical TATA sequence, and 80 nucleotides of the HIV-1 leader sequence encoding TAR. Correct transcription initiation on template −117/+80 generates an RNA of 356 nucleotides (Fig. 3B, lane 1). Template −117/+3, in which all HIV-1 sequences downstream of +3, including TAR, are removed, generates a 279-nucleotide runoff transcript (Fig. 3B, lane 2). These transcripts are not produced in the presence of 0.5 μg of α-amanitin per ml, demonstrating their dependence on RNA polymerase II (Fig. 3B, lanes 3 and 4).

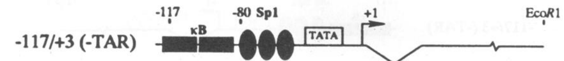
To examine the influence of astrocytic nuclear factors on HIV-1 transcription in vitro, HeLa transcription extracts were supplemented with astrocyte-derived nuclear extracts. In this and subsequent experiments, a lesser amount of template DNA was utilized to decrease basal transcription (as described in Materials and Methods). Supplementation of HeLa transcription extracts with nuclear extract from unstimulated U-87MG astrocytes had negligible influence on transcription from template −117/+80 (Fig. 3C, lanes 1 and 2). However, supplementation of HeLa transcription extract with nuclear extract from PMA-stimulated U-87MG glial astrocytes resulted in a transcriptional activation that strongly correlates with the induction of κB-binding activity (Fig. 3C, lanes 3 to 7). The greatest activation in transcription is seen upon addition of extract stimulated with PMA for 1 to 3 and 20 h (approximately eightfold ± twofold activation in multiple experiments, as determined by densitometry). Transcription from template

A.

Competitor: — — κB κB^mut.
Glial NE: — + + +




B.




Competitor: — — κB κB^mut.
Glial NE: — + + +

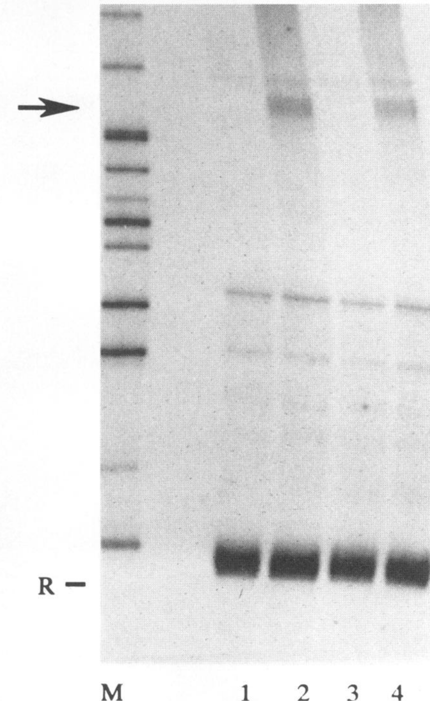



FIG. 4. Competition with wild-type κB oligonucleotides, but not mutant κB oligonucleotides, abolishes transcriptional activation by astrocytic factors. (A) Template −117/+80. The arrow indicates the 356-nucleotide transcript. The transcriptional activation seen in the presence of the 20-h PMA-stimulated U-87MG nuclear extract (compare lane 2 with lane 1) is blocked by preincubation with 1 μg of oligonucleotide containing the wild-type κB sequence (GGGACTTTCC) (lane 3) but not by 1 μg of oligonucleotide containing a mutated κB sequence (CTCACTTTCC) (lane 4). (B) Template −117/+3. The arrow points to the 279-nucleotide transcript (the lanes are as described in the legend to panel A). M, marker; R, nonspecific reference band used as a control for loading; NE, nuclear extract; $κB^{mut}$, mutated κB sequence; +, present; −, absent.

−117/+3 is similarly activated by PMA-stimulated astrocytic nuclear extract, demonstrating that this response is independent of the TAR element (Fig. 3C). However, deletion of the κB-binding sites, utilizing template −80/+3, abolishes the transcriptional enhancement, suggesting that κB-binding factors mediate the activation (Fig. 3C). The presence of astrocytic nuclear factors has no influence on transcription directed by the adenovirus major late promoter, demonstrating that activation is not a general phenomenon (Fig. 3C). These results suggest that the astrocyte-derived κB activity present in both the α and β complexes contributes to activation of HIV-1 transcription.

**Evidence that astrocyte-derived κB-binding factors are responsible for transcriptional activation.** To determine whether astrocytic κB-binding activity is responsible for the transcriptional activation observed upon supplementation of the HeLa system with astrocytic nuclear factors, oligonucleotide competitors were used to interrupt interaction of κB-binding factors with the promoter. The induction in transcription from template −117/+80 seen upon supplementation of the HeLa extract with stimulated astrocytic nuclear proteins (Fig. 4A, lanes 1 and 2) is inhibited by the presence of an excess of oligonucleotide containing the wild-type κB-binding sequence (GGGACTTTCC) (lane 3) but not by incubation with the mutant oligonucleotide (**CT**CACTTTCC) (lane 4). Neither wild-type κB oligonucleotide nor mutant κB oligonucleotide influences the basal transcription from −117/+80 in HeLa extract alone (unpublished results). This result is consistent with the supposition that κB-binding proteins are responsible for the observed transcriptional activation. As expected, the same κB-dependent induction in transcription is observed with template −117/+3, which does not contain the TAR element, demonstrating that the effect is not TAR dependent (Fig. 4B, lanes 1 to 4). Taken together, these results strongly suggest that the κB-binding activity present in the astrocytic nuclear extracts is responsible for the observed transcriptional activation.

**TAR-independent transactivation by Tat is dependent on**

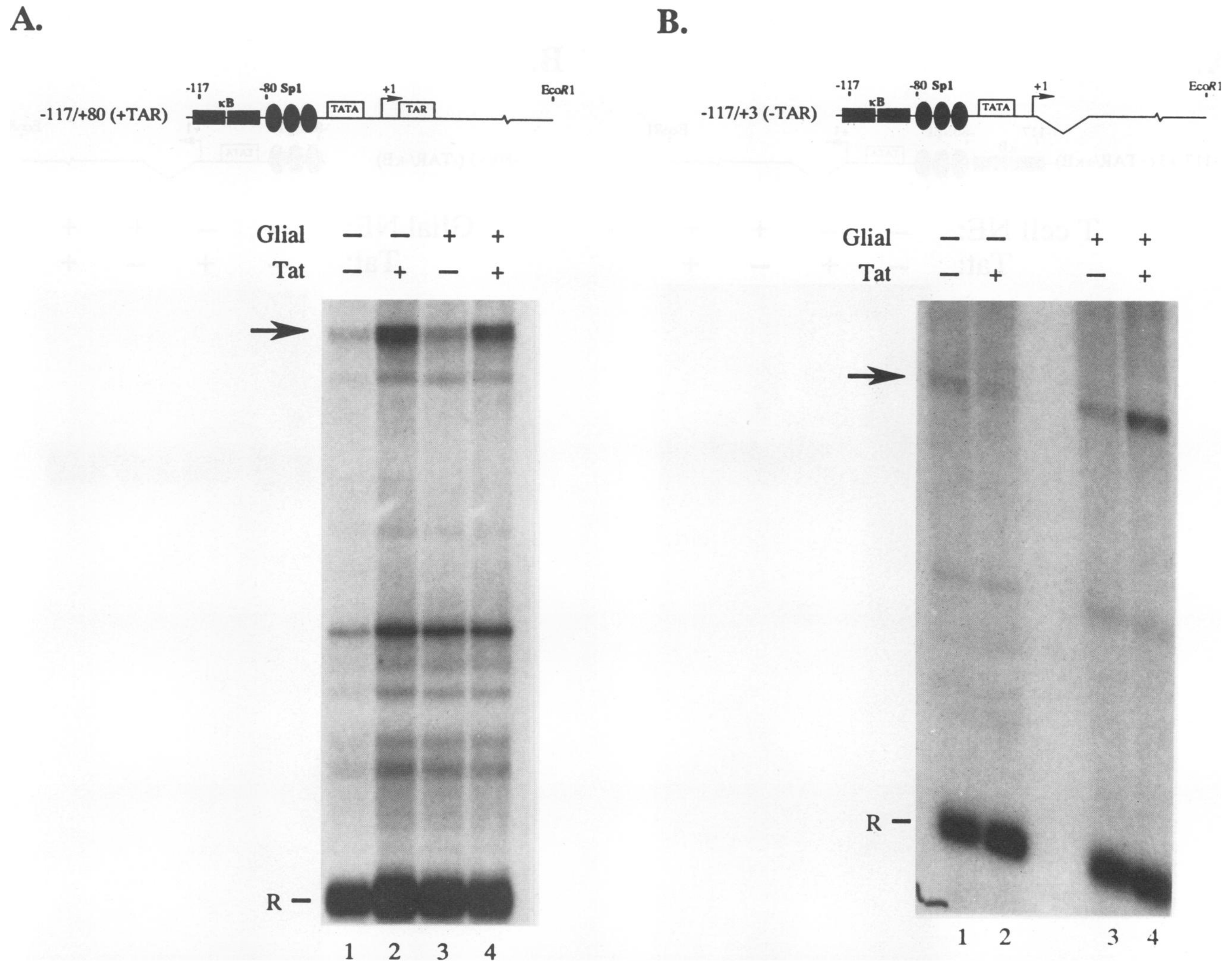



FIG. 5. TAR-independent activation of HIV-1 transcription by Tat in vitro requires astrocytic nuclear factors. (A) In vitro transcription of template −117/+80. The arrow indicates a runoff transcript of 356 nucleotides. Lanes: 1, basal transcription in HeLa transcription extract alone; 2, same as lane 1 with the addition of 1 μM synthetic Tat protein; 3, basal transcription in HeLa whole-cell extract supplemented with nuclear extract from unstimulated U-87MG astrocytes; 4, same as lane 3 with the addition of 1 μM synthetic Tat protein. (B) Template −117/+3. The arrow indicates a runoff transcript of 279 nucleotides. The lanes are as described in the legend to panel A. R, nonspecific reference band used as a control for loading; −, absent; +, present. The intensity of the bands was determined by densitometry of multiple experiments. Representative gels are shown.

**astrocyte nuclear factors.** TAR-dependent activation of HIV-1 transcription by Tat can be observed in vitro with the HeLa whole-cell system, as has been previously reported (29, 36). The addition of an optimal concentration (1 μM) of synthetic Tat results in approximately a sevenfold ± twofold increase in transcription from the −117/+80 template (Fig. 5A, lanes 1 and 2). The ability of Tat to activate HIV-1 transcription in the HeLa system is dependent on TAR, as demonstrated by the inability of Tat to activate transcription from template −117/+3, which has the TAR element removed (Fig. 5B, lanes 1 and 2). Our previous studies carried out in vivo indicated that astrocytes support TAR-independent activation of HIV-1 transcription by Tat (60). Supplementation of HeLa transcription extract with nuclear factors from unstimulated U-87MG astrocytes does not influence transactivation of template −117/+80 (Fig. 5A, lanes 3 and 4). However, the presence of nuclear factors from unstimulated astrocytes contributes to an approximately 4.5-fold ± 2-fold activation of transcription by Tat from template −117/+3 (Fig. 5B, lanes 3 and 4), demonstrating TAR-independent activation in vitro. Interestingly, when this experiment was performed with PMA-stimulated astrocytic nuclear extracts, the addition of Tat resulted in a decrease in transcription, possibly representing a squelching phenomenon. Similar decreases in transcription were observed with 3- and 20-h PMA-stimulated glial extract (unpublished observation).

Supplementation of the HeLa system with nuclear extracts prepared from unstimulated Jurkat T lymphocytes does not permit Tat activation of the TAR-deleted −117/+3 template (Fig. 6A), further suggesting that the ability to support TAR-independent Tat responsiveness is a feature unique to astrocytes. It has been reported that nuclear extract from PMA-stimulated Jurkat T cells activates HIV-1 transcription in vitro (33). It has also been reported that TAR-independent replication of HIV-1 is observed in PMA-stimulated Jurkat T cells (6, 26). Perhaps T-cell activation results in the expression of an

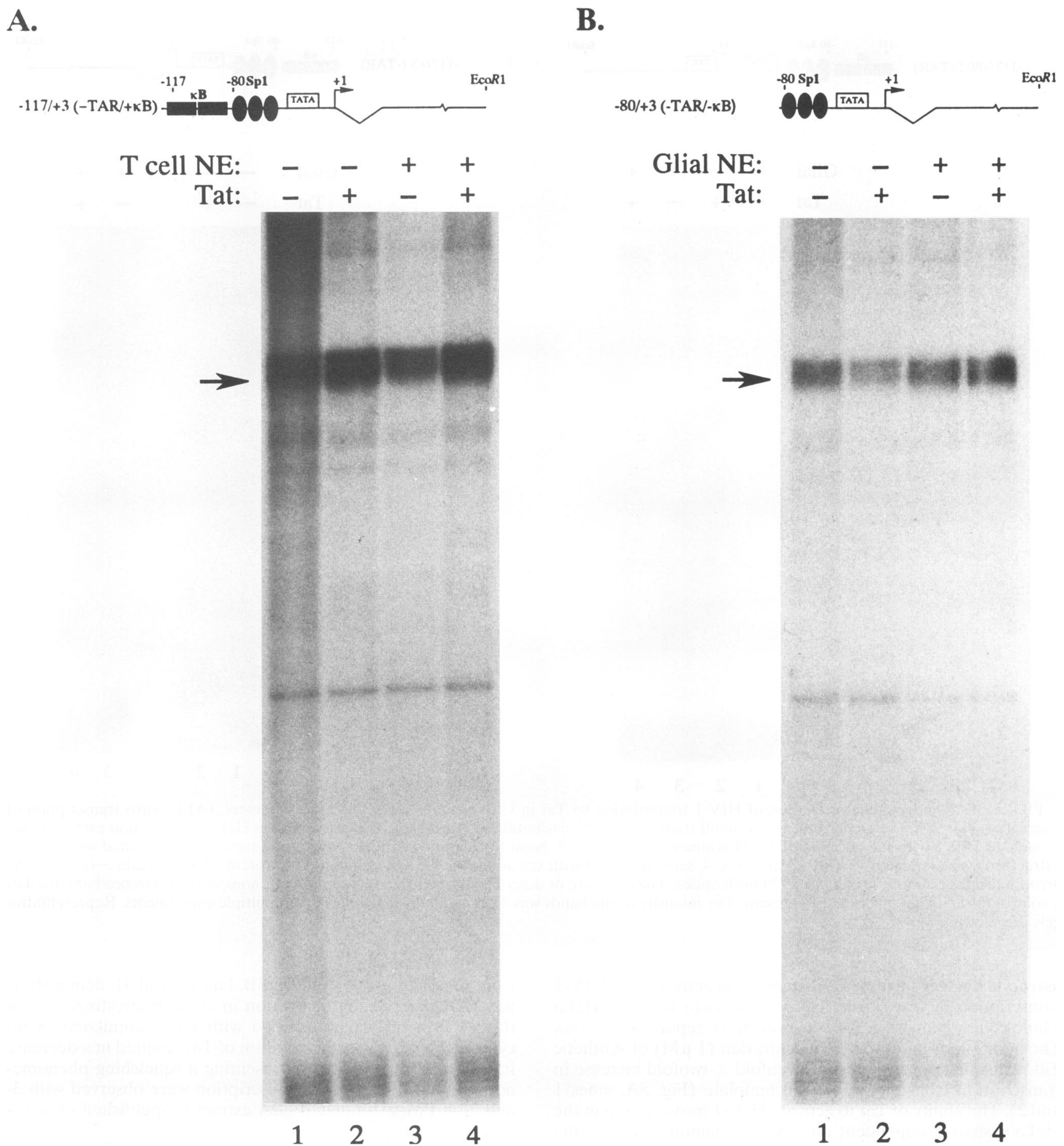



FIG. 6. (A) Jurkat T-cell nuclear factors do not support TAR-independent Tat transactivation. In vitro transcription of template −117/+3 is shown. The arrow indicates a runoff transcript of 279 nucleotides. Lanes: 1, basal transcription in HeLa whole-cell extract alone; 2, same as lane 1 with the addition of 1 μM synthetic Tat protein; 3, basal transcription in HeLa whole-cell extract supplemented with nuclear extract from unstimulated Jurkat T cells; 4, same as lane 3 with the addition of 1 μM synthetic Tat protein. (B) TAR-independent transactivation of HIV-1 transcription by Tat requires the κB-binding sites. In vitro transcription of template −80/+3 is shown. The arrow points to a correctly sized runoff transcript of 279 nucleotides. Lanes: 1, basal transcription in HeLa whole-cell extract alone; 2, same as lane 1 with the addition of 1 μM synthetic Tat protein; 3, basal transcription in HeLa whole-cell extract supplemented with nuclear extract from unstimulated astrocytes; 4, same as lane 3 with the addition of 1 μM synthetic Tat protein. NE, nuclear extract; −, absent; +, present. The intensity of the bands was determined by densitometry of multiple experiments. Representative gels are shown.

NF-κB-related activity that is common to, and enriched in, astrocytes. Our current studies are examining the ability of PMA-stimulated Jurkat T cells to support TAR-independent Tat activation in vitro and the relationship of this mechanism to that observed in astrocytes (unpublished data). TAR-independent activation of HIV-1 in vivo was found to be mediated by the κB domain of the viral LTR (6, 60). As expected, the ability of astrocytic nuclear factors to support TAR-independent Tat responsiveness in vitro requires a functional κB-binding site. Transcription from template −80/+3, in which both TAR and the κB-binding sites have been deleted, is not activated by Tat, implicating the astrocytic κB-binding factors as mediators of Tat activity (Fig. 6B). Further evidence for the involvement of κB-binding factors in mediating Tat transactivation comes from competition experiments. Excess oligonucleotide competitors were used to interrupt interaction of κB-binding factors with the promoter. Wild-type κB oligonucleotide, but not mutant oligonucleotide, inhibited Tat activation (unpublished data).

## DISCUSSION

Differential utilization of transcription factors, determined by different subunit combinations or interactions with modulatory activities, is a mechanism by which a cell can increase the functional diversity of a limited number of gene products. Such diversity is observed in the activity and specificity of the NF-κB family of transcription factors. In Jurkat T cells, the specific complex of p49-p65 works in concert with Tat to stimulate HIV-1 transcription, while a complex of p50-p65 may not (44). In U-87MG glial astrocytes, a newly recognized κB-binding activity is observed which is retained on a Tat affinity column and is capable of activating HIV-1 transcription. In addition, the data suggest that the astrocyte-derived κB-binding activity is responsible for mediating TAR-independent transactivation by Tat.

Prototypical NF-κB consists of two protein subunits, 50 and 65 kDa, termed p50 and p65, respectively. The cloning of the genes encoding p50 and p65 allowed recognition that they were both related by homology in their amino-terminal region to the product of the *rel* oncogene. In vivo, prototypical NF-κB consists of a heterodimeric complex of p50 and p65 which is sequestered in an inactive form in the cytoplasm by association of the p65 subunit with an inhibitory activity, IκB (3–5). NF-κB binding activity is rapidly induced by dissociation of IκB from the cytoplasmic complex, allowing nuclear translocation of p50 and p65 (3–5). Upon binding DNA, both p50 and p65 contribute to transcriptional activation (23). Activation of NF-κB, in most cell types, occurs independently of de novo protein synthesis and seems to involve phosphorylation of the inhibitor IκB (24, 27). Recently, a gene related to, but distinct from, p50 was cloned (51). The product of this gene, p49, is capable of forming a complex with p65 which activates HIV-1 transcription in Jurkat T cells (44, 51). Evidence suggests that the heterodimeric complex p49-p65, but not p50-p65, works in concert with Tat to stimulate HIV-1 transcription, demonstrating that functional differences result from different combinations of κB-related proteins (44).

The κB-binding factors present in astrocytes show characteristics of prototypical NF-κB, such as specificity of binding, inducibility by PMA, and the bimodal kinetics of this induction. However, electrophoretic mobility of the κB complexes derived from astrocytes differs from that of those seen in lymphocytes, demonstrating heterogeneity. The α complex derived from U-87MG cells comigrates with the B1 complex of Jurkat T cells and is induced with similar kinetics (Fig. 1) (38). Further, two-dimensional UV cross-linking experiments strongly suggest that astrocytic α and lymphocytic B1 complexes contain the same proteins in the same stoichiometry (unpublished data). Taken together, these observations suggest that the α complex is the U-87MG equivalent of the Jurkat B1 complex, both representing prototypical NF-κB. Most interesting, however, is the presence of a more slowly migrating κB-binding complex β in U-87MG cells which has no readily observable counterpart in Jurkat T cells (Fig. 1). It is the astrocyte-derived β complex which is retained on the Tat affinity column, a feature not observed for either the astrocyte-derived α complex or the Jurkat-derived NF-κB (Fig. 2). The β complex may be composed of novel, CNS-specific proteins of the Rel family of κB-binding factors. Preliminary observations suggest, however, that the β complex represents a supershifting of prototypical NF-κB by a CNS-enriched activity that is capable of interacting with Tat.

The κB-binding activities of U-87MG cells are demonstrated to activate transcription in a promoter-specific manner that is dependent on the presence of κB-binding sites (Fig. 3 and 4), which classifies these binding activities as transcription factors. It is likely that the ability of astrocyte-derived κB factors to interact with Tat is related to TAR-independent Tat transactivation which is mediated through the κB domain of the HIV-1 LTR (6, 26, 60). TAR-independent transactivation of HIV-1 transcription by Tat is observed in vitro and, as expected, is dependent on the presence of both astrocytic nuclear factors and κB-binding sites within the promoter (Fig. 5 and 6).

The ability of Tat to potently activate the rate of HIV-1 transcription has been attributed to increased efficiency of transcriptional elongation, though increased transcriptional initiation in the presence of Tat has also been observed (reviewed in reference 22). It has been proposed that Tat functions in the vicinity of the HIV-1 promoter to bias initiation towards the formation of more-processive transcription complexes. This model is consistent with results implicating the participation of the κB domain and the Sp1 domain in Tat transactivation (7, 30, 55), as well as kinetic studies which suggest that a transient interaction between Tat and nascent TAR RNA is sufficient for wild-type levels of transactivation (8, 28). In other systems, distinct classes of RNA polymerase II transcription complexes, with differing efficiencies of elongation, have been observed and studied (37), and evidence suggests that their formation may be determined by specific conditions of transcriptional initiation (56). While Tat is the first identified promoter-specific elongation factor, it would not be surprising to discover that this mechanism of transcriptional regulation is not unique to HIV-1.

In addition to its role in regulating HIV-1 transcription, Tat appears to play other roles in the pathogenesis of AIDS. This probably reflects the recently recognized ability of Tat to influence the expression of select cellular genes (11, 50, 58). Functional interaction between Tat and cellular transcription factors, such as those interacting with κB, may be involved in initiating the cascade of altered cellular gene expression that contributes directly to disease. Consistent with this model, Tat has been directly implicated in the development of immunodeficiency (11, 62), as well as the development of Kaposi's sarcoma (18, 63).

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