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Identification of a Cellular Protein That Binds to Tat-Responsive Element of TGFβ-1 Promoter in Glial Cells

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Abstract Tat is a transcriptional transactivator produced by the human immunodeficiency virus type 1 (HIV-1) and plays a pivotal role in enhancing expression of the viral genome in the infected cells. Although initial studies have suggested that interaction of Tat with the transactivation responsive element (TAR), located within the LTR, is essential for Tat function, subsequent studies indicated that Tat has the ability to augment transcription of viral and cellular genes by a TAR-independent mechanism. In early studies we demonstrated that HIV-1 Tat stimulates transcription of the transforming growth factor, TGFβ-1, gene in glial cells. In this study, we have identified a cellular protein that interacts with the Tat-responsive region located between nucleotides -323 to -453 of the regulatory sequence of the TGFβ-1 promoter. Results from footprinting analysis revealed association of cellular proteins with the 130 nucleotide sequence located in the Tat-responsive region. Analysis of the associated protein by UV-crosslinking suggested the involvement of a protein between 40–45 kDa in size which preferentially interacts with the GC/GA rich sequence of the TGFβ-1 Tat-responsive sequence in a single-stranded configuration. The ability of the previously identified 40 kDa protein, named Pur α to bind to the GC/GA sequence in the single-stranded configuration, similar to those from TGFβ-1 promoter prompted us to investigate its binding capacity to the TGFβ-1 sequence and its transcriptional activity on the TGFβ-1 promoter. Results from band shift studies indicated the association of the bacterially produced Pur α to the TGFβ-1 DNA sequences positioned within the Tat-responsive region. Overexpression of Pur α in glial cells constitutively producing Tat augmented transcription of the TGFβ-1 gene. These results are consistent with previous reports on the cooperative action of Pur α and Tat in modulating other eukaryotic promoters. The importance of these findings with regard to deregulation of other cellular genes by HIV-1 Tat is discussed. J. Cell. Biochem. 67:466–477, 1997.

Key words: Tat; TAR; HIV-1; TGFβ-1 promoter

Infection with the human immunodeficiency virus type 1 (HIV-1), an etiologic agent of AIDS, often leads to a broad range of neurological disorders [Spencer and Price, 1992], however, the pathogenesis of these disorders is not completely understood. The presence of the virus in the brain and peripheral nerves has been confirmed by a variety of techniques [Bagasra et al., 1996; Nuovo et al., 1994; Pumarola-Sune et al., 1987]. Within the brain, infection appears to be confined primarily to macrophages, microglial cells [Koenig et al., 1986; Koyonagi et al., 1987; Merrill et al., 1991], with evidence for limited infection of other cell types in the brain including astrocytes, oligodendrocytes, neurons, and vascular endothelial cells [Bagasra et al., 1996; Dewhurst et al., 1987; Moses et al., 1993; Saito et al., 1994]. The observed limited infection of CNS cells with HIV-1 and the large scope of histopathological abnormality of the AIDS brain has led to the assumption that an indirect pathway induced by the infected cells should be involved in the pathogenesis of AIDS neurological disorders.

In this respect, most attention had been focused on the secretory proteins from the infected cells including viral regulatory proteins

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and cytokines [Atwood et al., 1993]. In support of this notion, results from in vitro cell culture indicated that HIV-1 infected cells may release the viral regulatory protein, Tat, in the media, which in turn, can be taken up by neighboring uninfected cells [Frankel and Pabo, 1988; Marcuzzi et al., 1992]. Tat is a small peptide which has the ability to regulate transcription of a variety of viral and cellular genes upon binding to the RNA target sequence, named TAR, and/or interacting with a variety of transcription factors [Chowdhury et al., 1992; Gaynor, 1995; Kingsman and Kingsman, 1996; Liu and Latchman, 1997]. Earlier reports indicated that the level of transforming growth factor β-1 (TGFβ-1) is increased within the brain of HIV-1 infected individuals compared to the brains of noninfected controls [Morganti-Kossman et al., 1992; Wahl et al., 1991]. TGFβ-1 is a multifunctional polypeptide that functions as a regulator of proliferation, differentiation, and other functions of many cell types [Massague, 1990]. This immunomodulator is involved in the control of cell proliferation and is a stimulator of extracellular matrix formation. Results from transient transfection assays indicated that ectopic expression of Tat in human astrocytic cells increases transcription of the TGFβ-1 promoter [Cupp et al., 1993]. Furthermore, intracerebral inoculation of a recombinant replication-defective HSV-1 Tat expression vector stimulated expression of TGFβ-1 in the murine central nervous system [Rasty et al., 1996]. These observations strongly suggest that HIV-1 Tat stimulates transcription of the TGFβ-1 promoter in in vitro cell culture and in an in vivo animal system. To better understand the molecular mechanism involved in the induction of the TGFβ-1 promoter by HIV-1 Tat, we utilized mutant deletion constructs and identified a region between nucleotides -453 to -323 upstream of the major transcription start site of the TGFβ-1 which confers responsiveness to Tat induction.

Here, we performed a series of DNA binding studies and identified a nucleoprotein complex which forms with the Tat responsive sequence of the TGFβ-1 promoter. DNA structure requirement for interaction of the cellular protein with the TGFβ-1 promoter and the size of the associated protein led us to believe that a previously identified protein, Pur α, is involved in this interaction. Results from in vitro DNA binding studies utilizing highly purified Pur α, and in vivo transfection of glial cells with Pur α expression plasmid indicated that Pur α binds to TGFβ-1 and has the ability to increase TGFβ-1 promoter activity in astrocytic cells. Of particular importance, earlier studies indicated that Pur α, which exhibits a great affinity to the GC/GA-rich sequence in the single-stranded configuration, directly interacts with HIV-1 Tat protein. Thus, it is likely that cooperative interaction of Tat and Pur α leads to the stimulation of the TGFβ-1 promoter in CNS cells.

MATERIALS AND METHODS

Cell Culture

U-87MG, an established human astrocytic cell line, was obtained from American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY), supplemented with 10% (vol/vol) fetal bovine serum (Gibco) or SerumPlus (JRH Biosciences, Hazelton Biologics, Inc., Lenexa, KS) and 1% (vol/vol) Pen/Strep (Gibco). The Tat-producer cell line, 5–10, is a U-87MG derivative stably transfected with HIV-1 Tat under the control of the SV40 late promoter [Taylor et al., 1992]. This cell line was maintained as described above, with the addition of 0.3 mg/ml G418 (Gibco).

DNase 1 Footprinting

A 130 bp HincII-BstEII fragment of the phTG2 construct [Kim et al., 1989] was labeled at the BstEII site either by filling in the 3′ end with [α^{32}P]dCTP and Klenow (Boehringer Mannheim, Indianapolis, IN), or by labeling the 5′ end with [γ-^{32}P]ATP using T4 polynucleotide kinase. DNA-binding reactions and subsequent digestion with DNase 1 were carried out according to established protocols (Current Protocols in Molecular Biology).

Plasmids

phTG 2, 5, and 6 are a series of deletion constructs of the 5′ promoter region of the human TGFβ-1 gene fused to pGEM-SVOCAT (Kindly provided by Dr. Seon jin Kim and Dr. Anita Roberts, NIH/NCI, Bethesda, MD) [Kim et al., 1989]. pCSMA-Pur α represents a Pur α expressor plasmid driven by cytomegalovirus (CMV) promoter. For RT-PCR analysis of TGFβ-1 message, the construct λβC1 [Derynck et al., 1985], harboring TGFβ-1 cDNA was used.

Oligonucleotides

Oligonucleotides 5′ and 3′ represent the 5′ and the 3′ region of the protected DNA sequence within the 130 bp TGFβ-1 promoter, respectively. The structure of the oligonucleotides is depicted below.

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5′ oligo  5′ GGATTAAGCCTTCTCCGC 3′ (coding strand)
          3′ GCGGAGAAGGCTTAATCC 5′ (noncoding strand)
3′ oligo  5′ CTGGTCCTCTTTCTCTGGT 3′ (coding strand)
          3′ ACCAGAGAAAGAGGACCAG 5′ (non-coding
                                     strand)
MB3       5′ TGCCTTGCAGGATGCCCACCCAGCTGACCCAGGG 3′
          3′ ACGGAACGTCCTACGGGTGGGTCGACTGGGTCC 5′.
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Expression and Purification of Pur α From Bacteria

Glutathione-S-transferase (GST)-Pur α fusion protein was produced in bacterial expression vector using the plasmid GST-2T. The protein was expressed in *Escherichia coli* and purified on GST affinity column (Pharmacia, LKB Biotechnology). Protein concentration was measured by the Bradford assay (Bio-Rad, Hercules, CA), and the purity of the protein was examined by SDS-PAGE. The eukaryotic expression vector was constructed by inserting cDNA sequences for Pur α in frame into pCSMA vector [Bergemann et al., 1992].

Gel Mobility Shift Assay

Gel-shift assays with purified Gst-Pur α were performed essentially as described [Chen et al., 1995]. Binding reactions were performed in total volumes of 25 µl, containing 0.5 to 1 µg of Gst-Pur α, 1 µg of poly (dI:dC) in binding buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5% glycerol, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA for 20 min on ice, before the addition of the probe. For competition studies, proteins were incubated with unlabeled oligonucleotides on ice for 15 min prior to addition of probe DNA. Protein-DNA complexes were analyzed on a 6% polyacrylamide gel. In gel shift analysis using the purified protein, 1–3 µg of the bacterially purified Pur α was used.

UV Cross-Linking

Binding reactions were carried out according to the protocol described previously [Khalili et al., 1988]. The reaction mixture was subsequently cross-linked using a hand-held long wave UV light (305 nm) on ice for 15 min. Resulting complexes were denatured by heating for 7 min prior to analysis on an 8% SDS-PAGE.

Transient Transfection and CAT Assays

U-87MG cells were plated in 60 mm tissue culture plates for 24 h prior to transfection at a density of 5×10^5 cells per plate. Three h before the addition of DNA, cells were fed with DMEM supplemented with 10% vol/vol serum plus medium supplement (JRH Biosciences, Hazelton Biologics, Inc., Lenexa, KS). Transfection was carried out by the calcium phosphate/DNA coprecipitation method [Graham and Van der Eb, 1973]. Three h following transfection, cells were treated with 15% glycerol for 1–2 min, after which cells were maintained in the cell culture media. Forty-eight h following transfection, cell extracts were prepared and CAT assays were performed essentially as described [Gorman et al., 1982].

RT-PCR and Southern Analysis

Forty-eight h following transfection of cells with Pur α expression plasmid, total RNA was isolated by Acid-Guanidinium-phenol-Chloroform (AGPC) method [Chomczynski and Sacchi, 1987]. Reverse transcriptase (RT) reactions were performed using 2 µg of total RNA and 20 pmol oligo d(T) 12–18, heated at 65°C for 5 min, and annealed at 37°C for 5 min. The mixture was then incubated at 42°C for 60 min in 20 µl of buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM Mg Cl_2, 1 mM deoxynucleotide triphosphates, and 1.5 U/µl of Mo-MuLV-RT (Boehringer Mannheim). Reactions were terminated by heating at 95°C for 5 min. PCR contained 10 pmol of primer pairs and 1 µg of cDNA in 100 µl of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, and 0.05 U/µl of Taq DNA polymerase (Boehringer Mannheim), and subjected to 20 cycles at 94°C for 1 min., 55°C for 1.5 min, and 72°C for 1 min using a DNA Thermocycler (Perkin-Elmer, Norwalk, CT). DNA fragments were resolved by electrophoresis on 1.4% acrylamide gels and following denaturation and neutralization, were transferred to nylon membranes (Hybond N, Amersham, Arlington Hts, IL). The amplified DNA fragments were subsequently hybridized to [α^{32}P]-labeled TGFβ-1 probe.

RESULTS

Identification of the Protein-Binding Sequences Within the Tat Responsive Region of TGFβ-1 Promoter

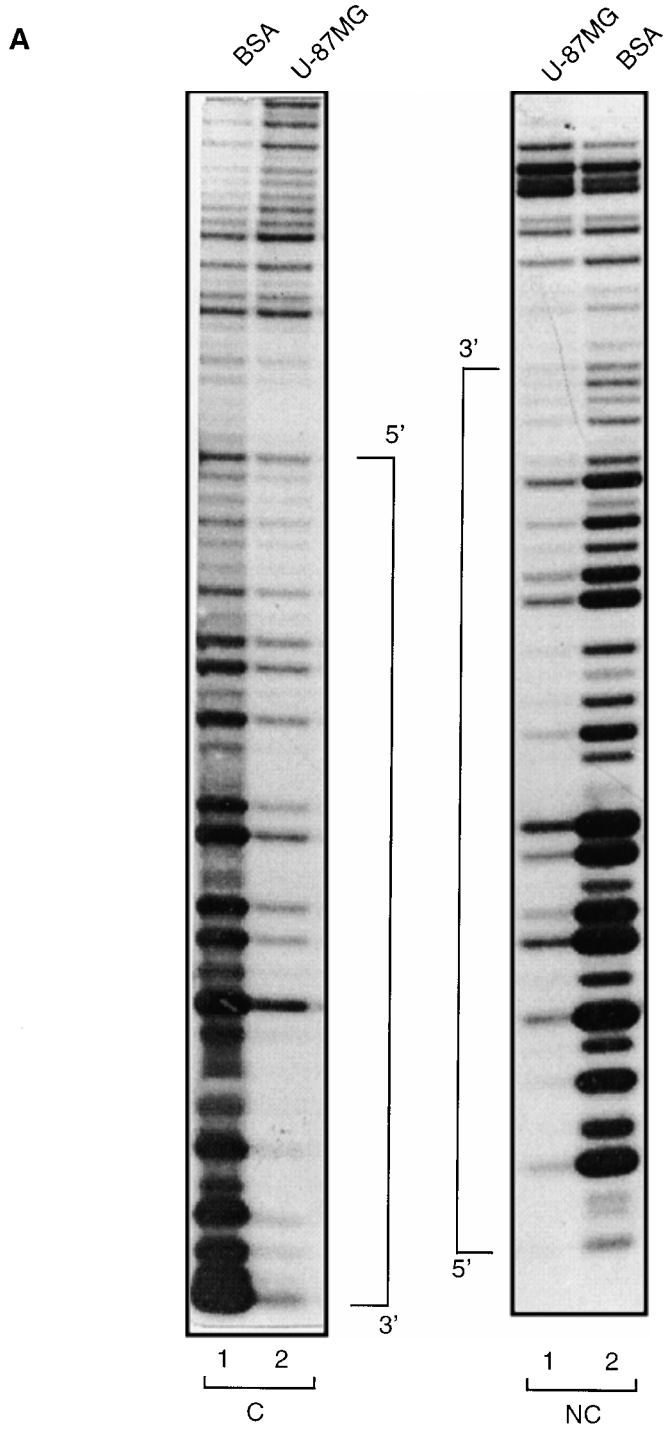
In the previous study, we utilized promoter deletion constructs and identified a 130 bp region between nucleotides -453 to -323 with respect to the transcription start site (position +1) which confers Tat-responsiveness to the TGFβ-1 promoter [Cupp et al., 1993]. Our repeated DNA binding experiments suggested that Tat may not directly associate with the 130 bp DNA sequence, suggesting that activation of the TGFβ-1 promoter by Tat may be mediated by a cellular protein(s) which interacts with the Tat-responsive region. In an effort to identify the participant cellular protein, we performed DNase I footprinting assay to locate the protein binding domain within the 130 bp sequence of the TGFβ-1 promoter. Toward this end, a 130 bp HincII-BstEII DNA fragment derived from phTG2 which contains the TGFβ-1 upstream regulatory sequences was labeled with [^{32}P] at the 5′-terminus. The labeled DNA fragment was incubated with nuclear protein extract prepared from human astrocytic cells, U-87MG, prior to partial digestion with DNase I. The cleavage products were analyzed by electrophoresis on denaturing polyacrylamide gels. As shown in Figure 1A, with both coding and non-coding strand probes, a broad region spanning between nucleotides -354 to -329 exhibited binding activity to the nuclear protein from U-87MG and was protected against DNase I digestion. Using similar protein concentrations and binding conditions, the control bovine serum albumin (BSA) showed no binding ability to either coding or non-coding strands. Figure 1B illustrates the position of the protected area within the TGFβ-1 Tat responsive region.

Detection of Nucleoprotein Complex Associated With the Tat-Responsive Region of TGFβ-1 Promoter

To gain more information regarding the protein which binds to the TGFβ-1 Tat-responsive element, synthetic oligonucleotides corresponding to the 5′ and the 3′ ends of the protected region were prepared and utilized in UV-crosslinking assay. In this assay, the double-stranded [^{32}P] labeled DNAs were separately incubated with nuclear protein from U-87MG and after complex formation, the reaction was irradiated with UV light for 10 min and resolved by 10% SDS-PAGE. One major complex with approximate molecular mass between 40 to 45 kDa was reproducibly observed with both 5′ and 3′ probes (Fig. 2, lanes 1, 5). To verify the specificity of the protein-DNA interaction, competition experiments were performed using unlabeled 5′ or 3′ oligonucleotides or the unrelated MB3 oligonucleotide which exhibited no significant sequence homology to the probes. As shown in Figure 2, inclusion of 50-fold molar excess of 5′-DNA competitor significantly diminished formation of a 40–45 kDa complex with the 5′ probe (compare lanes 1 and 2). Under identical conditions, control MB3 oligonucleotide showed a modest inhibitory effect on the formation of the complex (compare lanes 1 and 3), whereas the 3′-DNA competitor completely abrogated association of the nucleoprotein complexes (compare lanes 1 and 4). These results suggest that the 5′- and 3′-DNA oligonucleotides may interact with a common cellular protein and that the 3′-DNA may possess a higher binding affinity than the 5′-DNA. This notion was further supported by the results of competition experiments when binding of the 3′ probe to the protein was examined in the presence of unlabeled 5′, 3′, and the MB3 competitors. As shown in Figure 2, association of the 3′ probe to the nuclear protein was completely abrogated with unlabeled 3′-competitor (compare lanes 5 and 6), remained virtually unchanged with the MB3 (compare lanes 5 and 7), and showed a minor effect with the 5′-competitor (compare lanes 5 and 8).

Association of Pur α With the Tat Responsive Element of the TGFβ-1 Promoter

Close inspection of the DNA sequences corresponding to the Tat-responsive sequence which contains several GA and GC motifs, and association of a protein with approximately 40 to 45 kDa with DNA probe led us to speculate that Pur α may be associated with the TGFβ-1 regulatory sequence. We were also intrigued with earlier studies indicating that Pur α and Tat interact with each other, and stimulate transcription of human neurotropic JCV promoter in transfected cells [Krachmarov et al., 1996]. As such, in the next series of experiments we examined binding of highly purified bacterially produced Pur α which was fused to GST to the 5′- and the 3′- oligonucleotides of the TGFβ-1 promoter. Since Pur α exhibits a greater affinity



B

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-422 GCCTGACTCT CCTTCCGTTC TGGGTCCCCC TCCTCTGGTC -383
     CGGACTGAGA GGAAGGCAAG ACCCAGGGGG AGGAGACCAG

-382 GGCTCCCCTG TGTCTCATCC CCCGGATTAA GCCTTCTCCG -343
     CCGAGGGGAC ACAGAGTAGG GGGCCTAATT CGGAAGAGGC

-342 CCTGGTCCTC TTTCTCTGGT GACCCACACC GCCCGCAAAG -303
     GGACCAGGAG AAAGAGACCA CTGGGTGTGG CGGGCGTTTC
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Figure 1.

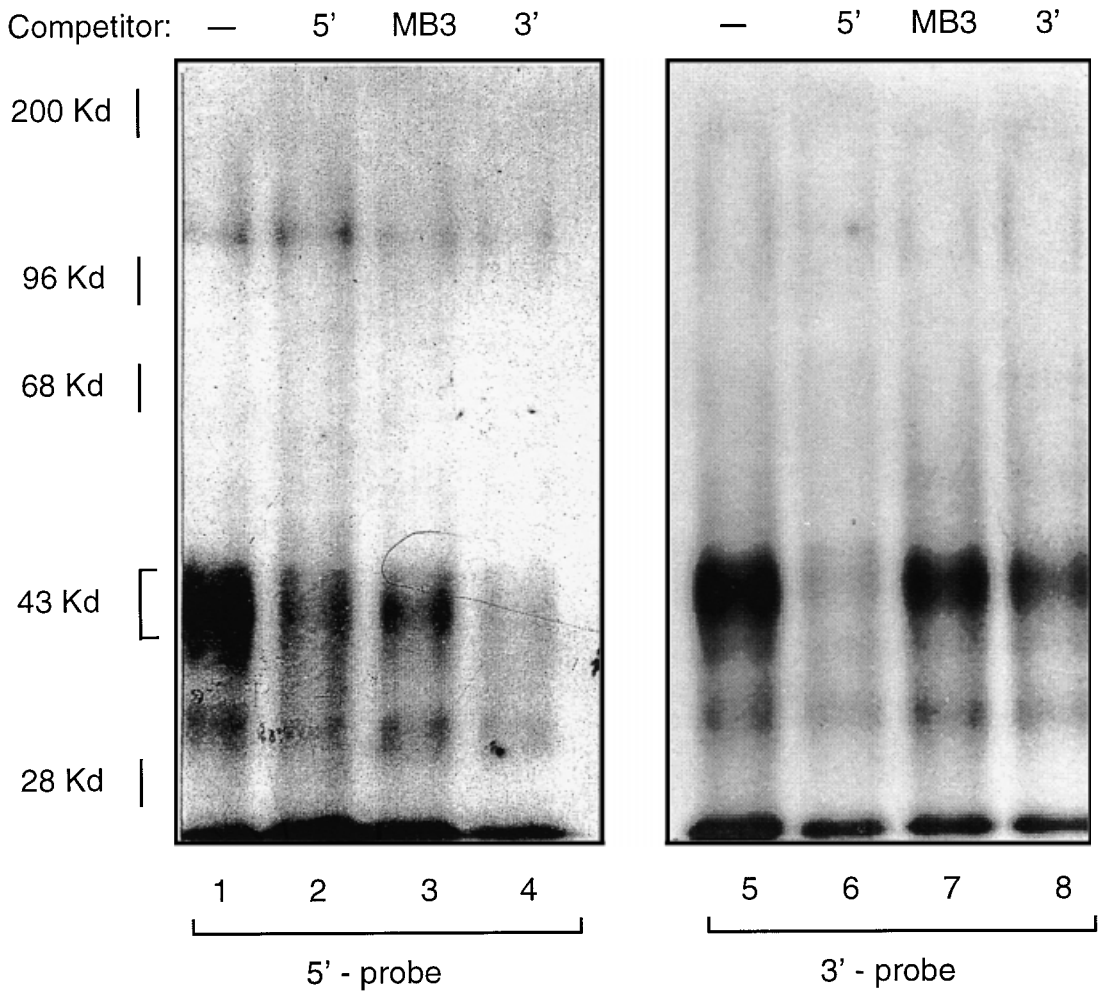

Fig. 2. UV-crosslinking and competition binding. The single-stranded oligonucleotide representing 5′ **(lanes 1–4)** or the 3′ **(lanes 5–8)** of the protected region (as depicted in Figure 1B) were uniformly labeled with BUdR and covalently cross-linked to proteins present in U-87MG cells in the absence (lanes 1 and 5) or presence of 50-fold excess of single-stranded competition DNAs. The numbers on the left represent the molecular size of the marker proteins.

Fig. 1. DNase 1 footprint analysis of the Tat-responsive region within TGFβ-1 promoter. **A:** DNase 1 footprint analysis was performed using the 130 bp region spanning nucleotides -453 to -323 of the TGFβ-1 promoter as a probe. In lanes depicted by C, the coding strand was labeled using [γ-^{32}P] ATP and T4 polynucleotide kinase and in lanes shown by NC labeling of the non-coding strand was done by filling in the 3′ end with [α^{32}P]dCTP and Klenow. Probes were incubated with 10 µg of cell extract from U-87MG glial cells or 10 µg of BSA followed by digestion with a 1:10 dilution of DNase 1 (Boehringer Mannheim) and electrophoresis on a 9% sequencing gel at 90 Watts. **B:** Primary sequence of the 130 bp representing the Tat-responsive region and the area of protection is highlighted by the brackets.

to the single-stranded DNA probes, these studies were performed with both double- and single-stranded DNA probes. As shown in Figure 3, Pur α showed moderate binding activity to the double-stranded 5′-probe and exhibited substantially higher binding affinity to the single-stranded coding strand, and lower affinity to the single-stranded noncoding strand of DNA (Fig. 3A, lanes 2, 4, 6, respectively). Under similar conditions, GST showed no binding affinity to the single- or double-stranded probe (Fig. 3A, lanes 1, 3, 5). Similar results were obtained with double- and single-stranded 3′-DNA probes, with the notion that binding of Pur α to the single-stranded noncoding probe was significantly higher than those observed with the other DNAs from the 3′ or the 5′

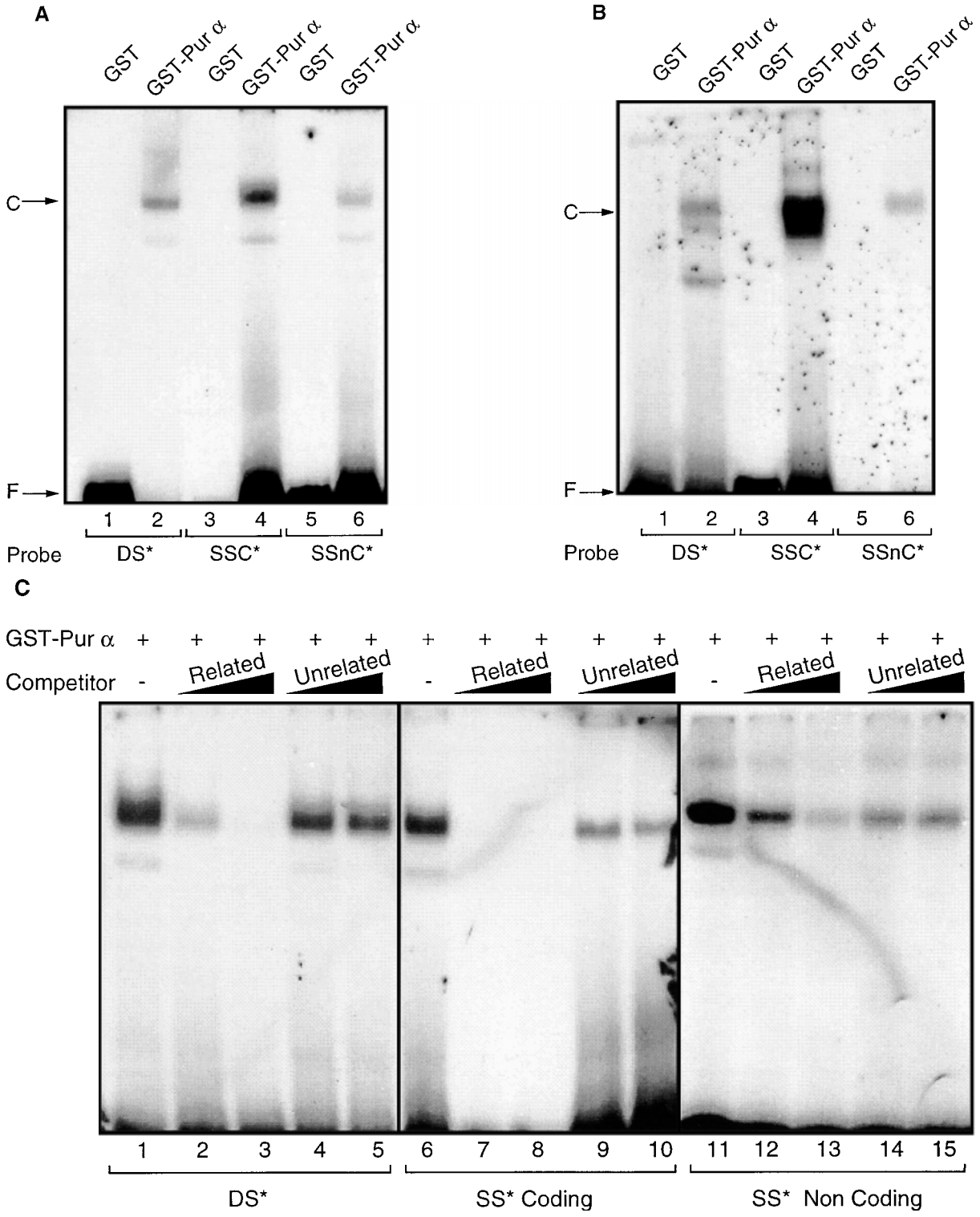

Fig. 3. Electrophoretic mobility shift analysis of Pur α binding to Tat-responsive elements within TGFβ-1 promoter. **A:** 5′-end-labeled single-stranded and duplex oligonucleotides from the 5′ region of TGFβ-1 Tat responsive element were analyzed for their binding to form complex with GST-Pur α or the control GST. **Lanes 1 and 2** present binding of the double-stranded probes (DS) to GST and GST-Pur α, respectively, whereas **lanes 3–6** present binding of GST and GST-Pur α to single-stranded coding (SSC) and noncoding (SSnC) strands. F and C depict positions of free probe and the major complex, respectively. **B:** Gel shift analysis of the 5′ end labeled single- and double-stranded oligonucleotides from the 3′ region of the TGFβ-1 Tat responsive element as described in (A). **C:** Specificity of Pur α binding to double- and single-stranded DNAs was examined by preincubation of GST-Pur α with 50 and 250 ng of unlabeled DNAs corresponding to probe (related), or the DNAs with limited GC/GA nucleotides (unrelated).

regions (Fig. 3B, lane 4). The sequence specificity of this interaction was determined by competition analysis with unlabeled oligonucleotides derived from the 5′ region of the TGFβ-1 Tat-responsive region (related) or the DNA with a limited GC content (unrelated). Results from these studies indicated that whereas the related competitors effectively blocked association of Pur α with double- and single-stranded 5′ probe (Fig. 3C), unrelated DNA showed no drastic effect in the double-stranded configuration and modestly affected, but not abrogated, binding of Pur α to the labeled probe. A similar set of competition experiments was performed to verify the specificity of the DNA:Pur α association using the 3′ region of the TGFβ-1 Tat-responsive region as a probe (data not shown). These data demonstrated that prokaryotically produced Pur α binds to the TGFβ-1 Tat-responsive sequence in a specific manner.

Activation of the TGFβ-1 Promoter by Pur α in Glial Cells

The ability of Pur α to modulate transcription of the TGFβ-1 promoter, and the importance of the Pur α binding site within the promoter region were evaluated by cotransfection of U-87MG cells. Three promoter reporter constructs containing 1132 nucleotides (phTG 2), 453 nucleotides (phTG5), and 323 nucleotides (phTG6) from the TGFβ-1 upstream regulatory sequence fused to the 5′ of the CAT gene (as shown in Fig. 4A) were introduced separately into U-87MG cells alone or together with a Pur α expression plasmid (pCMV-Pur α). As shown in Figure 4B, the basal transcriptional activity of phTG2 and phTG5 was increased in cells with ectopic expression of Pur α. Transcriptional activity of phTG6 which lacks Pur α binding sites between -453 to -322 remained virtually constant in the absence or presence of Pur α in the transfected cells. These results indicate that the Pur α binding site located within -453 to -323 is functionally active and responsive to Pur α activation in astrocytic cells.

Next, the capacity of Pur α to stimulate expression of the endogenous TGFβ-1 was examined by measuring the level of TGFβ-1 RNA with the RT-PCR technique. In this study, U-87MG cells that constitutively express HIV-1 Tat, 5–10 [Taylor et al., 1992], were transfected with pCMV-Pur α, and after 48 h, total RNA was prepared and subjected to RT-PCR utilizing oligonucleotide primers derived from the TGFβ-1 sequence. As shown in Figure 5, expression of the TGFβ-1 gene in Tat-producing cells was significantly increased upon overproduction of Pur α (Fig. 5A). Under similar conditions, the level of expression of the β-actin gene was not altered (Fig. 5C), indicating that the observed stimulating action of Pur α on expression of TGFβ-1 is specific and may not be a common event.

DISCUSSION

The narrow cell tropism of HIV-1 within the brain is insufficient to explain the complicated CNS pathology observed in AIDS-related neurological disorders known collectively as the HIV-1 associated cognitive motor complex. Of great interest is the extent to which the HIV-1 infected monocytes and macrophages of the brain contribute to the pathology. More specifically, considerable attention is being given to growth factors, immunomodulators (including cytokines and chemokines), and the viral structural and regulatory proteins including gp120 and Tat, respectively. Transforming growth factor β-1 (TGFβ-1) is a potent suppressor of the immune system [Massague, 1990] whose levels are significantly increased within the brain in response to infection with HIV-1 [Wahl et al., 1991], and may play a key role in the etiology of CNS dysfunction.

There have been numerous reports demonstrating the ability of Tat to alter expression of heterologous viral genes, including JCV and CMV, as well as several cellular genes [Cupp et al., 1993; Lotz et al., 1994; Roy et al., 1990; Tada et al., 1990; Taylor et al., 1992; Viscidi et al., 1989; Vogel et al., 1988, 1991; Zauli et al., 1992]. Detailed analysis of JCV promoter induction by Tat led to the identification of two Tat-responsive regions: one positioned within the leader of the viral late RNA species with extensive homology to the HIV-1 TAR; and the other which is enriched in a GA/GC sequence and located upstream of the JC virus late RNA start site termed upTAR [Chowdhury et al., 1992, 1993]. In a similar fashion, the response of tumor necrosis factor-β (TNFβ) to Tat requires a region located in the mRNA leader which is predicted to form a stem loop configuration resembling TAR [Buonaguro et al., 1994]. Close inspection of the eleven bases within the TGFβ-1 leader sequences showed no sequence homology to TAR and exhibited no stem-loop structure suggesting that activation of the

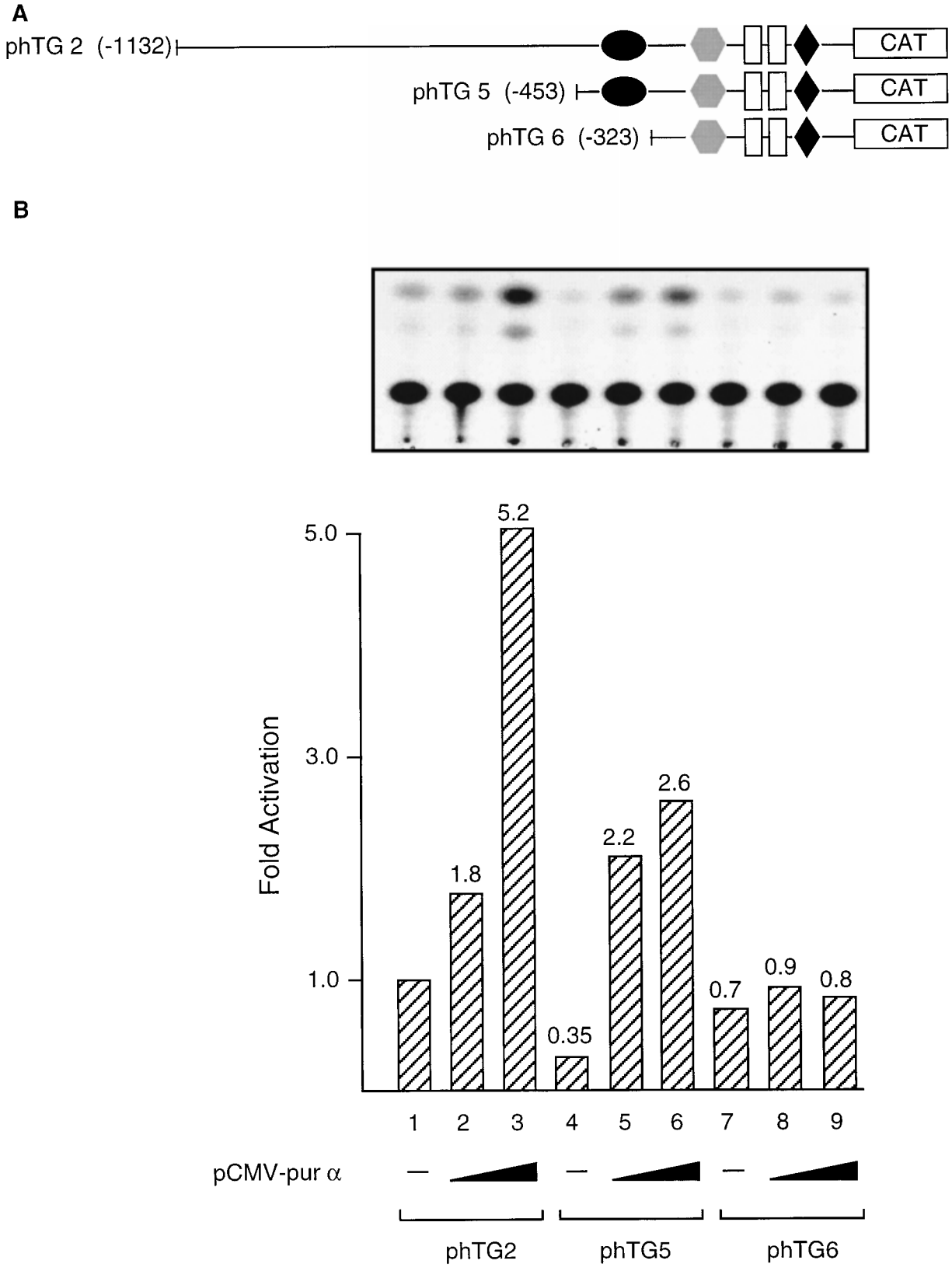

Fig. 4. Transactivation of the TGFβ-1 promoter by Pur α protein. **A:** Schematic representation of the TGFβ-1 promoter constructs. **B:** CAT-reporter plasmid constructs, phTG2, phTG 5, and phTG 6 were introduced into U-87MG cells by the Ca-phosphate precipitation method [Graham and van der Eb, 1973] in the absence and presence of increasing amounts (2.5 μg and 10 μg) of Pur α expressor plasmid, pCMV-Pur α. Forty h posttransfection, cell extracts were prepared and CAT enzymatic activity was measured as previously described [Gorman et al., 1982]. Each transfection was performed in duplicate and the average fold activation of the promoter by Pur α is shown by bar graphs. A representative of multiple transfection/CAT assays is presented.

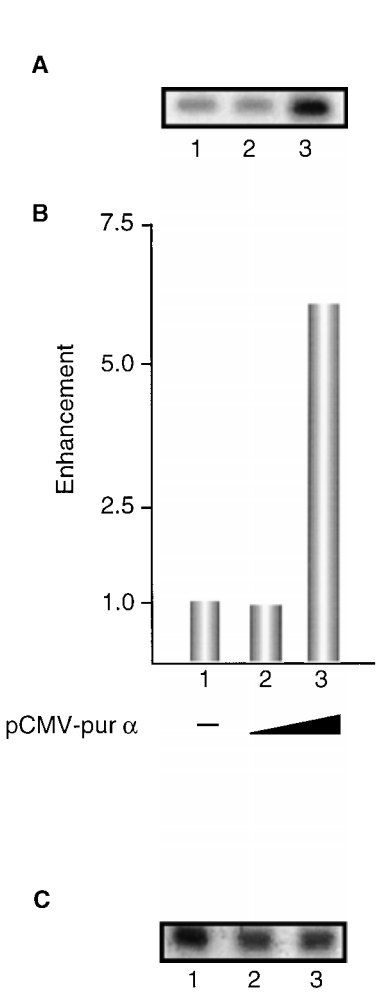

Fig. 5. Effect of exogenously expressed Pur α on TGFβ-1 mRNA production in Tat-producing cells. 5–10 is a derivative of U-87MG cells that continuously produce Tat protein [Taylor et al., 1992]. Approximately 10^7 cells were transfected with 2.5 μg and 10 μg of pCMV-Pur α and after 48 h, total RNA was prepared using acid-guanidinium thiocyanate-phenol-chloroform extraction method described previously [Chomczynski and Sacchi, 1987]. RT-PCR reaction followed by Southern analysis was performed as detailed in Materials and Methods. **A:** Represents TGFβ-1 RNA level in the cells transfected with empty control plasmids **(lane 1),** or transfected with 2.5 μg and 10 μg of pCMV-Pur α plasmids (**lanes 2** and **3,** respectively). The intensity of the band corresponding to TGFβ-1 mRNA was densitometrically measured and after normalizing to the levels of actin RNAs (shown in **C**), presented in the bar graphs **(B).**

TGFβ-1 promoter by Tat may not be mediated by direct interaction of Tat with a TAR-like RNA sequence, and may utilize a promoter binding protein.

Following identification of the Tat-responsive promoter regulatory elements of TGFβ-1 and the fact that Tat is unable to directly bind DNA, we sought to identify cellular factor(s) involved in the interaction. Footprinting analysis of the 130 bp region (encompassing Tat-response elements), identified a region which binds to cellular protein(s). UV-crosslinking gel mobility shift analysis identified a protein with similar molecular mass to the previously identified cellular regulatory protein, Pur α, whose consensus sequences (GC/GA) were repeated in the area protected against DNase I in footprinting assay. Furthermore, gel mobility shift assays using bacterially produced Pur α protein confirmed the involvement of this protein in regulation of the TGFβ-1 promoter through this region. These observations are consistent with the results from analysis of JCV upTAR which binds to Pur α and responds to Pur α/Tat induction.

Perhaps it should be noted that the Pur α binding site was identified in several other promoters including myelin basic protein [Haas et al., 1995]. However, our preliminary data showed no inducibility of the MBP promoter by Tat, suggesting that the structural organization of the promoter containing the Pur α binding site may be important in conferring Tat-responsiveness to the promoter activity.

Identification of cellular factors with the ability to interact with important viral proteins such as Tat is of particular interest as one may design a therapeutic modality based on the interaction of these two proteins to block HIV-1 gene expression by Tat and abrogate deregulating action of Tat upon cellular genes.

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