

# Control elements between –9.5 and –3.0 kb in the human tissue-type plasminogen activator gene promoter direct spatial and inducible expression to the murine brain

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## Abstract

Tissue-type plasminogen activator (t-PA) participates in the control of synaptic plasticity and memory formation in the central nervous system (CNS). Transgenic mice harbouring either 9.5, 3.0 or 1.4 kb of the human t-PA promoter fused to the *LacZ* reporter gene were used to assess t-PA promoter-directed expression *in vivo*. The 9.5 kb t-PA promoter directed expression to the brain, most notably to the dentate gyrus, superior colliculus, hippocampus, thalamus and piriform cortex. Staining was also observed in the retrosplenial and somatosensory cortex. The 3.0 kb t-PA promoter directed generalized and poorly defined expression to the cortex and hippocampus, while the 1.4 kb t-PA promoter directed expression selectively to the medial habenula. Intravenous administration of lipopolysaccharide into mice harbouring the 9.5 kb t-PA promoter resulted in an increase in reporter gene activity in the lateral orbital cortex and thalamus. Results of *in vitro* transfection experiments of NT2 cells with a series of t-PA promoter deletion constructs confirmed the presence of regulatory elements throughout the 9.5 kb promoter region. Finally, we describe a *cis*-acting element related to the NFAT recognition site that provides a protein-binding site and which may play a role in the selective expression of the 1.4 t-PA promoter in the medial habenula. These results indicate that elements between –3.0 and –9.5 kb of the t-PA promoter confer constitutive and inducible expression to specific regions of the CNS.

## Introduction

Tissue-type plasminogen activator (t-PA) is a serine protease that plays a major role in the fibrinolytic system, the enzymatic system responsible for fibrin digestion in the context of wound healing and tissue repair. It is the primary enzyme responsible for cleavage of plasminogen to plasmin and is considered to be the main plasminogen activator responsible for the removal of intravascular fibrin deposits (Bachmann, 1987).

Recently, it has become clear that t-PA is important in both the developing and mature central nervous system (CNS). t-PA contributes to neuronal plasticity during the learning process (Seeds *et al.*, 1995), while t-PA<sup>–/–</sup> strains exhibit delayed long-term potentiation and display some learning deficits (Frey *et al.*, 1996; Huang *et al.*, 1996; Calabresi *et al.* 2000). Conversely, transgenic animals which overexpress t-PA in neurons display accelerated learning capabilities (Madani *et al.*, 1999).

Although these studies implicate t-PA in a beneficial context in the CNS, there is also overwhelming evidence that t-PA is a crucial mediator of neurodegenerative events. t-PA<sup>–/–</sup> mice are resistant to excitotoxin-induced neuronal degeneration and seizure (Chen & Strickland, 1997), while infusion of active t-PA to the hippocampus of t-PA<sup>–/–</sup> mice restores the neurodegenerative effect (Chen & Strickland, 1997; Tsrka *et al.*, 1997). t-PA has also been shown to

modulate CNS function directly. For example, t-PA acts on the NMDA receptor in a plasmin-independent manner and promotes Ca<sup>2+</sup> influx and apoptosis in cultures of primary cortical neurons (Nicole *et al.* 2001).

t-PA is clearly one of the most important regulatory proteases in brain function, yet an understanding of how the human *t-PA* gene is regulated in this compartment is lacking. Previous studies have shown that the *t-PA* gene is expressed and regulated in a tissue- and cell type-specific manner (Costa & Medcalf, 1996; Costa *et al.*, 1998). Studies addressing the transcriptional regulation of the *t-PA* gene have described crucial regulatory elements within the proximal region and also at far upstream locations, thereby highlighting the complex nature of the t-PA promoter (Medcalf *et al.*, 1990) (Bulens *et al.*, 1995; Bulens *et al.*, 1997). t-PA expression in the brain is at least partly regulated by transcription control (Pecorino *et al.*, 1991), while the first 4.0 kb of the murine t-PA promoter has been shown to direct expression to various regions in the mouse brain *in vivo* (Carroll *et al.*, 1994).

Transgenic mice and rats expressing either 3.0 or 1.4 kb of the human t-PA promoter fused to the *LacZ* reporter gene have also been used to visualize t-PA promoter-driven expression during embryonic development (Theuring *et al.*, 1995). We have used the same transgenic mice to study expression patterns directed by the human t-PA promoter in the adult central nervous system. In addition, a third transgenic line incorporating 9.5 kb of the human t-PA promoter (9.5 tPA-LacZ) was developed for this study. Reporter gene expression

was assessed in all three lines under constitutive conditions and in mice treated with agents known to regulate t-PA transcription *in vitro*.

## Materials and methods

### Materials

Human neuron-like NT2 teratocarcinoma cells and rat pheochromocytoma PC12 cells were used (American Type Culture Collection; Rockville, MD, USA). Phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS; Serotype 0111 : 34) and 5-bromo-4-chloro-3-indolyl beta- $\beta$ -D-galactopyranoside (X-gal) were purchased from Sigma (St Louis MO, USA).  $\gamma$ - $^{125}$ I ATP and  $^{14}$ C-chloramphenicol were purchased from NEN (Boston, MA, USA).

### Generation of transgenic mice

The 3.0 tPA-LacZ and 1.4 tPA-LacZ transgenic mice have been described previously (Theuring *et al.*, 1995). Six individual founders of each line were generated and reporter gene activity assessed during embryonic development. One founder group was selected for each line and bred to homozygosity. In addition to these mice, transgenic mice harbouring the 9.5 kb t-PA promoter were generated for this study and are described below.

#### Generation of 9.5 kb t-PA LacZ transgenic mice

A plasmid containing 9.5 kb of the human t-PA promoter (t-PA 9578-CAT) (Bulens *et al.*, 1995) was kindly provided by Professor Désire Collen (Leuven, Belgium). The 9.5 kb promoter fragment was removed and inserted into the *SalI* and *SpeI* sites of plasmid pSVM-L containing the LacZ reporter plasmid (Theuring *et al.*, 1995) to generate plasmid 9.5 tPA-LacZ. Transgenic mice were generated by microinjecting fertilized mouse oocytes with plasmid 9.5 tPA-LacZ. Plasmids were introduced into the male pronuclei and viable eggs were then transferred into the oviducts of pseudopregnant surrogates. Transgenic pups were identified by Southern blot analysis of restriction enzyme-digested genomic DNA isolated from mouse tail samples. Founders were outbred with wild-type F1 mates to establish independent lines. Six independent 9.5 tPA-LacZ transgenic founder mouse lines were established. Reporter gene expression was assessed at various times during embryonic development. Some degree of variation was noted in the LacZ expression patterns between the individual lines, however, the differences related more to the intensity of expression, rather than to differences in location (data not shown). One of these lines was selected and bred to homozygosity and used for expression analyses in this study.

#### Staining for $\beta$ -galactosidase in mouse tissue

Experiments described in this study conformed to standards for experimentation on animals and had received approval from the Animal Ethics Committee (Physiology) of Monash University.

Transgenic animals, aged 8–12 weeks, were first anaesthetised with an intraperitoneal injection of pentobarbitone sodium (150 mg/kg), then perfused transcardially with phosphate buffered saline (PBS; 0.9% NaCl in 10 mM sodium phosphate, pH 7.0), followed by fixation with 4% paraformaldehyde in PBS for 10 min. Brains were removed, postfixed for a further 20 min, then incubated in 30% sucrose in PBS overnight at 4 °C. Brains were either kept intact or sectioned (100  $\mu$ m) using a freezing microtome. LacZ expression was detected by staining for  $\beta$ -galactosidase using X-gal as a substrate. Whole brain or brain sections was incubated in X-gal substrate solution (1 mg/mL X-gal in PBS containing, in mM: potassium ferrocyanide, 5; potassium ferricyanide, 5; MgCl<sub>2</sub>, 2) at 37 °C

overnight. The reaction was stopped by washing three times with PBS and the sections mounted on glass slides for examination.

### Co-localization experiments

Following staining for  $\beta$ -galactosidase, brain sections were washed in PBS and either counter-stained for neurons with neutral red (BDH, Kilsyth, Australia) or processed for immunohistochemistry of microglia and astrocytes. For immunohistochemistry, sections were first immersed in a solution of 3% H<sub>2</sub>O<sub>2</sub>/10% methanol for 5 min, followed by incubation with 5% normal goat serum for 60 min. Sections were then incubated overnight with either an anti-GFAP antibody (1 : 1000; Dako, Carpinteria, CA, USA) to detect astrocytes or an anti-MAC-1 antibody (1 : 1000; Serotec, Raleigh, NC, USA) to detect microglia. After rinsing, sections were incubated with either anti-rabbit or anti-rat biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA). This was followed by a final incubation in avidin/biotin complex (Vector Laboratories, Burlingame, CA, USA) for 60 min before visualization with 3,3'-diaminobenzidine/0.03% H<sub>2</sub>O<sub>2</sub>. Sections were then mounted on gelatin-coated slides, dried, dehydrated and coverslipped with permount.

### Lipopolysaccharide treatment

Transgenic animals were treated with LPS to investigate the regulation of t-PA promoter driven-LacZ expression *in vivo*. LPS (100  $\mu$ g; 1 mg/mL in normal saline) was injected i.v. through the tail vein. Animals were killed at 4, 12, and 24 h time points by i.p. injection of pentobarbitone sodium and the reporter gene expression pattern in brain sections determined by  $\beta$ -galactosidase staining as described above.

### Preparation of nuclear extracts

The preparation of nuclear extracts from NT2 and PC12 cells was performed as previously described (Costa & Medcalf, 1996). For extraction of protein from mouse brain, animals were first anaesthetised then perfused transcardially with 50 mL PBS. Brains were removed and cut into small pieces using a sterile blade, then added to 2 mL NP40 lysis buffer (in mM: 0.5% NP40, NaCl, 10; Tris (pH 7.4), 10; MgCl<sub>2</sub>, 3; DTT, 5; PMSF, 1). After 50 strokes of dounce homogenization, samples were briefly centrifuged and supernatants containing nuclei were collected and incubated on ice for 10 min. After centrifugation and washing with 1 mL NP40 buffer, pellets were resuspended in 100  $\mu$ L of Buffer C (in mM: NaCl, 420; HEPES (pH 7.9), 20; MgCl<sub>2</sub>, 1.5; EDTA, 0.2; 25% glycerol, DTT, 5; PMSF, 5). After incubation on ice for 20 min and centrifugation at 16 000 g at 4 °C for 10 min, the supernatants containing nuclear proteins were collected and stored at –80 °C.

### Electrophoretic mobility shift assay (EMSA)

Gel-purified oligonucleotides (100 ng) were labelled using T4 polynucleotide kinase and  $\gamma$ - $^{32}$ P ATP. Annealing was performed by adding a four-fold molar excess of the complementary strand and gel purified as previously described (Costa & Medcalf, 1996). After elution from native acrylamide gels, samples were precipitated with ethanol and finally resuspended in NaCl/Tris/EDTA buffer (in mM: NaCl, 100; Tris (pH 7.4), 10; EDTA (pH 7.4), 1) to approximately 100 cp/ $\mu$ L.

To perform the EMSAs, 4–10  $\mu$ g of nuclear protein or whole brain extracts in 4  $\mu$ L of Osborne buffer D (Osborne *et al.*, 1989) were incubated at 4 °C for 15 min with 1  $\mu$ L poly d[I-C] (1  $\mu$ g; used as a nonspecific competitor) (Boehringer Mannheim, Germany) and 3  $\mu$ L

of SMK buffer (12 mM spermidine, 12 mM MgCl<sub>2</sub>, and 200 mM KCl). 4 µL of <sup>32</sup>P-labelled probe (100 cp) diluted in Osborne buffer D was then added and the mixtures incubated on ice for a further 15 min before being applied to a native 5% polyacrylamide gel (Costa & Medcalf, 1996).

For competition titration experiments, extracts were incubated with 100 ng of unlabelled oligomers of either identical or unrelated sequence for 15 min prior to the addition of the probe. After electrophoresis, gels were fixed, dried and autoradiographed overnight at -70 °C with an intensifying screen. The sequences of the labelled oligomers and unlabelled competitors are provided in the figure legends.

#### *Transient transfection and analysis of chloramphenicol acetyltransferase (CAT) reporter gene activity*

To identify regions within the t-PA promoter important for conferring constitutive gene expression, a series of t-PA promoter deletion constructs (5 µg; range -9.5 kb to -0.196 kb) fused to the CAT reporter gene (Medcalf *et al.*, 1990; Bulens *et al.*, 1997) were transiently transfected into NT2 teratocarcinoma cells using the calcium phosphate precipitation method (Costa *et al.*, 1998). Cells were also cotransfected with plasmid pSV-β-galactosidase (Promega, Madison, WI, USA) and β-galactosidase activity was used as an internal control for transfection efficiency. Transfected cells were harvested 2 days later and cellular extracts then prepared. The CAT activity assay was performed by incubating 40 µL of protein extracts (usually 25 µg of protein), with 4 µL of 4.4 mM acetyl CoA and 1 µL of <sup>14</sup>C-chloramphenicol at 37 °C for 4 h. Samples were extracted with 1 mL ethyl acetate, vacuum dried, and finally dissolved in 20 µL of ethyl acetate. The samples were spotted onto TLC plates and processed using standard techniques. The degree of conversion of <sup>14</sup>C-chloramphenicol into its acetylated product was assessed by autoradiography. β-galactosidase activity in cell extracts was determined as previously described (Kirshenbaum *et al.*, 1993). The degree of CAT activity was normalized by adjusting for changes in β-galactosidase activity in the same samples.

## Results

### *Activation of the t-PA promoter in the murine brain*

LacZ reporter gene expression was initially visualized by β-galactosidase staining of whole brain mounts from adult transgenic mice expressing either the 9.5, 3.0, or 1.4 kb of the human t-PA gene promoter (Fig. 1A, i-iv). Extensive reporter gene expression was observed in the brain prepared from the 9.5 tPA-LacZ mouse, particularly in the cerebral cortex (panel i). Staining was most intense between the hemispheres in the retrosplenial cortex region (panel i, open arrow). A longitudinal striped pattern of staining was also seen on the dorsal surface of the cerebellum (the vermis; panel i, full arrow) and the lateral sides of the cerebellum. Staining was also apparent in the superior colliculus, but was notably absent in the inferior colliculus (broken arrow, panel i). As shown in the ventral view of the 9.5 t-PA LacZ brain (panel ii), specific dotted staining was also seen in ganglia within the olfactory tubercle (indicated by arrow). The brain stem displayed no reporter gene expression.

Brains of the 3.0 tPA-LacZ mice also displayed extensive staining throughout the cortex and cerebellum but there was no staining in the brain stem (Fig. 1A, iii). However the pattern of staining was generalized with no clear demarcation of expression as seen for the 9.5 tPA-LacZ brain, although relatively weaker staining, as seen in

the 9.5 tPA-LacZ mouse, was present in the inferior colliculus (panel iii). Finally, brains prepared from the 1.4 tPA-LacZ mouse did not express any detectable LacZ expression on the brain surface (panel iv).

### *Histochemical assessment of LacZ staining in the brains of transgenic mice*

To determine the specific regions within the brain expressing the transgene, a series of coronal sections (100 µm) were prepared from the three transgenic lines and stained for LacZ expression. As shown in Fig. 1B (i), the 9.5 kb t-PA promoter directed reporter gene expression to the primary motor cortex (M1), somatosensory cortex (S1), the barrel fields of the somatosensory cortex (S1BF), piriform cortex (Pir), and the olfactory tubercle (Tu). Weak staining was also seen in the basal ganglia caudate putamen and lateral septal nuclei. As shown in Fig. 1B (ii), very strong and well-defined reporter gene expression was found in the dentate gyrus (DG), medial habenula (MH), and the CA1 and CA2 fields of the hippocampus. No staining in the CA3 region was detected. Staining was also observed in the secondary somatosensory cortex (S2) and weak expression was noted in some thalamic nuclei.

As shown in Fig. 1B (iii and iv), the 3.0 kb t-PA promoter conferred generalized transgene expression to the cortex and basal ganglia; the pattern although intense, was diffuse, punctate and poorly defined compared with the expression pattern seen in the 9.5 kb tPA-LacZ mouse (panel iii). At the midbrain level (panel iv), the 3 kb t-PA promoter directed transgene expression to all CA fields of the hippocampus, weaker expression in dentate gyrus, stronger expression in thalamic nuclei dorsal lateral geniculate nuclei, ventral posterior thalamic nuclei (VP), but no expression in the MH.

In contrast, reporter gene expression directed by the 1.4 kb t-PA promoter was essentially absent from the brain (Fig. 1B, v) except for one notable exception; as shown in Fig. 1B (vi), strong reporter gene expression was detected in the MH. Fig. 1C provides a higher magnification (different brain) to better illustrate the well-defined expression directed by the 9.5 kb t-PA promoter to the hippocampus, DG and MH and to superficial layers of the retrosplenial cortex (RSC) (Fig. 1C, i), and the selective expression pattern directed by the 1.4 kb t-PA promoter to the MH (Fig. 1C, ii). Anatomical locations and nomenclature were based on Franklin & Paxinos (1997).

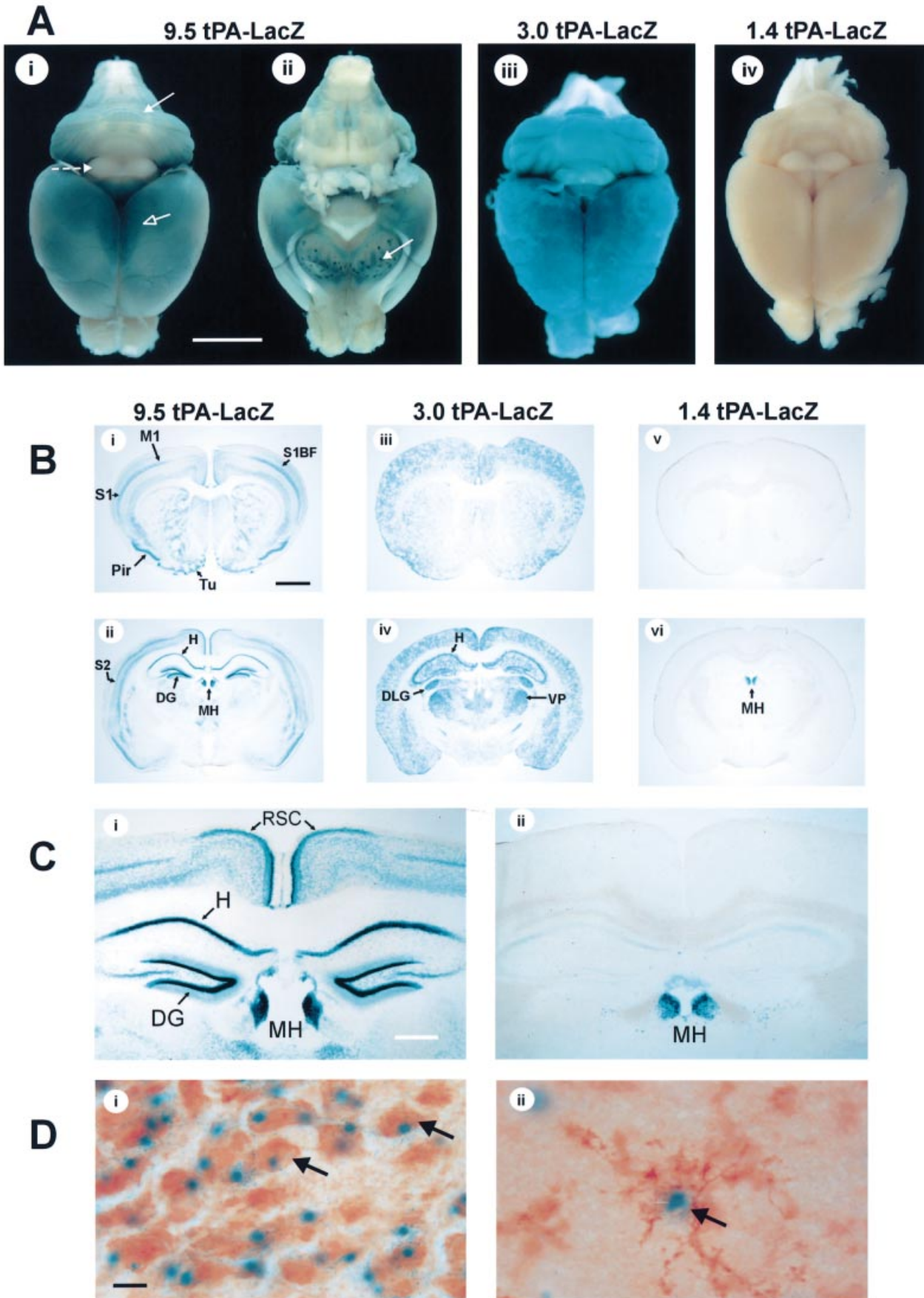
Co-localization experiments revealed that LacZ expression was present mainly in neurons (Fig. 1D, i) and in some microglia (Fig. 1D, ii) but not in astrocytes (data not shown).

Of the other organs investigated, transgene expression was found in horizontal cells of the inner nuclear layer of the retina, the cortex of the kidney and in the mantle zone of the spleen of the 9.5 tPA-LacZ mice (data not shown). Although t-PA is expressed in endothelial cells, we found no evidence for LacZ expression in any vascular compartment in any of the transgenic lines.

### *Lipopolysaccharide (LPS) treatment induces LacZ expression in vivo*

To determine whether the t-PA promoter-directed reporter gene expression pattern could be regulated, transgenic mice were injected i.v. with LPS (100 µg). LPS is a proinflammatory component from the outer cell wall of gram-negative bacteria and regulates endogenous t-PA gene expression *in vivo* (Moll *et al.*, 1994).

Treatment of 9.5 kb tPA-LacZ mice with LPS for 4 h resulted in an increase in expression in the lateral orbital cortex (LOC; Fig. 2B) and some thalamic sites (panel D) compared to the vehicle-treated controls (panels A and C). Induction by LPS was transient because



the increase in reporter gene expression was no longer evident after 24 h of treatment (data not shown). Of the other organs studied, a slight increase in reporter gene expression was seen in the cortical region of the kidney (data not shown). Mice expressing either the 3.0 or the 1.4 kb t-PA promoter did not show LPS induction in any compartment including the brain. These data suggest that important regulatory elements necessary to convey LPS-induced t-PA gene transcription reside between -3 kb and -9.5 kb upstream from the transcription start site.

#### Identification of a NFAT-like binding site in the t-PA promoter that may be related to transgene expression in the MH

We were particularly interested in the remarkable selective expression pattern produced in mice carrying the 1.4 kb t-PA promoter. In these mice, LacZ reporter gene activity was restricted exclusively to the MH. Intriguingly, transgenic mice harbouring a 168-bp fragment of the human insulin gene promoter (as well as 5.5 kb of intronic sequence) fused to the LacZ reporter gene, also displayed an essentially identical expression profile (Douhet *et al.*, 1995). We

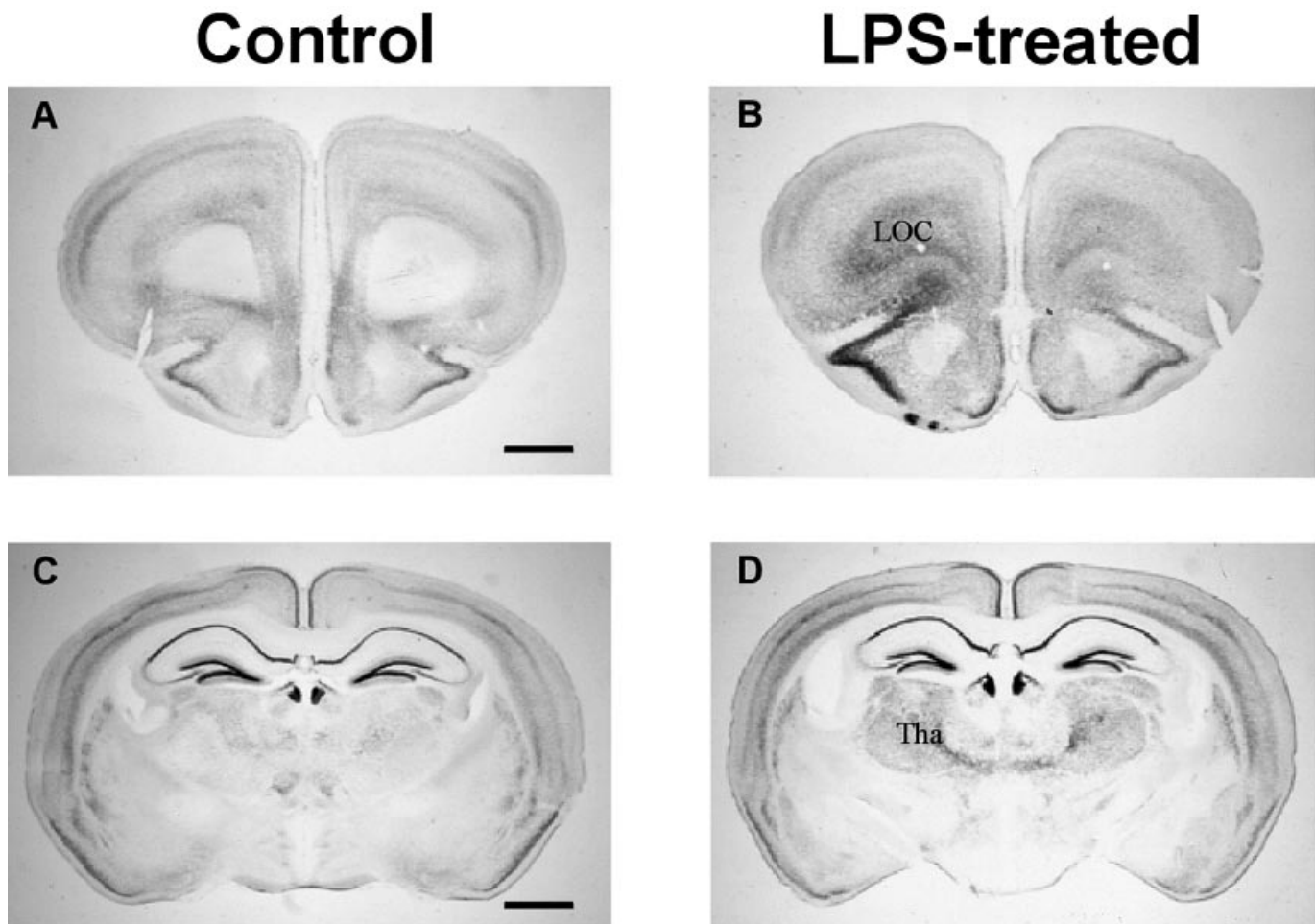


FIG. 2. LPS treatment increases tPA-LacZ transgene expression *in vivo*. 9.5 kb tPA-LacZ transgenic mice were treated *i.v.* for 4 h with either normal saline (A and B, frontal cortex) or 100  $\mu$ g of LPS (C and D, parietal region). LPS increased reporter gene expression in the lateral orbital cortex (LOC; B) and thalamus (Tha; D). Scale bar, 1.5 mm.

FIG. 1. (A) t-PA/LacZ gene expression in whole brains of three different transgenic mice. (i) Dorsal view of 9.5 tPA-LacZ mouse brain. LacZ reporter gene is strongly expressed in the retrosplenial cortex [broken arrow in (i) and as a striped pattern on the vermis of the cerebellum (arrow)]. (ii) Ventral view of 9.5 tPA-LacZ mouse brain: arrow indicates expression within ganglions in the olfactory tubercle. (iii) Dorsal view of 3.0 tPA LacZ LacZ mouse brain: LacZ expression pattern is generalized. (iv) Dorsal view of 1.4 tPA-LacZ mouse brain: no LacZ expression on the brain surface. Scale bar in A (i), 5 mm. (B) Localization of LacZ reporter gene expression. (i) Coronal section (frontal cortex) of brain from 9.5 tPA-LacZ transgenic mouse showing LacZ expression in primary motor cortex (M1), somatosensory cortex (S1), barrel fields of the somatosensory cortex (BFS1), piriform cortex (Pir) and olfactory tubercle (Tu). (ii) Midbrain view of same brain showing strong staining in the CA1 and CA2 fields of the hippocampus (H), dentate gyrus (DG), medial habenula (MH), and the secondary somatosensory cortex (S2). (iii) Diffuse and poorly defined staining was found in the forebrain of 3.0 tPA-LacZ transgenic mice, while at the midbrain level (iv) staining is found throughout the hippocampus, with weaker expression in the DG, stronger expression in the dorsal lateral geniculate nuclei (DLG), ventral posterior thalamic nuclei (VP), but no expression in the MH. (v) No staining is seen in the frontal cortex of brains of 1.4 tPA-LacZ transgenic mice. (vi) Staining is only detected in the MH in 1.4 tPA-LacZ transgenic mouse. Scale bar in B (i), 2.5 mm. (C) Higher magnification highlighting reporter gene expression directed by (i) the 9.5 kb t-PA promoter to the DG, hippocampus, retrosplenial cortex (RSC) and MH. (ii) The 1.4 kb t-PA promoter directs expression exclusively to the MH. Scale bar in C, 0.5 mm. (D) Representative photomicrographs of the hippocampal CA1 region illustrating colocalization of the 9.5 kb tPA promoter directed Lac-Z expression (blue stain) to many neurons counterstained with neutral red (i) indicated with arrows. Reporter gene expression is also observed in some microglia [see arrow in (ii)] as revealed by counterstaining with the anti-MAC-1 antibody. Scale bar in D, 20  $\mu$ m.

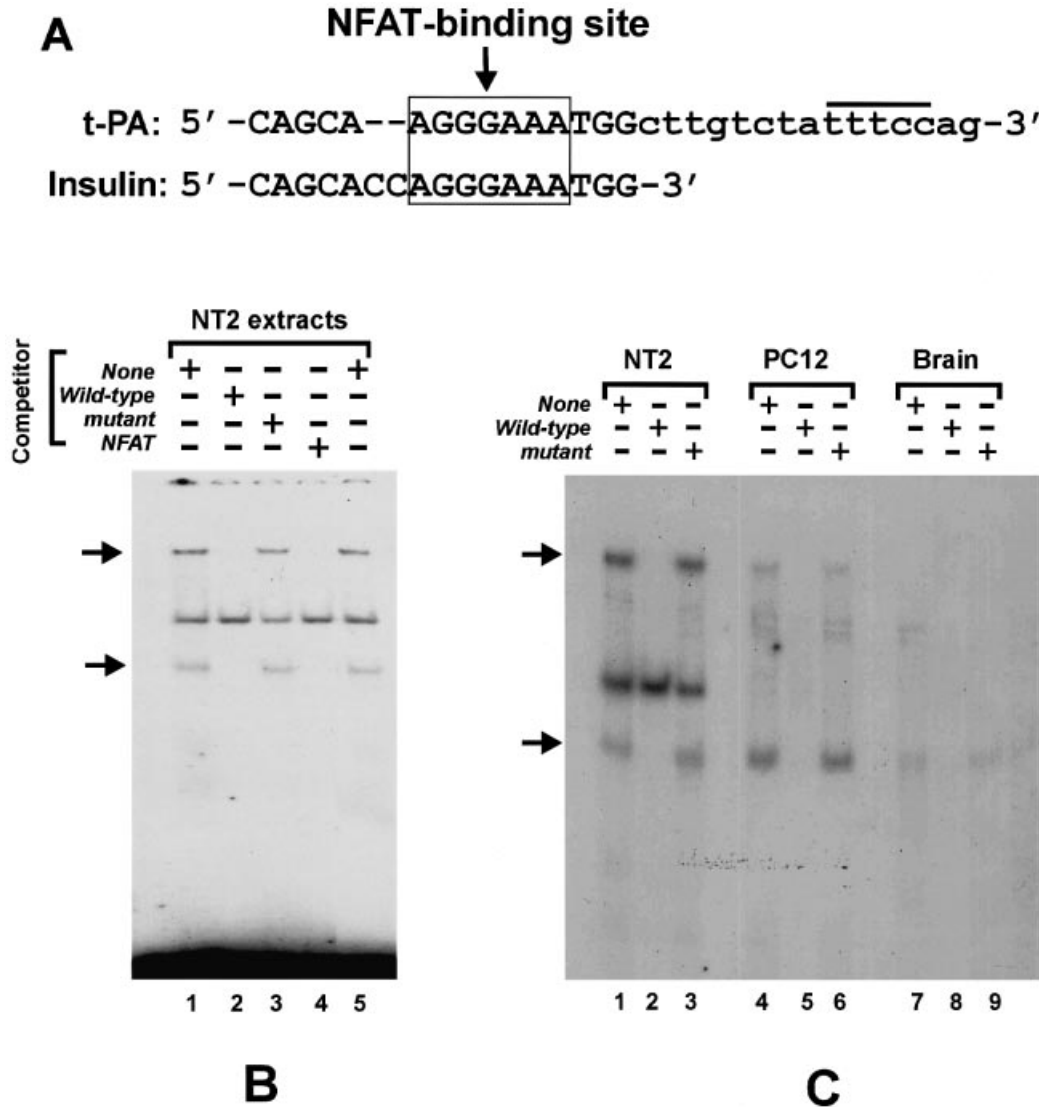


FIG. 3. Nuclear Proteins from NT2 cells recognize the NFAT-like binding site in the t-PA promoter. (A) Alignment of the first 1.4 kb t-PA promoter with the first 168 bp of the human insulin promoter reveals the presence of a NFAT consensus binding site between positions -707 and -693 in t-PA and between positions -152 and -136 in the insulin promoter (boxed region). A NFAT-like recognition site is also present on the other DNA strand in the t-PA promoter between positions -681 and -677 (solid line above sequence). (B) EMSA experiments performed using a labelled oligonucleotide probe containing the NFAT-like element in the t-PA promoter and nuclear proteins extracted from NT2 cells produced two specific complexes (lanes 1 and 5; indicated with arrows). Lane 2: specific complexes are abolished in the presence of 100 ng unlabelled oligonucleotide of same sequence as probe ('wild-type'), but not in the presence of 100 ng of a mutant sequence ('mutant'; lane 3). Inclusion of an oligonucleotide competitor containing the NFAT consensus sequence ('NFAT') also competes for binding (lane 4). Sequences of oligonucleotides used for EMSAs (upper strand only shown): t-PA NFAT-like sequence (wild-type), 5'-TCAGCAAGGGAAATGGCT-3'; t-PA NFAT mutant sequence, 5'-TCAGCCACTGCTATGGCTT-3' (substituted residues underlined); NFAT consensus sequence, 5'-CAAAGAGGAAAATTT-3'. (C) EMSAs performed using extracts prepared from NT2 cells (lanes 1-3), PC12 cells (lanes 4-6) and mouse brain (lanes 7-9). Specific complex formation is abolished in the presence of unlabelled wild-type competitor (lanes 2, 5 and 8) but not with mutant competitor (lanes 3, 6 and 9). Sequences of oligonucleotides used are shown in B.

reasoned that a habenula-specific DNA element that directs expression to this compartment might be present in both the insulin and t-PA promoters.

Alignment of the -168 bp human insulin promoter and -1.4 kb t-PA promoter revealed a region with striking sequence homology. This region was located between -707 and -693 in the t-PA promoter, and between -152 and -136 in the insulin promoter. Indeed, a 10 nt stretch of perfect identity was also apparent within this sequence of both promoters (AGGGAAATGG). This region was closely related to the consensus-binding site for the transcription factor 'nuclear factor of activated T cells' (NFAT, underlined sequence above) (Fig. 3A). NFAT family members regulate cytokine expression (Northrop *et al.*,

1994; Ho *et al.*, 1995) and play critical roles in the immune response in response to calcium signalling. In addition, one member of the NFAT family (NFATc4) is expressed in hippocampal neurons (Graef *et al.*, 1999). The NFAT-like element within the t-PA promoter provides a specific binding site for a brain-derived protein.

A series of EMSAs were performed to investigate whether transcription factors prepared from mouse brain and neuron cell lines could specifically recognize the NFAT-like binding site in the human t-PA promoter. Nuclear proteins extracted from neuron-like NT2 cells were incubated with labelled double stranded oligonucleotide probes harbouring the NFAT-like sequence identified in the t-PA promoter. EMSA experiments indicated that this sequence provides a

binding site for nuclear factor(s) extracted from NT2 cells (Fig. 3B, lanes 1 and 5). As shown, three DNA–protein complexes were produced which displayed different migration profiles. Competition experiments revealed that the fastest and slowest migrating complexes were specific (indicated with arrows) as formation of these complexes was abolished in the presence of an unlabelled oligonucleotide of the same sequence as labelled probe (lane 2). Furthermore, addition of a similar oligonucleotide containing a 5 nt substitution within the NFAT-like binding site failed to compete for protein binding (lane 3). Finally, an oligonucleotide harbouring the NFAT consensus sequence also competed for binding (lane 4). These data suggest that either NFAT (presumably NFATc4) or a factor with similar sequence specificity recognizes this region within the t-PA promoter. Also, a near identical formation of protein–DNA complexes occurred when using the NFAT consensus oligonucleotide as a probe, providing further support to suggest that NFAT recognizes this site (data not shown).

As shown in Fig. 3C, the specific binding seen with extracts prepared from NT2 cells (lanes 1–3) was also seen with nuclear proteins extracted from PC12 cells (Fig. 3C, lanes 4–6). Interestingly, only the fastest migrating complex was produced using extracts prepared from mouse brain (lanes 7–9). The factor(s) associating with this element have not been formally identified. However, given the sequence homology with the NFAT binding site, it is likely that a member of the NFAT family (possibly NFATc4) is part of the assembly of proteins that recognize this element in the t-PA promoter and may play a role in the activation of the t-PA promoter in the medial habenula.

In a related context, a second NFAT-like sequence is also present on the other DNA strand in the t-PA promoter situated 12 bp downstream of the t-PA NFAT-like site (indicated with line over sequence in Fig. 3A). Moreover, *in vivo* footprint analysis demonstrated that this precise region provides a binding site for nuclear proteins in three different neuronal cell lines (Wolf-Dieter Schleuning, unpublished data). The protein-binding potential of this downstream inverted NFAT element was not explored by EMSA analysis in this study.

Regulatory elements associated with t-PA regulation in neurons are located throughout the first 9.5 kb of the promoter region. To further define the location of gene regulatory elements within the 9.5 kb t-PA promoter that contribute to neuron-specific expression, we transiently transfected NT2 cells with a series of t-PA promoter deletion constructs (range 9.5 kb–0.196 kb) fused to the CAT reporter gene (Fig. 4A). As shown in panels B and C, the degree of reporter gene expression in transfected cells varied depending on the length of t-PA promoter used to drive reporter gene expression. Constructs harbouring 9.5 kb of the t-PA promoter directed high constitutive expression in NT2 cells. Similar high level expression was produced by the  $-7.1$  kb promoter fragment. Stepwise truncation of the promoter to  $-5.1$ ,  $-1.2$  and  $-0.63$  kb reduced levels of constitutive expression by approximately 36, 17 and 8%, respectively. Interestingly, further truncation to  $-0.41$  kb resulted in an increase in promoter activity. This is consistent with the possibility that repressor elements reside between  $-0.63$  and  $-0.41$  kb. It is of interest that repressor elements exist in the rat t-PA gene promoter at a similar position relative to the transcription start site (Leonardsson & Ny, 1997). These data indicate that regulatory elements, including enhancer and repressor regions, exist throughout the 9.5 t-PA promoter fragment. These data are also broadly consistent with the difference in the expression pattern of the different lengths of the t-PA promoter *in vivo*.

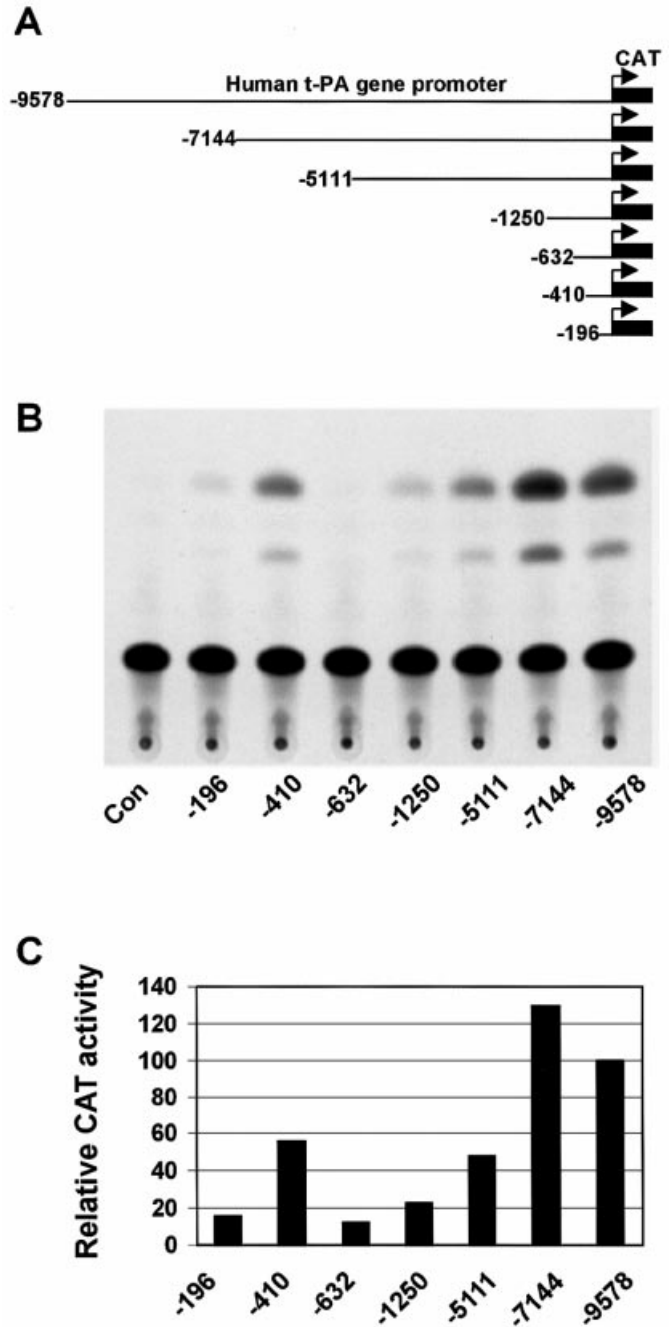


FIG. 4. Transient transfection of NT-2 cells with a series of t-PA promoter deletion constructs fused to the CAT reporter gene. (A) Schematic representation of the series of t-PA promoter deletion constructs (range  $-9.5$  kb to  $-0.196$  kb) fused to the CAT reporter gene used for transient transfection of NT2 cells. (B) Autoradiograph showing the degree of CAT activity in NT2 cells transfected with the t-PA promoter CAT constructs shown in A. (C) Quantitative of CAT activity in NT2 cells transfected with the series of t-PA promoter deletion constructs. Results presented have been adjusted for changes in  $\beta$ -galactosidase expression.

## Discussion

There is growing evidence that extracellular proteolysis is involved in developmental processes, synaptic plasticity, axonal remodelling and neurotoxicity in the adult CNS. t-PA is now well established as one of the major regulatory serine proteases involved in each of these events (see Indyk *et al.*, 1999 for review). t-PA is also an immediate early

gene expressed in response to events that involve neuronal plasticity, such as seizures and kindling (Qian *et al.*, 1993). Extracellular proteolysis initiated by t-PA may facilitate synaptic microremodeling, and thereby influence activity-dependent neuronal plasticity and learning (Qian *et al.*, 1993; Madani *et al.*, 1999; Calabresi *et al.* 2000).

Several molecular mechanisms are involved in the regulation of t-PA gene expression, such as transcription (Medcalf *et al.*, 1990; Pecorino *et al.*, 1991), translation and polyadenylation (Huarte *et al.*, 1992), and mRNA stability (Ouyang *et al.*, 1995). Although the t-PA gene is regulated at each of these levels, transcriptional mechanisms certainly play a significant role in the expression of the t-PA gene in all cell systems studied to date. We have focused on the transcriptional component of t-PA gene regulation using models of transgenic mice which express different lengths of the t-PA promoter fused to the LacZ reporter gene. Our results show that the 9.5 kb human t-PA promoter directs transgene expression in a clear and well-defined pattern in the CNS, most notably within the hippocampus, DG, Pir, MH, and specific layers of the RSC and S1. Truncation of the promoter to -3.0 kb resulted in a loss in precision of the expression pattern; expression was still relatively strong but was diffuse and regional demarcation was largely lost. Expression within the MH was lost entirely. It seems likely that tissue-specific repressor elements are missing from the 3.0 kb promoter allowing promiscuous expression. Truncation of the t-PA promoter to -1.4 kb removed most of the elements required to direct expression to the CNS, except for expression within the medial habenula. The reason why expression in the MH is lost in mice carrying the 3.0 kb t-PA promoter, and regained in mice harbouring the 1.4 kb promoter construct is unclear. It may be related to cryptic medial habenula-specific silencing element(s) between -1.4 kb and -3.0 kb that could be counteracted by elements between -3.0 and -9.5 kb. Although we cannot exclude the possibility that the loss of expression in the MH in 3.0 t-PA LacZ mice is related to the integration site of the transgene, it is unlikely that the integration site would have such a selective influence on spatial gene expression without having a more general effect. Nonetheless, it can be concluded from this *in vivo* promoter deletion analysis that many of the DNA elements essential for expression of t-PA in the CNS lie between -1.4 kb and -3.0 kb upstream of the t-PA transcription initiation site. However, elements required for precise spatial expression reside between -3.0 kb and -9.5 kb, because only the transgenic line carrying the 9.5 kb t-PA promoter displayed accurate spatial expression in the brain. The location of these DNA elements (repressors and enhancers) within this region involved in the regulation in the CNS is presently unknown. Interestingly, *in vitro* studies have identified a multihormone responsive enhancer at -7.0 kb in the human t-PA promoter (Bulens *et al.*, 1997). Whether this region plays a role in the expression of t-PA within the CNS remains to be determined.

The expression pattern seen in the 9.5 t-PA LacZ mouse is particularly striking. Apart from the strong reporter gene seen in the retrosplenial cortex (viewed in both whole mounts and in cross section), a remarkable stripped pattern of staining was seen on the dorsal surface and lateral sides of the cerebellum. The significance of this is unclear, but it nonetheless points to an intricate demarcation in t-PA expression that is not obviously defined by anatomical criteria. Also, the extensive staining of ganglions within the olfactory tubercle suggests a previously unsuspected role for t-PA in this compartment.

An important consideration that needs to be addressed is the pattern of gene expression conferred by the human t-PA promoter to the expression of the endogenous murine t-PA gene, as it could be argued that the murine system may lack some transcription factors

TABLE 1. Reporter gene expression in 9.5, 3.0 and 1.4 kb t-PA LacZ transgenic mice and endogenous t-PA gene expression

	9.5 kb	3.0 kb	1.4 kb	Endogenous t-PA*
Olfactory Tubercle	++++	+	-	++
Piriform Cortex	++++	-	-	++++
Cerebral Cortex	+++	+++	-	+++
Habenular nucleus, medial	++++	-	++++	+++
Hippocampus CA1, CA2	++++	+++	±	+++
Hippocampus CA3	+	+++	±	+++
Dentate gyrus	+++++	++	-	+++++
Thalamus	++	++++	-	+
Hypothalamus	+	++	-	++
Interpeduncular nuclei	+++	+	-	+
Pontine nuclei	++	+	-	++
Cerebellum lobules	++	+	-	++

\*The endogenous t-PA gene expression pattern is a summary of published results in the murine brain (Sappino *et al.*, 1993; Thewke & Seeds, 1996) and rat brain (Ware *et al.*, 1995).

necessary to convey faithful expression of the human t-PA promoter. However, endogenous t-PA mRNA has been found in many regions of the murine brain, which are spatially coincident with the LacZ expression pattern produced in the 9.5 t-PA-LacZ mice. For example, t-PA mRNA has been detected in the cerebellum (Ware *et al.*, 1995), olfactory system (Thewke & Seeds, 1996) including the olfactory tubercle, DG and Pir (Sappino *et al.*, 1993). Strong t-PA mRNA expression has also been visualized in the MH as well as in all pyramidal fields of the hippocampus (Sappino *et al.*, 1993). In our study, we observed very strong reporter gene expression within the CA1 and CA2 fields, but relatively weak expression within the CA3 field. Interestingly, transgenic mice expressing the first 4.0 kb of the murine promoter directed LacZ reporter gene expression pattern to all hippocampal fields (Carroll *et al.*, 1994). The reason why the human promoter directs relatively weak transgene expression to CA3 is unclear but may reflect differences between the murine and human promoter sequences or a limitation of the activity of the human t-PA promoter in the murine environment.

A comparison of the degree and location of LacZ expression observed in the three mouse lines used in our study, with that of endogenous t-PA expression is provided in Table 1. It can be concluded from this comparison that, with few exceptions, the expression pattern directed by the 9.5 kb human t-PA promoter closely reflects that of the endogenous murine t-PA gene.

The transgene expression pattern directed by the 1.4 kb t-PA promoter also raises a number of other issues. These same mice have previously been used to study t-PA promoter directed expression during development (Theuring *et al.*, 1995) and in this study, the 1.4 kb promoter directed widespread expression to the CNS. The fact that this is not observed in adult animal suggests that elements upstream of -1.4 are required to maintain continuous expression during development and into adult life while regulatory regions within the 1.4 kb promoter that direct expression to the CNS during development are subsequently silenced. We have not yet assessed the temporal changes in reporter gene expression in these particular transgenic mice during the neonatal period and early adulthood.

The unique transgene expression pattern directed by the 1.4 kb t-PA promoter also raises the question about MH-specific regulatory elements within this promoter region. The human insulin promoter also directs expression to this compartment and alignment of these two promoter sequences revealed a 15-bp region of identity. This

region included a sequence identical to the consensus NFAT binding site, suggesting that NFAT or a closely related protein may recognize this region and play a role in the expression of both the t-PA and insulin promoters in the MH. The evidence supporting this is circumstantial, however, it is interesting to point out that NFATc4 has been identified as a neuron-specific transcription factor that is expressed in hippocampal neurons (Ho *et al.*, 1994) and can activate genes upon changes in calcium levels (Ho *et al.*, 1995; Graef *et al.*, 1999; Crabtree, 2001). Although this region provided a binding site for proteins extracted from mouse brain as well as two neuronal cell lines, more direct studies are necessary to determine the role of this element, and its associated binding proteins, in the transcriptional regulation of the t-PA gene in the brain. It will also be interesting to explore the role of the other NFAT-like element located 12 bp downstream, but on the other DNA strand, in the t-PA promoter.

Our studies also show that the human t-PA promoter can be modulated by LPS treatment *in vivo*. LPS-mediated induction was transient and only observed in the 9.5 kb tPA-LacZ mice, indicating that regions within the t-PA promoter responsible for transcriptional induction by LPS reside between -3.0 kb and -9.5 kb. The molecular mechanisms underlying LPS-mediated induction of the t-PA promoter remain to be determined. However, it is interesting to point out that *i.v.* injection of LPS into rats has been shown to induce *c-fos* expression in the brain after 2 h (Elmqvist *et al.*, 1996). Members of the AP-1 transcription factor family are known to associate with key elements in the proximal human t-PA promoter (Costa & Medcalf, 1996), but the extent of AP-1 binding activity in the upstream promoter region is unknown. Nonetheless, it seems reasonable to speculate that LPS may be influencing t-PA expression via a *c-fos* related mechanism.

In summary, our results demonstrate that elements between -9.5 kb and 3.0 kb are required to direct spatial and LPS-inducible expression to various regions of the murine brain, while elements within the first 1.4 kb of the t-PA promoter direct expression exclusively to the MH. We also provide evidence for the existence of a NFAT-like element at position -707 in the t-PA promoter that provides a specific binding site for a brain-derived transcription factor(s). Based on circumstantial evidence, this region may also play a role in expression of t-PA in the medial habenula. Further studies are required to localize additional elements directing region specific t-PA expression in the CNS and the role of NFAT in this process.

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## Abbreviations

CAT, chloramphenicol acetyltransferase; DG, dentate gyrus; kb, kilobase pairs; LPS, lipopolysaccharide; M1, primary motor cortex; MH, medial habenula; NFAT, Nuclear factor of activated T-cells; PBS, phosphate buffered saline; RSC, retrosplenial cortex; S1, somatosensory cortex; S1BF, barrel fields of the somatosensory cortex; S2, secondary somatosensory cortex; t-PA, tissue-type plasminogen activator; Tu, olfactory tubule; VP, ventral posterior thalamic nuclei.

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