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In Vitro Analysis of the Tissue Plasminogen Activator Promoter Reveals a GC Box-Binding Activity Present in Murine Brain but Undetectable in Kidney and Liver

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Tissue plasminogen activator (t-PA) mRNA levels are high in murine brain, lower in kidney, and undetectable in liver. Differences in t-PA mRNA levels are regulated in part at the transcriptional level. Brain, kidney, and liver nuclear extracts direct regulated transcription from the murine t-PA promoter in a manner that reflects the relative levels of t-PA gene expression in these tissues in vivo. Analysis of mutants has defined two GC box motifs as important elements for regulated transcription in vitro. Upon investigation of protein-DNA binding, we detected an activity in brain extracts which was not detected in kidney or liver extracts. An Sp1-like factor also binds to this region in all three tissue types. DNA interference experiments show that the brain-enriched binding activity and the Sp1-like factor contact the same GC-rich sequences. These studies provide additional evidence that brain-enriched DNA-binding activities can interact with sequences also recognized by ubiquitous transcription factors.

In vitro transcription systems are valuable for defining sequences and identifying factors important for transcriptional regulation. Extracts which direct RNA polymerase II transcription have been prepared from *Drosophila* (35) and mammalian (9, 32) cell culture systems and from mammalian tissues (14). Such extracts have been used to (i) define *cis* elements (31) and *trans*-acting factors (29, 30) involved in the liver-specific transcription of the albumin gene and elements required for stage-specific regulation in *Drosophila* embryos (18) and (ii) study steroid hormone (6) and hormone receptor (23) enhancement of transcription. Thus, in vitro transcription systems have been used to dissect various elements involved in hormonal, temporal, and tissue-specific gene regulation.

The tissue plasminogen activator (t-PA) gene is regulated in many cells and tissues and is therefore useful for studying differential gene expression. For example, transcription of the mouse t-PA gene is specifically induced during in vitro differentiation of F9 teratocarcinoma cells (38). It was found that 190 bp of 5′-flanking sequence from the murine t-PA gene confers on reporter genes differentiation-specific expression in F9 cells in a manner similar to the regulation of the endogenous t-PA gene (39). However, differentiation-specific expression was not found in vitro, since transcription directed by the t-PA promoter was observed with use of undifferentiated F9 cell extracts as well as extracts prepared from differentiated cells (8). Thus, the F9 in vitro transcription system did not recapitulate the differential expression of the t-PA gene observed with F9 cells in vivo.

Here we report that differences in t-PA mRNA levels in adult murine brain, kidney, and liver (40) are in part regulated at the transcriptional level. Furthermore, in vitro transcription directed by the t-PA promoter demonstrates accurate initiation and tissue-specific expression in nuclear extracts prepared from brain, kidney, and liver in a manner that parallels that seen in vivo. Analysis of promoter deletion mutants suggests that multiple elements are involved in t-PA promoter-directed, tissue-specific in vitro transcription. We have analyzed in detail a 100-bp region of the t-PA promoter which contains GC boxes. Protein-DNA binding studies of this region have revealed an apparently novel factor, designated brain-derived GC box-binding factor (BGC), that is present in brain extracts but not in extracts prepared from kidney or liver.

MATERIALS AND METHODS

Plasmid and probe synthesis. The TAMCAT constructs, including the 5′ deletion mutants (39) and pE4-CAT (28), have been described elsewhere. A chloramphenicol acetyltransferase (CAT)-specific oligonucleotide was end labeled with polynucleotide kinase and [γ-^{32}P]ATP (42) for primer extensions. Mutations within the Sp1 consensus sequences (8; note that the previously used terms box 4 and box 5 have been changed to GC box 1 [GC1] and GC box 2 [GC2] in this report) or within the NF1 consensus sequence (from the parental deletion Δ11) were introduced by oligonucleotide-directed mutagenesis (27). A 280-bp *Stu*I-*Pst*I fragment of the t-PA promoter was used for DNase footprinting as described previously (8). The *Hin*dIII-*Pst*I fragments of Δ16 TAMCAT and GC1− TAMCAT were used as probes in mobility shift assays (MSA) as indicated. The appropriate plasmid was digested with *Hin*dIII and labeled with Klenow fragment and [α-^{32}P]dATP. The probe was released by digestion with *Pst*I.

Tissue extracts. Nuclear extracts were prepared from CD1 mouse tissues at 4°C as described previously (14), with minor modifications. Protease inhibitors and phosphatase inhibitors (2.5 kallikrein inactivator units of aprotinin per ml, 2.5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 20 μg of soybean trypsin inhibitor per ml, 1 μg each of leupeptin, antipain, chymostatin, and pepstatin per ml, 1 mM $NaMoO_4$, and 5 mM NaF) were included in all buffers except dialysis buffer. Tissues (routinely 10 g of brain and kidney and 25 g of liver) were adjusted to 55 ml with homogenization buffer without glycerol and homogenized

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with a Teflon-glass homogenizer. Two 27-ml aliquots of homogenate were layered over two 10-ml cushions (homogenization buffer with 10% glycerol and 1.7 M sucrose) and centrifuged at 24,000 rpm for 1 h at 0°C in an SW27 rotor. Proteins were extracted from the pelleted nuclei (35) and were resuspended in Manley dialysis buffer (MDB; 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 100 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 17% glycerol, 2.0 mM dithiothreitol). Nuclear extract was dialyzed twice for 2 h against 400 ml of MDB. Protein was determined by a dye binding assay (Bio-Rad Laboratories). Extract (usually 5 mg/ml) was aliquoted and frozen in liquid nitrogen. HeLa whole-cell extracts were prepared as described previously (32).

Nuclear run-on transcription. Nuclei were obtained as described above. After the first centrifugation step, nuclei were resuspended in 10 ml of lysis buffer (60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM β-mercaptoethanol, 15 mM Tris [pH 7.4]) containing 10 μg of RNase A per ml, incubated on ice for 30 min, and then spun through a 5-ml cushion of 30% sucrose in lysis buffer at 1,500 rpm for 10 min at 4°C. The pellet was resuspended in nuclei storage buffer at 10^8 nuclei per ml and stored at −70°C. Transcription from isolated nuclei was performed (15). Albumin cDNA was provided by S. Tilghman, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was provided by M. Piechaczyk.

RNA blot analysis. Total cellular RNA preparation and Northern (RNA) analysis were carried out according to Sambrook et al. (42). The ^{32}P-radiolabeled DNA probes for t-PA mRNA (pEE2) (38) and for 18S RNA (1) were synthesized by random priming (12). Specific activities were between 1×10^9 and 3×10^9 cpm/μg.

MSA. Protein-DNA complexes were resolved as described previously (3), with a few modifications. A typical binding reaction mixture (16 μl) contained 0.5 volume of nuclear extract in MDB, 4 μl of gel shift buffer (10 mM HEPES [pH 7.9], 50 mM NaCl, 2 mM $MgCl_2$, 0.1% Triton), 2.25 μg of poly(dI-dC), and end-labeled DNA probe (10 fmol; 20,000 cpm). After incubation on ice for 20 min, 4 μl of 4× dye (20% glycerol–40 mM dithiothreitol–0.1% bromophenol blue in 1× Tris-borate-EDTA [TBE]) was added, and samples were run on a nondenaturing 3% polyacrylamide gel (30:1, acrylamide/bisacrylamide ratio) containing 0.1% Triton in TBE buffer at 4°C (20 to 30 mA). DNA competitors were included with probe prior to addition of extract. Sp1 antiserum or preimmune serum was added prior to extract in antibody perturbation experiments (26).

In vitro transcription assays. In vitro transcription reactions were performed as described previously (17), with a few modifications. A half reaction volume consisted of protein extract in MDB. Supercoiled DNA template (the quantity of which did not exceed the linear range of the assay), 500 μM ATP, CTP, GTP, and UTP, 10 U of RNAguard (Pharmacia), and 10 mM creatine phosphate were added to extracts (final volume, 25 μl) and incubated at 30°C for 1 h. Transcription was terminated with stop buffer (100 mM Tris-HCl [pH 7.5], 0.5% sodium dodecyl sulfate, 300 mM NaCl, 10 mM EDTA, 70 μg of tRNA per ml) and phenol extracted twice. Nucleic acids were precipitated with ethanol twice, rinsed with 70% ethanol, and dried by vacuum. Primer extensions were performed (34) with use of a CAT-specific primer. Products were denatured and fractionated by electrophoresis on an 8 M urea–6% polyacrylamide (19:1, acrylamide/bisacrylamide ratio) gel and visualized by autoradiography.

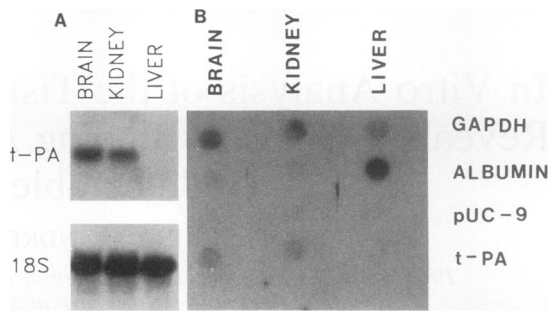

FIG. 1. Analysis of t-PA expression in brain, kidney, and liver. (A) Steady-state levels of t-PA mRNA in selected tissues (see also reference 40). Northern blot analysis was performed with RNA (40 μg) extracted from brain, kidney, and liver. After immobilization on nitrocellulose, RNA was hybridized with a random-primed ^{32}P-labeled probe for t-PA. Below is the identical blot reprobed with a ^{32}P-radiolabeled probe for 18S rRNA. The positions of the t-PA and 18S transcripts are indicated. (B) Rate of t-PA transcription in isolated nuclei from brain, kidney, and liver. Nuclei isolated from the three tissues were used to elongate run-on transcripts in vitro in the presence of [^{32}P]UTP. An equal number of counts of radiolabeled RNA from each tissue type was used to hybridize specific sequences (1.6 μg of GAPDH, 0.8 μg of albumin, 1.6 μg of pUC-9, and 1.6 μg of t-PA) immobilized on nitrocellulose.

DNase I protection assay. For footprint analysis (20), the protein-DNA binding reaction mixture (30 μl) contained 50 fmol of probe (1×10^4 to 3×10^4 cpm per tube), poly(dI-dC) (750 ng), and extract (20 μg) or purified NF1 factor (60 ng) in MDB containing 0.1% Triton. Samples were digested with DNase I (0 to 40 ng) for 1 min at 23°C. The reaction was extinguished, and the products were purified before electrophoresis on an 8 M urea–6% polyacrylamide gel. Products generated by chemical cleavage of the probe (33) were used as markers.

DNA interference experiments. The lower strand of the mutant GC1− t-PA promoter fragment was end labeled at the upstream *Hin*dIII site as described above and partially modified with diethyl pyrocarbonate (44). A large-scale binding reaction mixture (10× final volume) containing 75 μg of nuclear extract and 500,000 cpm of probe was incubated on ice for 25 min. Products were fractionated by electrophoresis on a nondenaturing 3% polyacrylamide gel. Free probe and complexed probe were localized by autoradiography overnight, excised from the gel, and electrophoresed onto an NA 45-DEAE membrane (Schleicher & Schuell) (2). DNA was eluted from the membrane (41), phenol-chloroform extracted, ethanol precipitated, and treated with piperidine. Equal counts (1,200 cpm) were electrophoresed on an 8 M urea–12% polyacrylamide gel. Maxam and Gilbert sequencing was performed on unmodified (G/A and C/T ladders) and diethyl pyrocarbonate-modified (A>G ladder) DNA, and the products were used as markers.

RESULTS

Differences in t-PA mRNA in murine brain, kidney, and liver are due, at least in part, to transcriptional control. t-PA mRNA is variably expressed in a wide variety of adult murine tissues. In particular, t-PA mRNA is observed at high levels in brain, lower levels in kidney, and undetectable levels in liver (Fig. 1A; 40). To investigate whether the differential accumulation of t-PA mRNA was due to transcriptional regulation, purified nuclei isolated from adult

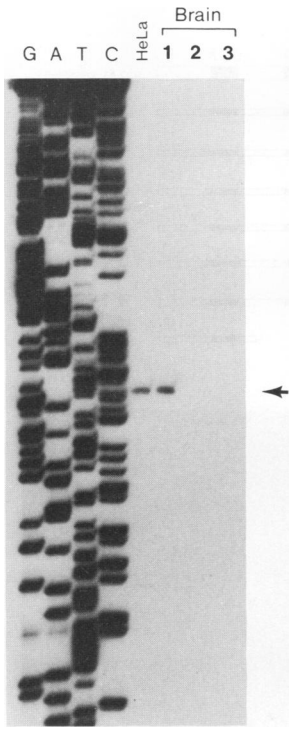

FIG. 2. In vitro transcription from 2.0 TAMCAT in brain extracts. HeLa whole-cell extract (125 μg) or brain nuclear extract (40 μg) was used to direct the in vitro transcription from a supercoiled template, 2.0 TAMCAT (21 nmol). RNA synthesized in vitro was analyzed by primer extension, and products were electrophoresed next to a dideoxy sequence of 2.0 TAMCAT (lanes G, A, T, and C). The arrow marks the position of the proximal initiation site of transcription for the t-PA promoter in vivo. Reactions were also performed in the presence of α-amanitin (4 μg/ml; lane 2) or in the absence of exogenously added template (lane 3).

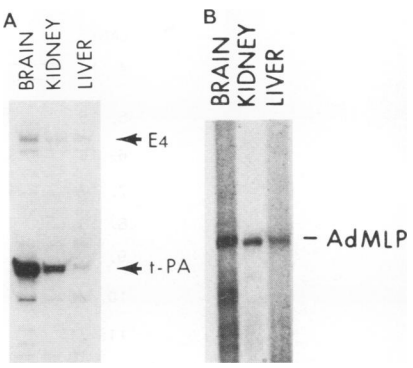

FIG. 3. In vitro transcription from the t-PA promoter in tissue extracts. (A) Nuclear extracts from brain, kidney, and liver (40 μg each) were used to direct transcription from the 2.0 TAMCAT template. RNA was analyzed by primer extension. E4CAT (equimolar with the TAMCAT template [21 nmol]s) was included in the reactions as an internal control. Transcription from TAMCAT, when normalized with respect to transcription from E4CAT, occurred in the ratio of 20:6:1 in brain, kidney, and liver extracts, respectively. (B) Transcripts synthesized in vitro from the AdMLP. The supercoiled template (1 μg) which consisted of the AdMLP upstream of a G-free cassette was used to synthesize radiolabeled transcripts in nuclear tissue extracts (75 μg of protein) in the presence of 3′-*O*-methyl-GTP (0.1 mM). The products of the reaction were run directly on a sequencing gel without primer extension (14). The position of the AdMLP-specific transcript is indicated.

brain, kidney, and liver were used in nuclear transcription experiments (Fig. 1B). The elongated radiolabeled RNA was used to probe a nitrocellulose filter on which t-PA plasmid DNA was immobilized. The level of t-PA transcription in the three nuclear preparations directly paralleled that of the steady-state levels of t-PA mRNA with the exception that a low level of transcription was detectable in liver nuclei, in contrast to undetectable steady-state levels of t-PA mRNA. The discrepancy may be due to RNA instability in vivo or perhaps to the different sensitivities of the two assays. As expected, transcription of GAPDH sequences was relatively constant among the three tissue types, since its regulation occurs at the posttranscriptional level in many tissues (36) and albumin transcription was specifically high in liver. No significant signal was observed for pUC-9 sequences. These results demonstrate that tissue-specific t-PA expression is regulated at least in part at the transcriptional level.

t-PA is accurately transcribed in vitro in brain nuclear extracts. To determine whether the murine tissues were an appropriate system for studying differential expression of t-PA, we tested whether extract prepared from brain tissue could accurately initiate transcription from the t-PA promoter. Nuclear extracts were prepared from adult brain (14), and whole-cell extracts were prepared from HeLa cells (32). The template consisted of 2 kb of 5′-flanking sequences of the t-PA promoter fused to the CAT gene (2.0 TAMCAT). RNAs were synthesized in vitro, and the RNA products were subjected to primer extension. The products of these primer extension reactions were electrophoresed next to a sequencing ladder generated from the template in order to identify the initiation site(s) of transcription (Fig. 2). Two start sites of transcription have previously been determined during analysis of RNA extracted from various murine tissues (39). However, in vitro, both HeLa and brain extracts directed t-PA transcription from only the proximal start site. No transcription was detected in the presence of α-amanitin (lane 2) or in the absence of exogenously added template (lane 3).

Regulated in vitro transcription is observed for the t-PA promoter in brain, kidney, and liver nuclear extracts. To investigate whether the t-PA promoter could function in a tissue-specific manner in vitro, transcription reactions were performed with nuclear extracts prepared from brain, kidney, and liver. The adenovirus E4 promoter fused to CAT reporter sequences (E4CAT; 28) was used as an internal control in addition to the test template, 2.0 TAMCAT. RNA was analyzed by primer extensions, and the products were quantitated after electrophoresis by densitometric scanning. As can be seen in Fig. 3A, transcription from 2.0 TAMCAT occurred in a tissue-specific manner, in a ratio of 20:6:1 (brain/kidney/liver), when normalized with respect to E4CAT.

To support the quantitation of regulated t-PA promoter-directed transcription in vitro, we tested another control promoter template to determine whether all three extracts were similarly transcriptionally competent. A plasmid containing the adenovirus type 2 major late promoter (AdMLP) fused to a G-free cassette (43) was used to direct transcription of radiolabeled RNA with a predicted size of approximately 200 bp. The absence of G residues between the promoter cap site and the G-free cassette allows specific termination at the first G residue flanking the cassette in the absence of GTP. Figure 3B shows that the AdMLP tran-

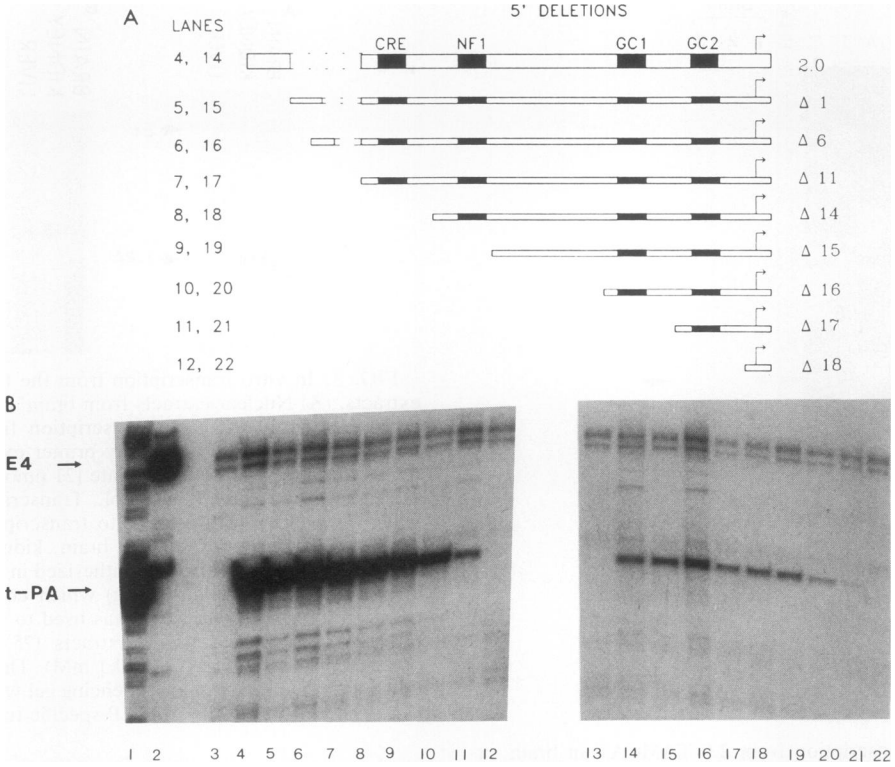

FIG. 4. In vitro transcription from 5′ deletion mutants of the t-PA promoter in tissue extracts. (A) Diagrammatical representations (center) and numerical designations (right) of the t-PA promoter mutants (also see reference 39). Regions of sequence homology to the CRE, NF1, and Sp1 consensus sequences are indicated by black rectangles (distances are not to scale). The lanes in which each mutant was used as a template for in vitro transcription reactions are shown on the left. (B) Primer-extended products of in vitro transcription reactions using the promoterless construct pUCCATpA (lanes 3 and 13) or the t-PA 5′ deletion mutants (21 nmol each) in brain nuclear extract (40 μg; lanes 3 to 12) and liver nuclear extract (40 μg; lanes 13 to 22). E4CAT was included with the 5′-deleted templates (in equimolar amounts) as an internal control (lanes 3 to 22). 2.0 TAMCAT alone (lane 1) and E4CAT alone (lane 2) were used to direct in vitro transcription in HeLa whole-cell extracts as positional markers, labeled as indicated. Minor bands are most likely the result of inappropriate initiation of transcription from vector sequences or incomplete primer extension. Quantitation by densitometry yields the following ratios (t-PA/E4) for lanes 4 to 12, respectively: 6.00, 7.92, 6.80, 8.45, 8.75, 7.88, 6.2, 0.78, and 0.10.

scribes with comparable efficacy in all three tissues. Quantitation by densitometry revealed that transcription in brain extract was approximately twofold higher than transcription in liver extract. Since all three extracts direct similar quantities of transcripts from either the E4 promoter or AdMLP, the enhanced expression from the t-PA promoter in brain extract strongly suggests that elements responsible for this regulated expression lie within the t-PA promoter.

5′ promoter deletion analysis suggests that the GC box motifs are important for regulated in vitro transcription. To investigate sequences of the t-PA promoter which are important for tissue-specific expression in vitro, a series of 5′-deleted t-PA promoter fragments fused to the CAT reporter gene was used as templates in brain and liver nuclear extracts, with an equimolar amount of E4CAT as a control (Fig. 4). This experiment also illustrates that the E4 promoter directs approximately equivalent transcription in the two extracts. Both 2.0 TAMCAT and E4CAT were transcribed alone in HeLa cell extracts (lanes 1 and 2, respectively) to determine respective plasmid-specific transcripts. The promoterless construct pUCCATpA (lanes 3 and 13) does not direct any transcripts of appropriate length. In brain nuclear extracts, there are small changes in transcription from mutant TAMCAT templates as the deletions proceed toward GC1 and GC2 (see figure legend for quantitation). A dramatic decrease in transcription (approximately 10-fold) is seen from the 5′ deletion mutants which remove these sites (lanes 11 and 12). In contrast, the level of transcription from mutant TAMCAT templates in liver extracts remains relatively constant as the deletions approach GC1 and GC2. A dramatic decrease in t-PA promoter-directed transcription is also observed in liver extracts upon deletion of GC1 and GC2. Thus, the data suggest that the GC boxes are important for (i) basal activity, since their removal greatly reduces the level of transcription in liver extracts, and (ii) regulated transcription, since a mutant containing only these two boxes (Δ16) exhibits brain-enriched transcription.

Several site-directed mutants (8), created to alter sequences in the GC boxes known to be important for Sp1 binding, were tested for their effects on t-PA promoter-driven transcription in tissue extracts. A mutant designed to interfere with either GC1 (GC1−) or GC2 (GC2−) binding was transcribed with an efficiency similar to that of a deletion mutant containing only GC2. The double mutant (GC1−/2−) directed transcription from the t-PA promoter at the very low level of a deletion mutant lacking both boxes. A mutant (GC2+) designed to make GC2 a more perfect Sp1 consensus sequence resulted in levels of transcription similar to those of the parental template (Δ16) containing intact GC boxes (Fig. 5). These results paralleled those obtained with F9 cells (8) and suggest that the GC boxes are important elements for transcription in vitro.

	DNA Sequence	Footprint	Transx
NF1 consensus sequence:	5' NTTGGCN NNN NGCCAAN		
wt t-PA sequence:	5' ACTGGCG GCA AGCCAAG	+	+
NF1+ mutant:	5' ATTGGCG GCA AGCCAAG	+	+
NF1- mutant:	5' ACTTGCG GCA AGCCAAG	-	+
NF1-- mutant:	5' ACTTGCG GCA AGCAAAG	-	+

		In vitro Transcription[a]
Sp1 consensus sequence:	5' G/A T/C T/C CCGCCC A/C	
wt t-PA Box GC1	5' A C C CCGCCC A	
mutant GC1-	5' A C C TTGCCC A	≈Δ17 (GC2)
wt t-PA Box GC2	5' G C C CCACCC C	
mutant GC2+	5' G C C CCGCCC C	≈Δ16 (GC1,GC2)
mutant GC2-	5' G C C TTACCC C	≈Δ17 (GC2)
mutant GC1-/GC2-	contains GC1- and GC2-	≈Δ18 (none)

FIG. 5. Summary of in vitro analysis of site-directed mutants in brain extract. NF1 site-directed mutants were created within a Δ11 background. GC box site-directed mutants were created within a Δ16 background. Nucleotides in bold type denote wild-type (wt) sequences that deviate from the consensus sequence. Site-directed mutations are underlined. [a], For quantitative comparison, the 5′ deletion mutant yielding roughly equivalent in vitro transcription is indicated. GC boxes present in each 5′ deletion mutant are shown in parentheses (see Fig. 4).

Several nuclear factors from tissue extracts bind the t-PA promoter. Overall protein-DNA binding activity in tissue extracts was examined by DNase I footprinting. DNA footprinting failed to reveal any obvious tissue-specific factors (Fig. 6), since extracts from all three tissues similarly bound three regions. Weak footprints were observed over sequences that correspond to GC1 and GC2. The third footprint was located over the NF1 consensus sequence. Purified human NF1 (41) protected the same region (lane 2) as did factors contained in brain, kidney, and liver nuclear extract (lanes 3, 4, and 5, respectively). Several site-directed mutants were created in the NF1 consensus sequence, two of which abolished the footprint. However, these mutants had no effect on t-PA promoter-directed transcription in vitro (Fig. 5). Thus, NF1 binds the t-PA promoter in vitro but does not play a significant role in the in vitro transcription of t-PA (compare 5′ deletion mutants with and without the NF1 consensus sequence; lanes 8, 9, 18, and 19 in Fig. 4). Whether NF1 binding to the t-PA promoter contributes in vivo has yet to be determined.

MSA reveal subtle differences in GC box-binding activity among different tissue extracts. MSA (13) were used to examine further the DNA-protein interactions occurring at the GC box sequences. Unexpectedly, a radiolabeled probe containing GC1 and GC2 incubated in nuclear extracts prepared from brain (Fig. 7a, lane 1) produced a more complex pattern of bands than did those prepared from kidney and liver (lanes 7 and 13), although some bands were common to all three extracts. MSA were carried out in the presence of increasing amounts of homologous competitor DNA or nonhomologous competitor DNA in an attempt to determine the specificities of the shifted complexes. The shifted complexes in all three tissue extracts were competed for with a 50-fold (lanes 2, 8, and 14), a 100-fold (lanes 3, 9, and 15), and a 200-fold (lanes 4, 10, and 16) molar excess of unlabeled homologous DNA. Note that the faster-migrating complexes were more resistant to competition. A 200-fold (lanes 5, 11, and 17) and a 500-fold (lanes 6, 12, and 18) molar excess of unlabeled nonhomologous DNA did not result in competition of the shifted complexes in any of the three extracts. Therefore, we conclude that the shifted complexes result from sequence-specific protein-probe interactions.

The presence of two GC boxes in the MSA probe led to a complexity of the shifted bands which was difficult to analyze. To simplify the pattern of shifted bands, we used site-directed mutants which disrupt either one GC box, both GC boxes, or neither GC box, in addition to wild-type DNA, as probes in the MSA. A reduction of complexity in the shifted bands in all three tissues was observed (Fig. 7b). More importantly, an obvious difference became apparent among tissue extracts when either of the single site-directed mutants, GC1− or GC2−, was used as a probe in the MSA. A band of slower mobility (complex 2) was present in brain extracts (lanes 6 and 14) but absent in kidney and liver extracts. Some complexes were common to all extracts (complexes 1 and 4). A minor complex slower in mobility than complex 2 (complex 3) was present only in kidney and liver extracts (lanes 7, 8, 15, and 16). Use of the mutant GC2+ probe (lanes 10 to 12), designed to improve the Sp1 consensus sequence, resulted in a pattern of shifted bands identical to that of the wild-type probe. Shifted complexes were absent in MSA with use of the double site-directed mutant. The MSA was also performed with various amounts of total protein extract (data not shown). These experiments demonstrated that the brain-enriched GC box-binding activity was observed with 3 μg of brain extract protein but not with 15 μg of liver extract protein. This result establishes a fivefold minimum enrichment of the binding activity in brain extract versus liver extract.

All complexes formed with the GC box contact identical nucleotides. To determine whether the brain-enriched activity (complex 2) bound to the same or overlapping DNA sequences, binding interference experiments (44, 45) were performed (Fig. 8). The GC1− probe was partially modified by diethyl pyrocarbonate treatment and used in MSA. Complex 2 had contact points identical to those of complexes 1 and 4 in brain extracts. In fact, all shifted complexes in brain extracts contacted the same residues. All shifted complexes in liver extract also contacted identical nucleotides even though the interference pattern was shifted approximately 1.5 bp compared with that of brain extract. The cause of this anomaly is not yet known. The results of the binding interference experiments demonstrate that the factor ob-

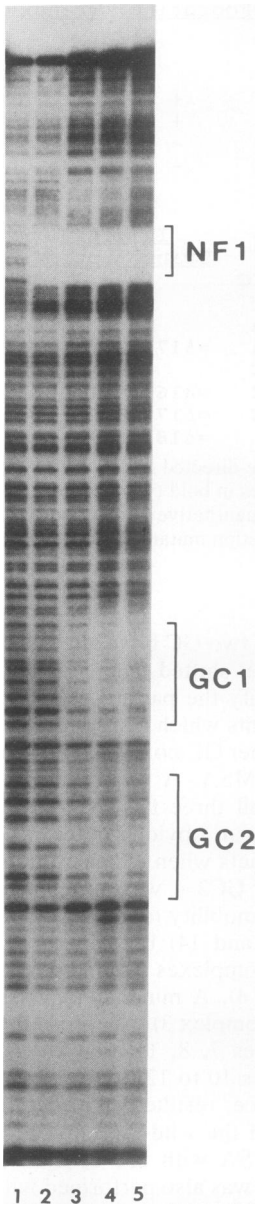

FIG. 6. Protein binding to the t-PA promoter. Proteins in the tissue nuclear extracts were assayed for DNA-binding activity by DNase I protection. A probe containing t-PA sequences downstream from Δ11 was made by end labeling the upper strand. The probe was digested with DNase I (25 to 35 ng) in the absence of extract (lane 1) or in the presence of purified human NF1 factor (60 ng; lane 2), brain nuclear extract (20 μg; lanes 3), kidney nuclear extract (20 μg; lane 4), or liver nuclear extract (20 μg; lane 5). Regions of protection are indicated at the right and correspond to the consensus sequence for two GC boxes and a NF1-binding site.

served only in murine brain extract recognizes the Sp1-binding site.

The presence of Sp1 antibody in MSA does not perturb the brain-enriched DNA-binding activity. To ascertain whether transcription factor Sp1 was a component of any of the shifted bands, a polyclonal antibody that recognizes Sp1 was used (Fig. 9). As a control, probe was subjected to MSA using purified human Sp1 (lane 1). The addition of Sp1 antibody caused a supershift (lane 2), while preimmune serum did not perturb the shift (lane 3). Preimmune or immune serum was also included in the MSA binding reaction using tissue extracts. The antibody abolished the shifts common to all three extracts, suggesting that their formation was due to the interaction of a murine Sp1-like factor with the single GC box probe (lanes 5 and 7). Most interestingly, the complex with the slower mobility unique to brain extracts (complex 2) was not significantly affected by the antibody (lane 5). Our results demonstrate that a DNA-binding activity identified in brain extract, designated BGC, is antigenically distinct from Sp1 yet interacts with at least an overlapping part of a GC box.

DISCUSSION

In this report, we demonstrate that the t-PA gene is regulated at least in part at the transcriptional level in vivo. This work prompted us to examine the expression of the t-PA promoter in vitro by using extracts prepared from murine tissues. Results obtained from studies using 5′ deletion mutants and site-directed mutants suggest that a 100-bp fragment of t-PA sequences (including the start sites of transcription) plays a role in the regulated expression observed in tissue extracts. Further investigation of proteins binding to this promoter fragment revealed two important characteristics of the t-PA promoter. First, the 100-bp fragment contained two GC boxes that bound a factor specifically recognized by an Sp1 antiserum in all three extracts. Second, a particular DNA-binding activity (complex 2) was detected by MSA in brain extract but not in kidney and liver extracts. Although this factor, BGC, was shown by interference experiments to recognize the same DNA sequence as does the Sp1-like factor, several lines of evidence suggest that it is distinct from Sp1: (i) the BGC-DNA probe complex was not affected by an Sp1 antiserum in MSA, (ii) this complex had a mobility slower than that of a complex recognized by the Sp1 antiserum, and (iii) the formation of this complex was tissue selective, in contrast to a complex containing Sp1, which is considered to be ubiquitous.

The molecular aspects governing gene regulation in the brain likely involve brain-enriched *trans*-acting factors, some of which may interact with common DNA elements as shown here. For example, BETA, a brain-specific transcriptional activator, interacts with the same DNA-binding site as does the ubiquitous factor NF-κB (24). In addition, a sequence comparison of several brain-specific promoter fragments (aldolase c, γ-enolase, and creatine kinase) does not reveal any shared brain-specific *cis*-acting elements. Instead, the presence of GC boxes was a common characteristic (46).

GC-rich sequences are common *cis*-acting elements regulating the expression of many genes. Recently, it has become apparent that many factors can interact with the same DNA sequence. Like other DNA sequences, such as the AP1 site, which binds a family of at least six different members (reviewed in reference 25), and the CRE site, which binds at least seven (16), the GC-rich sequences seem to be a binding site for a family of transcription factors. Different types of factors, including positive, negative, and inducible factors, have been reported to bind GC-rich sequences. To the best of our knowledge, BGC is distinct from GC box-binding factors previously identified (4, 5, 19, 21, 22, 47). Our results from Sp1 antibody experiments suggest that BGC is antigenically distinct from the best-characterized GC box factor, Sp1 (7, 10, 11). One may speculate that BGC is a form of Sp1 which has undergone modification such that antibody no

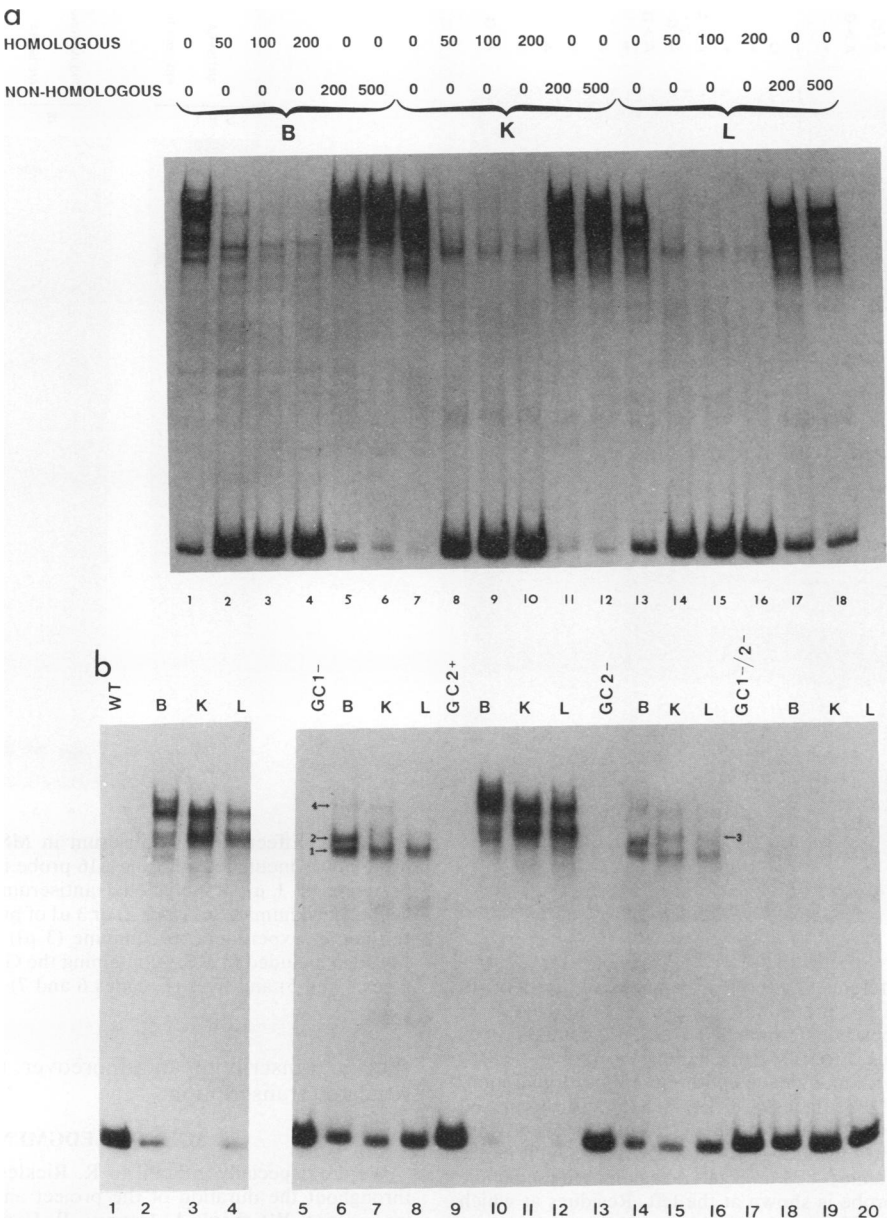

FIG. 7. Mobility shift assays of a GC box-containing t-PA fragment. (a) MSA competitions with unlabeled DNA fragments. A molar excess of homologous (same as probe) or nonhomologous (sequences upstream of Δ16) fragment was included as indicated in reaction mixtures containing 7.5 μg of brain (B; lanes 1 to 6), kidney (K; lanes 7 to 12), or liver (L; lanes 13 to 18) nuclear extract. (b) Site-directed GC box mutants in MSA. The wild-type (WT; lanes 1 to 4), mutant GC1− (lanes 5 to 8), mutant GC2+ (lanes 9 to 12), mutant GC2− (lanes 13 to 16), and mutant GC1−/2− (lanes 17 to 20) probes were incubated in the absence (lanes 1, 5, 9, and 13) or presence of brain (lanes B), kidney (lanes K), or liver (lanes L) nuclear extract. Complexes 1 to 4 are indicated.

longer recognizes it. Alternatively, BGC could represent a complex involving Sp1 and a brain-enriched coactivator (37) which interferes with the recognition of Sp1 by the antibody. In evaluating these possibilities, one must consider that a polyclonal antibody against Sp1 did not recognize the brain-specific complex.

Mechanism. The DNA-binding activities of BGC and the Sp1-like factor seem to be similar in brain extracts. Although the biochemical and functional relationship of BGC and Sp1 has yet to be elucidated, several possibilities may be proposed for their mode of action. In erythroid extracts, an erythrocyte-specific factor, NF-E1, displaces the binding of Sp1 to the ζ-globin gene promoter (48). The authors suggest that Sp1 could act as a transcriptional repressor of the ζ-globin gene in nonerythroid cells. A similar hypothesis could be put forth for the tissue-specific expression directed from the t-PA promoter; i.e., brain-enriched BGC may displace Sp1. Within the context of the t-PA promoter, BGC could be a better transcriptional activator than Sp1 and perhaps synergize more efficiently with general factors that bind the t-PA promoter further upstream. Alternatively, BGC may interact with other brain-enriched factors that bind the t-PA promoter upstream, which have not yet been detected in our assays, perhaps because of their low abun-

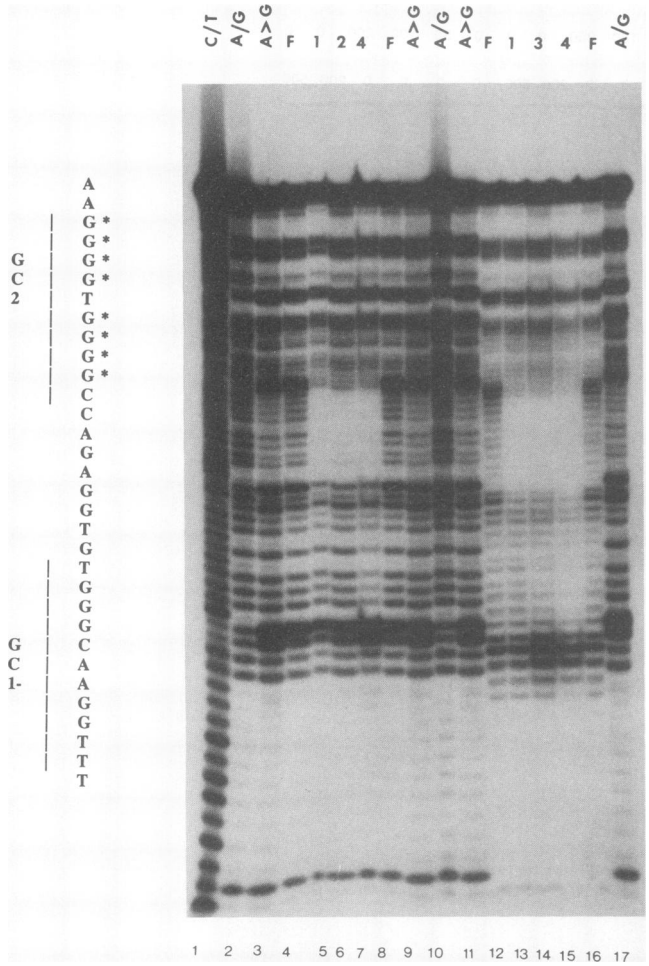

FIG. 8. DNA interference patterns of complexes formed between the mutant GC1− probe and factors in brain and liver extracts. Diethyl pyrocarbonate-modified free and bound probes (see Fig. 7b, complexes 1 to 4) were separated by native electrophoresis, eluted, purified, and cleaved with piperidine. Equal numbers of counts of free DNA (lanes 4, 8, 12, and 16) and complexed DNA (brain complexes 1, 2, and 4 in lane 5, 6, and 7, respectively; liver complexes 1, 3, and 4 in lanes 13, 14, and 15, respectively) were run on a 12% polyacrylamide–8 M urea gel. A region of the sequence of the mutant GC1− probe is shown at the left. Residues at which modification interfered with protein binding are indicated by asterisks.

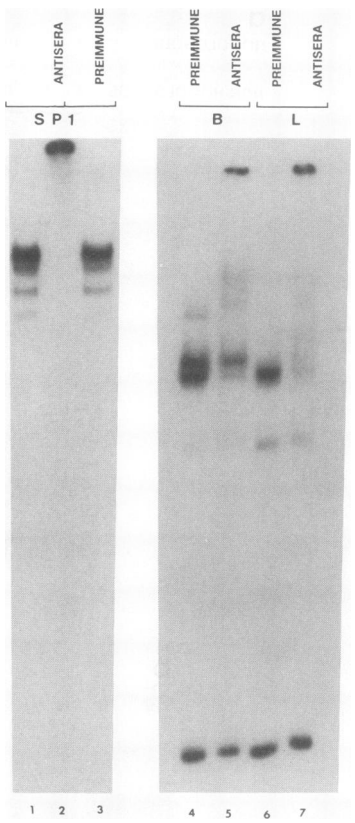

FIG. 9. Effect of Sp1 antiserum in MSA. Purified human Sp1 protein was incubated with the Δ16 probe in the absence (lane 1) or presence of 3 μl of polyclonal antiserum raised against *E. coli*-synthesized human Sp1 (lane 2) or 3 μl of preimmune serum (lane 3). In another experiment, preimmune (3 μl) or Sp1 (3 μl) antiserum was also included in MSA containing the GC1− probe and brain (B; lanes 4 and 5) and liver (L; lanes 6 and 7) extracts.

dance. The results from the deletion analysis support these models, since they clearly demonstrate that sequences upstream of the GC boxes are important for increased transcription in brain extracts but have little effect on transcription in liver extracts.

One intriguing possibility for multiple tissue-specific genetic programs is that they are conferred by tissue-enriched *trans*-acting factors which act at common DNA elements. We have shown that GC box sequences are important *cis* elements for tissue-specific in vitro transcription from the t-PA promoter and that brain contains both a ubiquitous Sp1-like GC box-binding activity in addition to an apparently distinct brain-enriched GC box-binding activity. Future investigations will resolve whether the findings reported here elucidate general mechanisms applicable to describe brain-specific transcription and, moreover, mechanisms of tissue-regulated transcription.

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