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Glial Fibrillary Acidic Protein Transcription Responses to Transforming Growth Factor-β1 and Interleukin-1β Are Mediated by a Nuclear Factor-1-Like Site in the Near-Upstream Promoter

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Abstract: Elevated expression of glial fibrillary acidic protein (GFAP) is associated with astrocyte activation during responses to injury in the CNS. Because transforming growth factor-β1 (TGF-β1) and interleukin-1β (IL-1β) are released during neural responses to injury and because these cytokines also modulate GFAP mRNA levels, it is of interest to define their role in GFAP transcription. The increases of GFAP mRNA in response to TGF-β1 and decreases in response to IL-1β were shown to be transcriptionally mediated in rat astrocytes transfected with a luciferase-reporter construct containing 1.9 kb of 5′-upstream rat genomic DNA. Constructs containing sequential deletions of the rat GFAP 5′-upstream promoter identified a short region proximal to the transcription start (−106 to −53 bp) that provides full responses to TGF-β1 and IL-1β. This region contains an unusual sequence motif with overlapping nuclear factor-1 (NF-1)- and nuclear factor-κB (NF-κB)-like binding sites and homology to known TGF-β response elements. Mutagenesis (3-bp exchanges) in −70 to −68 bp blocked the induction of GFAP by TGF-β1 and the repression by IL-1β. Gel shift experiments showed that the DNA segment −85 to −63 bp was bound by a factor(s) in nuclear extracts from astrocytes. The concentrations of these DNA binding factors were increased by treatment of astrocytes with TGF-β1 and decreased by IL-1β. Binding of these nuclear factors was blocked by mutation of −70 to −68 bp. Despite homology to NF-1 or NF-κB binding sites in the GFAP promoter at segment −79 to −67 bp, anti-NF-κB or anti-NF-1 antibodies did not further retard the gel shift of the nuclear factors/DNA complex. Moreover, astrocytic nuclear proteins do not compete for the specific binding to NF-1 consensus sequence. Thus, nuclear factors from astrocytes that bind to the −85- to −63-bp promoter segment might be only distantly related to NF-1 or NF-κB. These findings are pertinent to the use of GFAP promoter constructs in transgenic animals, because *cis*-acting elements in the GFAP promoter are sensitive to cytokines that may be elaborated in response to expression of transgene products. **Key Words:** Glial fibrillary acidic protein promoter—Transforming growth factor-β1—Interleukin-1β—Nuclear factor-1 binding site.
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This study analyzed the transcriptional regulation of glial fibrillary acidic protein (GFAP) by transforming growth factor-β1 (TGF-β1) and interleukin-1β (IL-1β). These and other cytokines are implicated in the regulation of GFAP expression during neurodegenerative diseases and experimental brain lesions (Duguid et al., 1989; Steward et al., 1990; Logan et al., 1992). Modulations of GFAP expression are implicated in the regulation of astrocyte–neuron interactions during responses to brain injury (Steward et al., 1990; Laping et al., 1994*a*,*b*) and during the estrous cycle (Stone et al., 1998). The sensitivity of GFAP expression to even mild neuronal impairments (Canady and Rubel, 1992) has led to the use of GFAP expression as a general marker for neurodegenerative changes (May et al., 1997). The functional role of GFAP is controversial because mice lacking GFAP (GFAP-KO) develop normally, are fertile, and respond to stab wound injury (Gomi et al., 1995; McCall et al., 1996). However, GFAP-KO mice showed impairments in long-term potentiation and long-term depression, which may reflect alterations in astrocyte–neuron interactions (McCall et al., 1996; Shibuki et al., 1996).

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Abbreviations used: AP-1, activator protein-1; BSA, bovine serum albumin; GFAP, glial fibrillary acidic protein; IL-1β, interleukin-1β; NF-1, nuclear factor-1; NF-κB, nuclear factor-κB; TβRE, transforming growth factor-β1 responsive element; TGF-β1, transforming growth factor-β1.

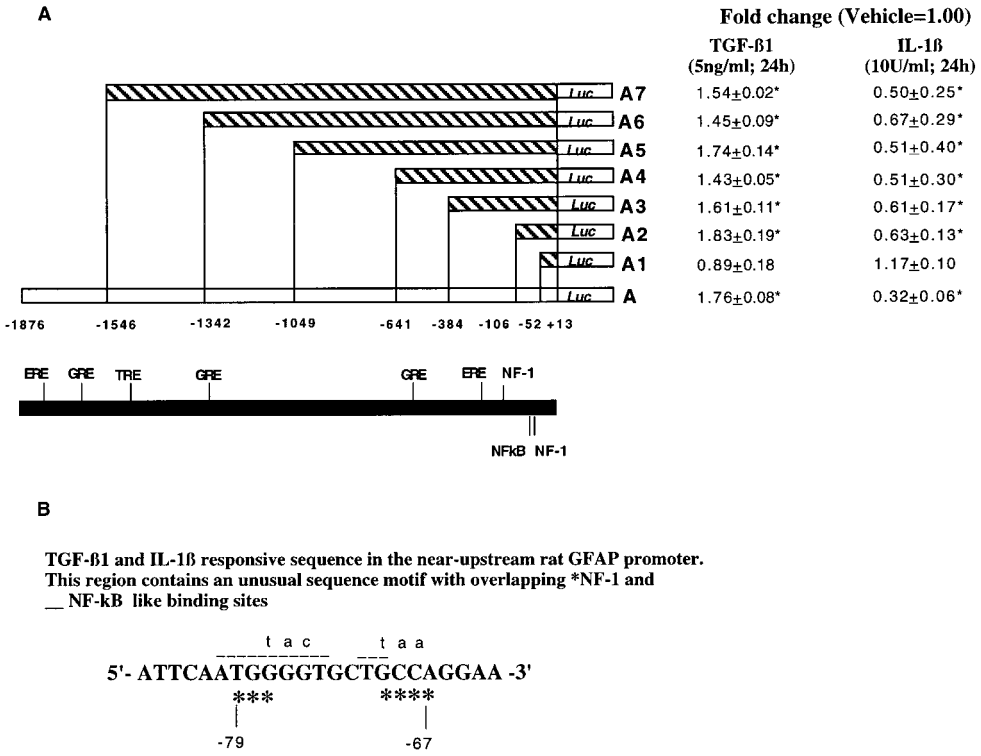

FIG. 1. A: The rat GFAP promoter constructs and relative luciferase activity affected by TGF-β1 and IL-1β in cultured astrocytes. We show several response elements in the 5′-upstream region of the rat GFAP: NF-1, NF-κB, ERE (estrogen response element), GRE (glucocorticoid response element), and TRE (AP-1 binding site; 12-*O*-tetradecanoylphorbol 13-acetate response element) (Laping et al., 1994*b*). Luciferase activity was normalized to total cellular protein and β-galactosidase to control for transfection efficiency. Data are mean ± SEM values, expressed as fold change from values for vehicle controls (vehicle = 1.00) in three independent experiments. *$p < 0.05$. **B:** TGF-β1 and IL-1β response motif in the near-upstream rat GFAP gene. Mutated bases are shown above in lowercase letters.

Among the cytokines induced during neurodegeneration that also modify GFAP expression are TGF-β1 and IL-1β (Laping et al., 1994*a*; Krieglstein et al., 1995). By the nuclear run-on assay, TGF-β1 increases GFAP transcription (Laping et al., 1994*a*). However, GFAP may also be regulated posttranscriptionally (Selmaj et al., 1991; Laping et al., 1994*b*). Because the GFAP promoter is often used to drive the expression of transgenes, e.g., of TGF-β1 (Galbreath et al., 1995), it is important to learn more about *cis*-acting elements that regulate its responses to cytokines. We therefore also examined whether the repression of GFAP expression by IL-1β (Selmaj et al., 1991; Oh et al., 1993) is mediated transcriptionally.

The 5′-upstream promoter of GFAP contains numerous *cis*-acting regulatory elements (Brenner et al., 1994; Laping et al., 1994*b*), consistent with increased transcription in response to deafferentiating lesions (Brenner et al., 1994; Laping et al., 1994*b*) and to TGF-β1 (Laping et al., 1994*b*). Among possible *cis*-acting sequences that might mediate responses to TGF-β1 are nuclear factor-1 (NF-1)- and nuclear factor-κB (NF-κB)-like binding sites, as well as TGF-β1 responsive elements (TβREs), which occur in the near 5′-upstream rat GFAP promoter (Laping et al., 1994*b*). NF-1-like binding sequences mediate transcriptional influences of TGF-β1 in a collagen promoter (Rossi et al., 1988; Ritzenthaler et al., 1993). We determined the activity of the NF-1/TβRE-like site in the near-upstream rat GFAP promoter that mediates the induction of GFAP by TGF-β1 in rat primary cultured astrocytes.

MATERIALS AND METHODS

GFAP promoter luciferase contructs

Of the rat GFAP 5′-upstream region, 1.9 kb was directionally subcloned into *Kpn*I and *Nhe*I sites of the pGL2 Basic plasmid (Promega, Madison, WI, U.S.A.; Fig. 1) and confirmed by sequencing. Construct A contains the full 1.9-kb (−1,876 bp to +13 bp) 5′-upstream rat genomic sequence (Rozovsky et al., 1995; Morgan et al., 1997). Other constructs contained consecutive deletions numbered serially (A1–A8) in relation to upstream length (Fig. 1). Fragments were obtained by internal restriction sites (Fig. 1) in combination with *Kpn*I, *Sma*I, and *Apa*I sites in the pGL2 Basic vector and were blunt-ended using T4 DNA polymerase for religation and subcloning into the pGL3 Basic vector (Promega). Fragment A1 was constructed using one of the internal *Xho*II sites in combination with *Bgl*II restriction. Because we noted possible confounds with the widely used pGL2 reporter, which contains an active activator protein-1 (AP-1) site, all data presented here are obtained with GFAP constructs in the pGL3 Basic vector.

Site-directed mutagenesis

Construct A2 was subcloned into pALTER (Promega) and engineered by site-directed mutagenesis using Altered Sites IITM (Promega) and oligonucleotides containing three or six base mutations described below. Mutations were confirmed by sequencing.

Cell culture and transfection

Primary neonatal astrocytes were originated from cerebral cortexes of 1–3-day-old F344 rat pups by mechanical dissociation, as described by McCarthy and de Vellis (1980). Cells were plated in plastic culture dishes at 2×10^5 cells/cm^2 and cultured in Dulbecco's modified Eagle's medium/F12 culture medium (Cellgro) supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY, U.S.A.), 100 U/ml penicillin, and 50 U/ml streptomycin (Sigma, St. Louis, MO, U.S.A.) at 37°C in a 5% CO_2/95% air incubator, with medium renewal every 2–3 days until confluence (8–12 days). Confluent cultures were purified from contaminating microglia and oligodendrocytes by a standard shaking procedure (Giulian and Baker, 1986). This purification usually yields cultures in which 98% of the cell population are GFAP-immunopositive astrocytes (Rozovsky et al., 1995). Purified astrocytes (primary cultures) were replated in six-well plates (1×10^6 cells per well) for transient transfection. Therefore, the first passage of monotypic astrocyte cultures were used in these studies.

Promoter constructs were transiently transfected into astrocytes by lipofection (Superfect TM; Qiagen, Santa Clarita, CA, U.S.A.). To evaluate efficiency of transfection, cells were cotransfected with an SV40 promoter-driven β-galactosidase gene (pSV-β-galactosidase control vector; Promega). At 48 h after transfection cells were treated with TGF-β1 (5 ng/ml) and IL-1β (10 U/ml) in serum-free medium. Porcine platelet TGF-β1 and human recombinant IL-1β (R&D, Minneapolis, MN, U.S.A.) were reconstituted in serum-free medium with 2 μg/ml bovine serum albumin (BSA; Sigma) as recommended by the supplier, and this BSA-containing serum-free medium was used for vehicle controls. Cells were harvested after 1–48 h treatment as indicated. After cell lysis, luciferase activity was assayed using the luciferase assay system (Promega), and activity was normalized to total protein content (Coomassie protein assay; Pierce). Data are expressed as mean ± SEM percentages of vehicle controls from three or four independent experiments. Statistical significance was evaluated by ANOVA (Abacus Concepts, Berkeley, CA, U.S.A.).

Gel-shift analysis with nuclear extracts

To isolate nuclear proteins (Dignam, 1983; Abmayr and Workman, 1995), cultured astrocytes were homogenized in hypotonic buffer, and nuclei were separated by centrifugation at 3,300 *g* for 10 min. Nuclei were extracted in two steps with continuous gentle mixing for 30 min in high salt buffer containing 200 or 400 m*M* KCl, respectively. The supernatant of nuclear extracts with 400 m*M* KCl was diluted with binding buffer [10 m*M* HEPES (pH 7.9), 2 m*M* $MgCl_2$, 50 m*M* KCl, 1 m*M* EDTA, 5 m*M* dithiothreitol, 10% glycerol, and 0.2 m*M* phenylmethylsulfonyl fluoride] and concentrated (Centricon 10 microfilters; Amicon).

Complementary 23-bp oligonucleotides containing the putative NF-κB and NF-1 binding site of the wild-type GFAP promoter and 3-bp mutations for the respective sites were synthesized: WT (wild type), ATTCAATGGGGTGCTGCCAGGAA (coordinates −63 to −85 nucleotides); mutNF-κB, ATTCAATGtacTGCTGCCAGGAA; and mutNF-1, ATTCAATGGGGTGCTtaaAGGAA. A complementary 34-bp oligonucleotide containing 3-bp mutations for both sites was synthesized: the double mutant dmut, GGCTATTCAATGtacTGCTtaaAGGAAGTCAGGG (coordinates −56 to −89 nucleotides). This mutant oligonucleotide DNA contained additional bases adjacent to the mutations to allow annealing effectively in the site-directed mutagenesis of the luciferase constructs.

Double-stranded oligomers were constructed by annealing cDNA in saline Tris-EDTA after heating to 95°C/5 min, with slow cooling to 4°C. The wild-type DNA fragment was then labeled in a polynucleotidetransferase reaction using [^{32}P]dATP. As one of the controls, we also used the NF-1 consensus oligonucleotide (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), which was labeled as wild-type DNA GFAP fragment.

Human recombinant p50 protein (6.9 ng; Promega) characterized as the DNA binding form of NF-κB (Baeuerle and Baltimore, 1989) or up to 10 μg of nuclear extract was incubated in binding buffer [10 m*M* HEPES (pH 7.9), 2 m*M* $MgCl_2$, 50 m*M* KCl, 1 m*M* EDTA, 5 m*M* dithiothreitol, 10% glycerol, 0.1% Na_3PO_4, 200 mg/ml BSA, and 0.2 m*M* phenylmethylsulfonyl fluoride] with 0.12 pmol of ^{32}P-labeled wild-type DNA and 0.06–12 pmol of unlabeled wild-type or mutant NF-κB or NF-1 competitor oligonucleotide. After 20 min, samples were separated on an 8% low-cross-link polyacrylamide gel (20 cm) at 350 V for up to 4 h. After drying, ^{32}P was evaluated by phosphorimaging. For "supershift" assays 2 μl of anti-NF-1 (N-20) or anti-NF-κB (NLS) (both from Santa Cruz Biotechnology) was added 30 min before addition of the probe and incubated at 4°C. As a positive control, p50 protein was used.

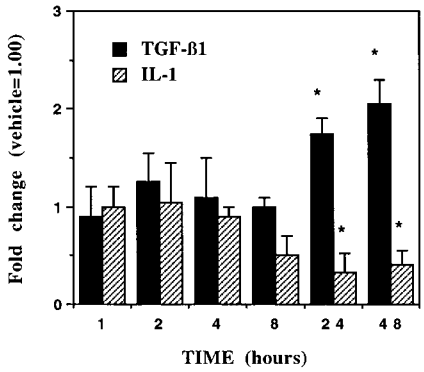

FIG. 2. Induction of luciferase activity for the full length (1.9 kb) of the rat GFAP upstream promoter in pGL3 Basic after treatment with TGF-β1 and IL-1β in cultured astrocytes. Data are means ± SEM, expressed as fold change from the vehicle control, defined as 1.00, of four independent experiments.

RESULTS

Responses of the GFAP promoter to TGF-β1 and IL-1β

Activity of a 5′-upstream construct containing 1.9 kb of rat GFAP genomic DNA (Fig. 1, construct A) with a luciferase reporter was induced by TGF-β1 in serum-free medium. After a lag, increased transcription was detected at 24 h, reaching a maximum increase of 100% by 48 h (Fig. 2). In contrast, treatment with IL-1β resulted in 70% decreased GFAP transcription (Fig. 2).

TABLE 1. *TGF-β response elements in various promoters*

Gene	Sequence	Reference
Rat GFAP promoter	5′-TGGGGTGCT GCCAGGAA-3′	Present study
Rat α1(I) collagen	5′-TGCCCACG GCCAAG -3′	Ritzenthaler et al. (1993)
Mouse α2 (I) collagen	5′-TCGCCCTT GCCAAG -3′	Rossi et al. (1988)
Human elastin	5′-TCCCCCAG GCCTCC -3′	Marigo et al. (1994)
Human type I plasminogen activator inhibitor	5′-TGGCTGCAT GCCCT -3′	Riccio et al. (1992)
Human growth hormone	5′-TGGCCTGCG GCCAG -3′	Courtois et al. (1990)
Human α2 integrin	5′-TGGCTAGGGC GCCA -3′	Zutter et al. (1994)
Human perlecan	5′-TGGCCCGGCG GCCC -3′	Iozzo et al. (1997)
Human α2(I) collagen	5′-AACGAGTCAGAGTTT -3′	Chung et al. (1996)
NF-1 consensus	5′-TGGMNNNNN GCCAR -3′	Gil et al. (1988)
AP-2 consensus	5′-CGCCCGC GGCCCGT -3′	Williams and Tjian (1991)
AP-1 consensus	5′-TGAGTCA -3′	Angel et al. (1987)
NF-κB consensus	5′-GGGRNNYYC -3′	Supakar et al. (1995)
TβRE consensus	5- TGGCC N_{3-5} GCC -3′	Iozzo et al. (1997)

These data [partially reproduced from Iozzo et al. (1997)] are modified and completed with the rat GFAP sequence. The homologous sequences are boldface, the NF-1, TβRE, and AP-2 consensus are boldface and underlined, and the AP-1 consensus is underlined. R, purine; Y, pyrimidine; N, R or Y; M, A or C. Spaces are introduced for the alignment.

Regulation of promoter constructs

Sequential deletions of the 5′-upstream promoter (Fig. 1) identified regions that might mediate transcriptional response to TGF-β1 and IL-1β. Constructs A2–A7 gave responses to TGF-β1 that were equivalent to the full-length promoter. For IL-1β, the responses of constructs A2–A7 were slightly lower compared with those to the full-length promoter. Construct A2 (123 bp at the 3′ end of the promoter) was the shortest to retain full induction by TGF-β1 and repression by IL-1β. Construct A1 (20 bp 5′ from the TATA box) did not respond to either TGF-β1 or IL-1β; however, the basal activity of this construct was fourfold above background (activity of promoterless pGL3 Basic vector).

Site-directed mutagenesis of putative NF-κB and NF-1 binding sites

The above data indicate that *cis*-regulatory elements within the near-upstream region −53 to −106 bp mediate TGF-β1 and IL-1β responses. Motifs at −79 to −66 bp resemble binding sites for NF-1 and NF-κB (Table 1) (Gil et al., 1988; Novak et al., 1991; Laping et al., 1994*b*). Mutations in the putative NF-1 binding site abolished TGF-β1-induced transcription (Fig. 3, mNF-1 and dmut). In contrast, mutations in the putative NF-κB site did not block induction by TGF-β1 (Fig. 3, mNF-κB).

Similar effects were observed for IL-1β repression. Mutations in the putative NF-κB site (Fig. 3, mNF-κB) did not affect IL-1β repression. Mutations in the putative NF-1 site (Fig. 3, mNF-1) or mutations in both sites (Fig. 3, dmut) attenuated the repression by IL-1β. However, this reduction was not statistically significant for the dmut construct. These mutated constructs showed the same basal activity as wild-type A2 construct. Basal activity of A2 (no treatment) was 15-fold above the background.

DNA binding of nuclear proteins by gel shift

Nuclear extracts from rat cultured astrocytes were evaluated for the presence of factors that interacted with oligonucleotide sequences in the GFAP promoter using gel-shift assays. The wild-type oligonucleotides showed retarded migration by DNA binding factor(s) in the nuclear extracts. The amount of DNA binding by nuclear factors was increased by TGF-β1 treatment of astrocytes (Fig. 4); relative band intensity was 43% above that for the vehicle-treated control. DNA binding by nuclear factors decreased by IL-1β treatment (38% below that for the vehicle-treated control). Competitor DNA at 20- and 100-fold higher concentrations reduced binding of nuclear proteins to oligonucleotide DNA (Fig. 5, lanes 3

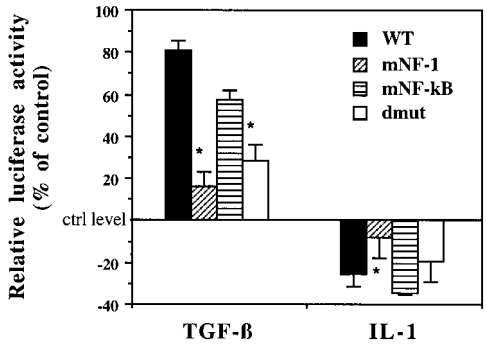

FIG. 3. TGF-β1 (5 ng/ml) and IL-1β (10 U/ml) responses of the wild-type GFAP promoter construct A2 (WT; 106 bp) and A2 mutated constructs with luciferase reporter after transfection into cultured astrocytes: mNF-1, a 3-bp mutation affecting the putative NF-1 binding site in construct A2; mNF-κB, a 3-bp mutation in the putative NF-κB binding site in construct A2; and dmut, mutations at both sites (see Materials and Methods). Luciferase activity was normalized to total cellular protein and β-galactosidase to control for transfection efficiency. Data are means ± SEM, expressed as a percentage of untreated controls (ctrl), from three independent experiments. $^*p < 0.05$ (WT vs. mutants).

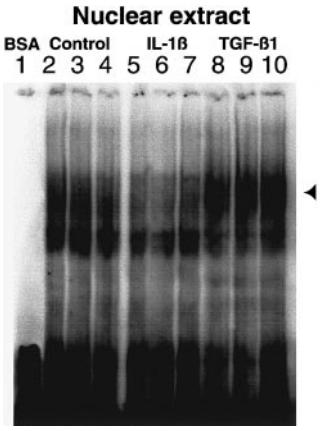

FIG. 4. TGF-β1 and IL-1β regulation of nuclear proteins that bind the wild-type sequence (−63 to −85 bp). Mobility-shift results show a nuclear protein/DNA complex (arrowhead) in nuclear extract from untreated cultured astrocytes (lanes 2–4, triplicates) that decreased in level after IL-1β treatment (lanes 5–7, triplicates) and increased in level after TGF-β1 treatment (lanes 8–10, triplicates). Lane 1, BSA in binding buffer. Mobility-shift experiments were repeated with similar results.

and 4; 20-fold, 10% and 100-fold, 7% binding compared with binding without competitor, respectively). A 3-bp mutation (mNF-κB; Fig. 5, lanes 5 and 6) competed almost as well (20-fold, 38%; 100-fold, 8% binding). However, competition by a 20- and 100-fold excess of unlabeled oligonucleotide containing a 3-bp mutation in the NF-1 like site (mNF-1) did not alter binding of the

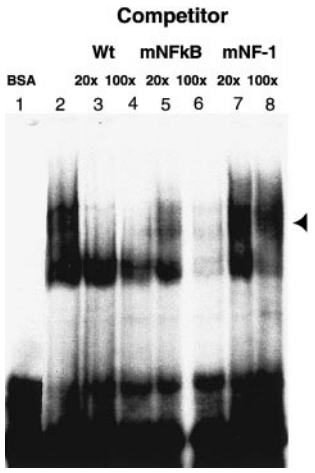

FIG. 5. Mobility shift of a nuclear protein/DNA complex from nuclear extracts of cultured astrocytes after TGF-β1 treatment. A 20- and 100-fold excess of unlabeled wild-type (Wt) oligonucleotide DNA (−63 to −85 bp of the GFAP promoter) effectively competes for the labeled nuclear protein/DNA complex (at 20×, 10%; at 100×, 7% binding) (arrowhead, lanes 3 and 4). A 3-bp mutation (mNF-κB, lanes 5 and 6) competed almost as well (20×, 38%; 10×, 8% binding). In contrast, an oligonucleotide DNA containing a 3-bp mutation in the NF-1-like site (mNF-1) was much less effective for binding the nuclear protein (20×, 98%; 100×, 96% binding) (lanes 7 and 8). Lane 1, BSA in binding buffer; lane 2, no competitor.

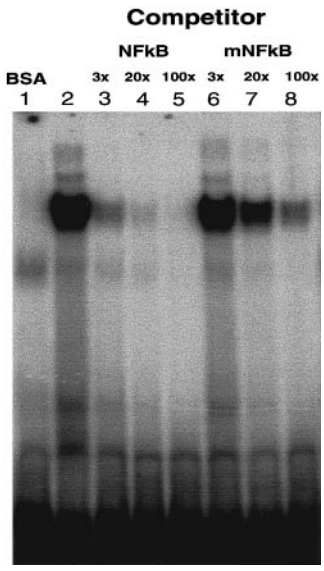

FIG. 6. Human recombinant NF-κB (p50 protein) binding to the wild-type NF-κB-like site or a mutagenized site (mNF-κB). Labeled 23-bp DNA containing the putative NF-κB site in the GFAP promoter was incubated with human recombinant p50 protein or BSA for control. Complexes of p50 protein and DNA were resolved by gel electrophoresis. Unlabeled 23-bp DNA containing wild-type NF-κB was used as a competitor in the binding reaction. Mobility-shift results show the titration of wild-type 23-nucleotide DNA/p50 protein complexes (lanes 2–8) with unlabeled oligonucleotides containing wild-type DNA (NF-κB, lanes 3–5) or mutant DNA (mNF-κB, lanes 6–8). Lane 1, no p50 protein; lane 2, no competitor. Mobility-shift experiments have been repeated with similar results.

nuclear protein to wild-type DNA (Fig. 5, lanes 7 and 8; 20-fold, 98% and 100-fold, 96% binding).

As further controls, we examined gel shift with recombinant p50, an NF-κB binding protein that contains the specific DNA binding domain (Baeuerle and Baltimore, 1989). Competitor DNA at equimolar and higher concentrations decreased levels of the labeled p50 protein/DNA complex (Fig. 6, lanes 3–5). The mutated oligonucleotide containing a 3-bp mutation in the putative NF-κB site (mNF-κB) competed 10-fold less for binding p50 protein than wild-type (Fig. 6, lanes 6–8).

To characterize the proteins in nuclear extracts from astrocytes treated with TGF-β1 that bind to the NF-1/NF-κB region in the rat GFAP promoter, we preincubated nuclear extracts with antibodies to NF-1 or to NF-κB (see Materials and Methods) and then analyzed binding to the wild-type oligonucleotide used for the gel-shift assay. There was no further retardation ("supershift") of the nuclear protein/DNA complex with either antibodies to NF-1 (data not shown) or antibodies to NF-κB (Fig. 7, lanes 5 and 6). In contrast, the positive control for NF-κB showed a clear supershift (lane 8). Because purified NF-1 protein is not available to use as a positive control, we used commercially prepared nuclear extracts from HeLa cells treated with phorbol ester as a source of NF-1 protein (Santa Cruz Biotechnology). However, a pilot experiment using this material did not

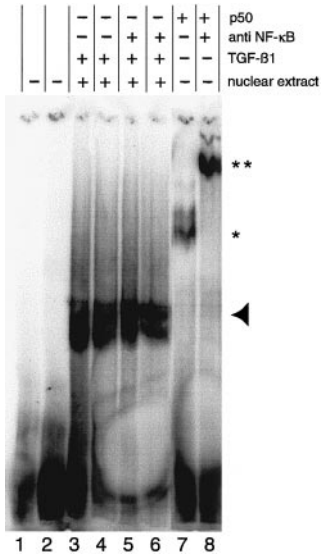

FIG. 7. Supershift of a nuclear protein/DNA complex from nuclear extracts of cultured astrocytes after TGF-β1 treatment by antibodies to NF-κB. Nuclear extracts were preincubated with anibodies to NF-κB and then analyzed for the binding to the wild-type oligonucleotide used for the gel-shift assay: lane 1, BSA in binding buffer; lane 2, rabbit serum; lanes 3 and 4, nuclear extract from TGF-β-treated cells, no antibodies (duplicates); lanes 5 and 6, plus anti-NF-κB (duplicates); lane 7, binding of p50 protein (positive control for NF-κB) to DNA fragment; and lane 8, p50 plus antibodies to NF-κB. There was no further retardation ("supershift") of the nuclear protein/DNA complex with antibodies to NF-κB (lanes 5 and 6). In contrast, p50 protein showed a clear supershift (lane 8). The arrowhead marks the retarded bands of nuclear protein/DNA complex. One star marks the band of retarded p50/DNA complex, and two stars marks the supershifted p50/DNA band.

"supershift" when incubated with NF-1 consensus oligonucleotide and antibodies to NF-1 (data not shown). Therefore, as a further control we examined the competitive binding of nuclear extracts from astrocytes treated with TGF-β and nuclear extracts from phorbol ester-treated HeLa cells to NF-1 consensus oligonucleotide (Fig. 8). Competitor DNA (50-fold) decreased the amount of labeled DNA/protein complex if HeLa cells were used (Fig. 8, lanes 2 and 3). However, there was no specific competition when nuclear extracts of astrocytes were used (Fig. 8, lanes 4 and 5).

DISCUSSION

These experiments show that 1.9 kb of the rat 5′-upstream GFAP promoter contains *cis*-acting elements that suffice to mediate induction by TGF-β1 and repression by IL-1β, when transfected into rat primary astrocytes. These responses exactly model the directions and extent of changes of levels of endogenous GFAP mRNA in cultured astrocytes in response to TGF-β1 (Laping et al., 1994*a*) and IL-1β (Oh et al., 1993). However, run-on studies show a twofold increase in GFAP transcription after TGF-β1 treatment in cultured astrocytes (Laping et al., 1994*a*), which is much faster (2 h) than the 24-h delay observed with the 1.9-kb construct. This difference could be due to activity of downstream *cis*-acting elements. In contrast to TGF-β1, IL-1β repressed GFAP transcription: the reductions were detected by 8 h, which matches the schedule of decrease in level of GFAP mRNA by IL-1β in cultured astrocytes (Selmaj et al., 1991; Oh et al., 1993).

Deletion constructs showed that both cytokine responses are mediated by a near-upstream site at −106 to −53 bp that is very close to the TATA box and that contains an overlapping NF-1- and NF-κB-like binding motif with homology to other TGF-β1 responsive sites (Table 1).

The segment −70 to −68 bp in the near-upstream GFAP promoter is necessary for induction of GFAP by TGF-β1 and repression by IL-1β based on the following: (a) A 3-bp mutation in this segment abolished induction by TGF-β1 and repression by IL-1β of GFAP promoter activity (Fig. 3). (b) In gel-shift studies, nuclear extracts from astrocytes treated with TGF-β1 showed increases in factor binding to the upstream segment −85 to −63 bp, whereas nuclear extracts from astrocytes treated with IL-1β showed less binding (Fig. 4). (c) Mutations in the segment −70 to −68 bp blocked binding of the astrocyte nuclear factor(s), whereas mutations in the NF-κB-like binding motif did not (Fig. 5). The agreement between induction of transcription and gel-shift studies with mutations in the −70 to −68-bp segment strongly indicates that this site mediates the induction of GFAP by TGF-β1 and repression by IL-1β. In contrast, mutation of the NF-κB-like binding site did not block the cytokine responses or binding of the astrocyte nuclear factor(s). Furthermore, no supershift with anti-NF-κB antibodies was detectable, despite efficacy of the positive control

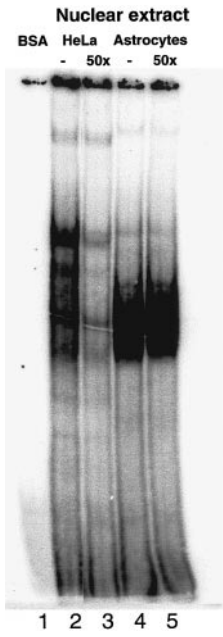

FIG. 8. Mobility shift of nuclear protein/DNA complex from nuclear extracts of HeLa cells treated with phorbol ester and of astrocytes treated with TGF-β1. Lane 1 shows the BSA control. In lanes 2 and 3, a 50-fold excess of unlabeled NF-1 consensus oligonucleotide effectively competes for the labeled nuclear protein/NF-1 DNA complex of HeLa cells. In lanes 4 and 5, there was no competition for the specific binding of nuclear proteins of astrocytes to NF-1 consensus oligonucleotide.

with p50. The mutated NF-κB site, however, showed much weaker binding of p50, a recombinant protein that contains the DNA binding site of NF-κB (Baeuerle and Baltimore, 1989). The characterization of nuclear factors from astrocytes with respect to NF-κB merits further study because the release of TGF-β1, IL-1β, and other cytokines may also be stimulated by oxidative stressors. Such stressors induce NF-κB, which is observed in injured brain and during brain lesions (Kaltschmidt et al., 1993). Therefore, the NF-κB binding site in the GFAP promoter remains of interest. Our observation that the same element mediates opposite transcriptional responses of GFAP to TGF-β1 and IL-1β is a precedent for the convergence of multiple stimuli on the same *cis*-acting sequence.

Although the segment −70 to −68 bp is homologous to part of a NF-1-like site, the failure to demonstrate a competitive binding of astrocytic nuclear factors with the NF-1 consensus sequence suggests that (a) factors in the nuclear extract from TGF-β1-treated astrocytes responsible for the induction might only be distantly related to NF-1 and (b) these factors may be completely distinct from NF-1 proteins. In addition, removal of a NF-1-like site at positions −118 to −104 bp (Miura et al., 1990) that is conserved among the human, rat, and mouse did not affect TGF-β1 induction or IL-1β repression, which further argues against a NF-1 action.

TGF-β1 modifies transcription through various transcription factors in other genes. In the α1(I) collagen promoter, Ritzenthaler et al. (1991) characterized a transforming growth factor-β activation element (TAE)—TGCCCAGGCCAAG—which includes overlapping NF-1 and AP-2 sites. Other TGF-β1-regulated genes with overlapping NF-1-like sites are cited in Table 1. No study has yet identified the nuclear protein that binds to the NF-1-like sites, although the functionality of these NF-1 sites for induction by TGF-β1 and for binding of nuclear proteins is consistently shown in mutagenized constructs (Ritzenthaler et al., 1993; Iozzo et al., 1997; Ogata et al., 1997). Quite different sequences mediate regulation of another collagen family gene, α2(I), by TGF-β1. Induction of α2(I) collagen by TGF-β1 in fibroblasts is mediated by a complex sequence containing an overlapping AP-1 and NF-κB-like sequence but that was not sensitive to competition by AP-1 or NF-κB sequences (Chung et al., 1996). In lung carcinoma cells (A-549), the autoinduction of TGF-β1 transcription is mediated by an AP-1 (Jun-Fos) complex (Kim et al., 1990). Recent studies characterize the Smad family of transcription factors (especially Smad-2 and Smad-3) as potent transducers of TGF-β1 signaling (Heldin et al., 1997). Because a DNA binding domain has not been identified so far, it is hypothesized that these proteins form complexes with other DNA binding proteins to activate transcription. Smad proteins also may mediate early steps in TGF-β1 signaling (Vindevoghel et al., 1998).

The near-upstream region of the GFAP promoter lacks certain other motifs implicated in transcriptional responses to TGF-β1 (Table 1 and Fig. 1), including AP-1 in the α2(I) collagen promoter (Chung et al., 1996), AP-2 in the α1(I) collagen promoter (Ritzenthaler et al., 1993), and the transforming growth factor-β inhibitory element (TIE) of the transin/stromelysin promoter (GNNTTGGtGA), which includes a required AP-1 site that binds Fos (Kerr et al., 1990). Sequential deletion (Fig. 1) did not indicate activity, with respect to TGF-β1, of the further upstream AP-1 site at −1,509 bp.

These findings are also pertinent to the increasing use of GFAP promoter constructs in transgenic animals. The present findings on the *rat* GFAP promoter raise the possibility that the *human* and *murine* GFAP promoters used in transgenic studies might also be subjected to cytokines; if so, then it would be important to consider possible confounds from cytokine induction on the promoter activity. For example, constructs of the full 5′-upstream GFAP region with a *lacZ* reporter showed vigorous induction in response to wounding (Mucke and Rockenstein, 1993; Brenner et al., 1994). Because levels of both TGF-β1 and IL-1β are increased by wounding (Lindholm et al., 1992; Logan et al., 1992), we suggest that *cis*-acting elements that mediate TGF-β1 and IL-1β effects could also mediate induction of GFAP expression by brain injury. However, the brain regional differences in responses to stab wounds (Mucke and Rockenstein, 1993) might represent local differences in the amount of cytokines released. Because GFAP transcription is induced by TGF-β1 and repressed by IL-1β, local cytokine concentrations could determine the net response of GFAP expression. Lastly, we note that Galbreath et al. (1995) observed the variable penetrance of hydrocephalus in transgenic mice that overexpress TGF-β1 under the control of the GFAP promoter. Individual differences in TGF-β1 mRNA levels that paralleled the degree of hydrocephalus in mice, all of which carried the germ line transgene, could reflect local variations in cytokine concentrations. Thus, mutagenesis to inactivate certain cytokine and hormone response elements from GFAP promoter constructs being used to drive overexpression in astrocytes might reduce interanimal variations.

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