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Ciliary Neurotrophic Factor Activates JAK/Stat Signal Transduction Cascade and Induces Transcriptional Expression of Glial Fibrillary Acidic Protein in Glial Cells

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Abstract: In recent reports, ciliary neurotrophic factor (CNTF) has been implicated as an injury factor involved in regulating astrogliosis in the CNS. In this study, we used a rat oligodendroglial progenitor cell line that is highly responsive to CNTF to examine CNTF-induced alterations that may play a role in activation of the glial fibrillary acidic protein (GFAP) gene. We determined that CNTF induces the transient translocation of Stat1α/p91 to the nucleus. This nuclear translocation was followed by GFAP promoter activation and an up-regulation of GFAP mRNA and protein. Levels of CNTF-α receptor mRNA, however, were unaffected by addition of the ligand. Transfection studies using an upstream 5′-flanking, 1.9-kb rat GFAP promoter linked to a luciferase reporter gene revealed CNTF-induced transcriptional activation within 1 h of ligand exposure. Moreover, serial-deleted constructs identified a distal (−1,857 to −1,546 bp) and a proximal (−384 to −106 bp) region as being important for CNTF-induced GFAP promoter activation. These two regions showed a strong degree of overlap for CNTF- and serum-induced activation of the GFAP gene. Analysis of the two regions revealed several *cis*-elements that are thought to be involved in GFAP regulation and/or the regulation of other genes by members of the interleukin-6 family of cytokines. Moreover, we are the first to report the presence of several putative CNTF-responsive elements within our identified distal and proximal regions in the GFAP gene promoter. **Key Words:** Ciliary neurotrophic factor—Ciliary neurotrophic factor-responsive elements—Ciliary neurotrophic factor-α receptor—Glial fibrillary acidic protein—JAK/Stat—Transcription.
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Glial fibrillary acidic protein (GFAP) is a highly conserved intermediate filament protein that is found in astrocytes within the CNS. Astrocytes are thought to play a key role in physiological processes, including maintenance of extracellular ion homeostasis, formation of tight junctions within the blood–brain barrier, regulation of neurotransmitter levels, and specialized immune functions (Federoff and Vernadakis, 1986; Kimelberg and Norenberg, 1989). Trauma to the CNS often results in an up-regulation of GFAP, hypertrophy, and/or hyperplasia of astrocytes (Bignami and Dahl, 1995). Reactive astrogliosis is a hallmark of most CNS disorders, including acquired immunodeficiency syndrome (AIDS) neuropathy, ischemia/anoxia, multiple sclerosis, head trauma, epilepsy, and encephalitis (Mokuno et al., 1989; Benveniste, 1995; Bignami and Dahl, 1995; Farrell et al., 1995). The exact role of reactive astrocytes, however, remains unresolved. Moreover, it is unclear what mechanism(s) trigger the resulting gliosis. Recent studies suggest that cytokines, such as ciliary neurotrophic factor (CNTF), may play a pivotal role.

CNTF was originally isolated as a neurotrophic factor supporting the survival of chick ciliary ganglion neurons (Manthorpe et al., 1986). More recent studies indicate that CNTF has pleiotropic actions on many neurons and glia (Ernsberger et al., 1989; Oppenheim et al., 1991; Louis et al., 1993; Barres et al., 1993; Kahn and de Vellis, 1994; Kahn et al., 1995). CNTF has high sequence homology to interleukin-6 (IL-6)

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Abbreviations used: AP-1, activator protein-1; CG-4, Central Glia-4; CHX, cycloheximide; CNTF, ciliary neurotrophic factor; CNTF-αR, ciliary neurotrophic factor-α receptor; CNTF-RE, ciliary neurotrophic factor-responsive element; CRE, cyclic AMP-responsive element; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GAS, interferon-γ-activated site; GFAP, glial fibrillary acidic protein; GRE, glucocorticoid-responsive element; HBS, HEPES-buffered saline; IL-6, interleukin-6; LIF, leukemia inhibitory factor; O-2A, oligodendroglia type-2 astrocyte; PBS, phosphate-buffered saline; SRE, serum-responsive element; Stat, signal transducer and activator of transcription.

family members, including IL-6, leukemia inhibitory factor (LIF), and oncostatin M (Bazan, 1984; Ip et al., 1992; Patterson, 1992). Members within this family share in a common signal transduction pathway; however, each cytokine has distinct mediators that are necessary for individual cytokine-induced activation. The CNTF receptor contains a unique CNTF-α receptor (CNTF-αR) component. In addition, the receptor also contains an LIFβ subunit and gp130, originally characterized as the β component for the IL-6 receptor complex. Binding of CNTF to the CNTF-αR component results in heterodimerization of the LIFβ and gp130 subunits and activation of the signal transduction cascade (Ip et al., 1992; Davis et al., 1993).

The CNTF receptor is structurally related to the interferon (IFN) receptor (Bazan, 1990), suggesting the possibility of a similar signaling pathway. Indeed, using a human neuroblastoma cell line, CNTF and IFN-γ were found to phosphorylate a 91-kDa protein and trigger its translocation to the nucleus. Moreover, phosphorylated p91 binds to an interferon-γ-activated site (GAS) on the gene promoter, resulting in the transcription of both IFN-γ- and CNTF-inducible genes (Bonni et al., 1993). An IL-6 DNA promoter site has been found to be highly homologous to GAS (Wegenka et al., 1993); thus, it is not surprising that CNTF-induced gene activation might also operate through a GAS-like sequence.

In a previous study, we examined the effects of intracerebral injections of CNTF on glial cells in vivo (Kahn et al., 1995). We found that CNTF evokes a strong astrogliotic response in addition to an up-regulation of microglia, while having no appreciable effect on other intermediate filament genes examined (Kahn et al., 1995, 1997). Moreover, the CNTF mRNA level is known to increase following a lesion to the CNS (Ip et al., 1993; Mata et al., 1993). These data support a role for CNTF in the CNS injury response. In vitro findings have shown that CNTF increases GFAP content in oligodendroglia type-2 astrocyte (O-2A) progenitor cells derived from rat optic nerve (Hughes et al., 1988; Lillien et al., 1990) and from an O-2A cell line, Central Glia-4 (CG-4) (Louis et al., 1993; Kahn and de Vellis, 1994). Although type-1 astrocytes in vitro express a functional CNTF-αR (Rudge et al., 1994), exposure to CNTF does not result in activation of the GFAP gene (Hughes et al., 1988). It is possible that because type-1 astrocytes already express high levels of GFAP, factors that might normally regulate the gene in vivo are refractory. Unlike type-1 astrocytes, the majority of CG-4 cells do not express GFAP, but the gene is up-regulated by CNTF or serum-derived factors. Considering the evidence suggesting that CNTF plays a role in regulating GFAP, we believe that CG-4 cells provide for an in vitro model system.

Our present study therefore has examined the CNTF-induced activation of the GFAP gene in CG-4 cells in vitro. We are the first to report that in glial cells, CNTF rapidly activates p91, resulting in its subsequent translocation to the nucleus. Activation of p91 was followed by an increase in levels of GFAP mRNA and protein, but no change in CNTF-αR mRNA expression was observed after ligand exposure. Further examination of CNTF-induced GFAP activation was obtained through transiently transfected CG-4 cells using the upstream 5′-flanking, 1.9-kb rat GFAP promoter DNA linked to a luciferase reporter gene. We are the first to identify specific distal and proximal regulatory regions, relative to the transcription start site, that are important for CNTF activation of the GFAP gene promoter. Moreover, we also report the presence of seven putative and novel CNTF-responsive elements (CNTF-REs) within the proximal and distal regions of the rat GFAP promoter. Considering the strong evidence suggesting that CNTF is an injury factor responsible for regulating GFAP in the CNS, we believe that our present study provides important and novel information on the molecular mechanisms governing CNTF activation of the GFAP gene.

MATERIALS AND METHODS

Cell culture

CG-4 cells (passages 12–18) were plated on 100-mm-diameter Petri dishes for northern analysis, 35-mm-diameter Petri dishes for transfection analysis, or 9-mm^2 glass coverslips for immunocytochemistry. All surfaces were treated with poly-L-ornithine (Sigma, St. Louis, MO, U.S.A.). The cells were propagated in growth medium consisting of 70% differentiation medium [DNB containing Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY, U.S.A.), N1 supplement (50 μg/ml transferrin, 5 μg/ml insulin, 100 mM putrescine, 20 nM progesterone, and 30 nM selenium), and biotin (10 ng/ml)] plus 30% B-104 neuroblastoma-conditioned medium (Schubert et al., 1974). All chemicals were purchased from Sigma. As previously described (Louis et al., 1993; Kahn and de Vellis, 1994), removal of the B-104 mitogenic source results in quiescent cells that express mature oligodendrocyte markers in the differentiation medium. Cell culture medium was changed every 2 days, and 1–2% fetal calf serum (FCS; Irvine Scientific, Santa Ana, CA, U.S.A.) was added to the medium to enhance cell survival. CG-4 cells were maintained in a humidified atmosphere of 95% O_2 and 5% CO_2. Cells were maintained as self-renewing O-2A progenitor cells by routine passage at confluency; cells were trypsinized (1.25%) for 5 min at 37°C and plated at 100 cells/mm^2. Recombinant rat CNTF (Genzyme, Cambridge, MA, U.S.A.) or cycloheximide (CHX; Sigma) was added for the times specified.

Light microscopy and immunocytochemistry

Identification of p91 was obtained through slight modifications of a previously characterized protocol (Schindler et al., 1992). In brief, CG-4 cells were rinsed twice in phosphate-buffered saline (PBS) and fixed in methanol/acetone (1:1 vol/vol) for 2 min at 4°C. Cells were air-dried for 20 min, washed twice in PBS, and blocked for 25 min in PBS containing 0.01% Tween-20 (Sigma) and 3% bovine serum albumin. Anti-p91 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) was prepared in blocking buffer for 1 h at room temperature. Cells were washed three times for 5 min each in PBS and incubated with a biotinylated secondary

antibody (1:200; Vector Laboratories, Burlingame, CA, U.S.A.) for 1 h at room temperature. Cells were washed three times for 5 min each in PBS and incubated with an avidin-fluorescein isothiocyanate (FITC) conjugate (1:200; Southern Biotechnology Associates, Birmingham, AL, U.S.A.) for 30 min at room temperature. Cells were washed three times for 5 min each in PBS, rinsed once with double distilled water, and mounted with Fluoromount (Southern Biotechnology Associates) onto glass slides.

Determination of CG-4 cell phenotype was obtained through fixing the cells in 4.0% paraformaldehyde for 25 min. Cells were washed three times for 5 min each in PBS and extracted in 0.1% Triton X-100 for 30 min. Primary antibodies—polyclonal myelin basic protein (1:200; a gift from Dr. Anthony Campagnoni)—or monoclonal GFAP (1:1,000; ICN Biomedicals, Irvine, CA, U.S.A.) was applied for 12–18 h in a humidified chamber at 4°C. Cells were washed three times for 5 min each in PBS and treated with secondary antibodies (1:200; Vector) linked to a specific fluorochrome—7-amino-4-methylcoumarin-3-acetic acid (Jackson Immunoresearch Laboratory, West Grove, PA, U.S.A.), Texas Red, or FITC (Southern Biotechnology Associates)—for 1 h at room temperature. Cells were washed three times for 5 min each in PBS and mounted as described above.

Northern analysis

Total RNA from cells grown to 90% confluency was extracted by the acid guanidinium–thiocyanate–phenol–chloroform technique (Chomczynski and Sacchi, 1987). Fifteen micrograms of RNA was electrophoresed on a 1.0% formaldehyde denaturing agarose gel and blotted onto nitrocellulose. The filters were probed with the following cDNAs: 1.1-kb mouse GFAP (a gift from Dr. Sally Lewis) and a 0.43-kb rat CNTF-αR (*Pst*I fragment from a 1.5-kb rat cDNA; a gift from Dr. George Yancopoulos, Regeneron Pharmaceuticals, Tarrytown, NY, U.S.A.) labelled by random priming with ^{32}P.

GFAP plasmid constructs

The DNA fragment (−1,857 to +13 respective to the transcription start site at +1) of the rat GFAP upstream promoter was generated by oligonucleotide-directed polymerase chain reaction methods (Huang et al., 1992, 1993) using a GFAP DNA template (a gift from Dr. Noboru Sueoka) (Kaneko and Sueoka, 1993). These fragments were cloned into a Bluescript vector (Stratagene, San Diego, CA, U.S.A.), and sequences were confirmed using the dideoxy chain-terminating method. The fragment was subcloned into a promoterless pGL-2-Basic plasmid containing a luciferase gene (Promega, Madison, WI, U.S.A.) and verified by DNA sequencing and restriction endonuclease mapping. 5′-Serial-deleted GFAP promoter constructs were generated by endonuclease cutting at specific restriction enzyme sites on the GFAP and pGL-2-Basic plasmids. Further cuts on the GFAP promoter generated G2 (−52 to +13), G3 (−106 to +13), G4 (−384 to +13), G5 (−641 to +13), G6 (−1,049 to +13), G7 (−1,342 to +13), and G8 (−1,546 to +13). Some of these constructs were kindly provided and exchanged with colleagues at the laboratory of Dr. Caleb Finch (University of Southern California, Los Angeles, CA, U.S.A.). Plasmid DNA used for transfection was purified as outlined by Qiagen (San Diego, CA, U.S.A.).

Transfection and enzyme assays

Transfections were achieved using the calcium phosphate method and reagents provided by Promega. Although Promega recommends using cells at 30–60% confluency, CG-4 cells were grown to 75% confluency for 1–2 days before transfection. The high density enhanced their survival following exposure to calcium phosphate. In brief, $CaCl_2$, double distilled water, and 2× HEPES-buffered saline (HBS) solutions were equilibrated to room temperature. The DNA and 2× HBS were prepared in separate sterile tubes. A DNA reaction mixture was prepared by adding 12.6 μl of $CaCl_2$ to 4.1 μg of DNA, and the final volume was brought up to 300 μl with double distilled water. The 2× HBS was vortex-mixed, and the DNA solution was added slowly, dropwise, to the 2× HBS. For all transfection studies, positive and negative control plasmids (pGL-2-Control and pGL-2-Basic, respectively; Promega) were included in sister wells. Moreover, 1 μg of pCAT-Control plasmid DNA (Promega) was included in all wells to determine transfection efficacy.

DNA solutions were incubated at room temperature for 30 min. The resulting mixture was vortex-mixed and added to cells dropwise. The plates were swirled and returned to the incubator. After 24 h, the medium was changed to fresh growth medium to let the cells recover. After the 24-h recovery, the medium was changed to DNB containing 2% FCS and incubated with and without CNTF (20 ng/ml) or 20% serum. After 6 and 24 h, cells were harvested in 250 μl of 1× lysis buffer, frozen at −20°C, and thawed, and luciferase activity was measured by adding 30 μl of cell extract to 100 μl of luciferase assay reagent (Promega). Luciferase activity was measured using a luminometer (Monolight 2010; Analytical Luminescence Lab, San Diego), and protein content was determined using the method of Bradford (1976).

RESULTS

To examine some of the early CNTF-induced signal transduction events, CG-4 cells were allowed to proliferate in the growth medium until 50% confluency and then transferred into the differentiation medium containing 2% FCS for 24 h. Expression of p91 was determined through immunocytochemistry at various time points after addition of CNTF to immature and progenitor-like CG-4 cells. In untreated cells, p91 is diffusely expressed within the cytoplasm (Fig. 1A and B). On exposure to CNTF, however, p91 is rapidly translocated to the nucleus within 5 min (data not shown), and nuclear localization is still apparent after 30 min of exposure to the ligand (Fig. 1C and D). After 1 h, however, the nuclear-localized p91 was absent, and the expression pattern mimicked that observed for the control (data not shown). Cells in the absence of primary antibody showed no immunofluorescence signal (data not shown).

For northern analysis of CNTF-induced mRNAs, CG-4 cells were allowed to proliferate in the growth medium until 90% confluency and then transferred into the differentiation medium containing 2% FCS for 24 h. Cells were grown in the presence and absence of 20 ng/ml CNTF and/or 10 μg/ml CHX and harvested at 12- and 24-h time points. CNTF-induced GFAP mRNA was observed at 12 and 24 h; CHX did not

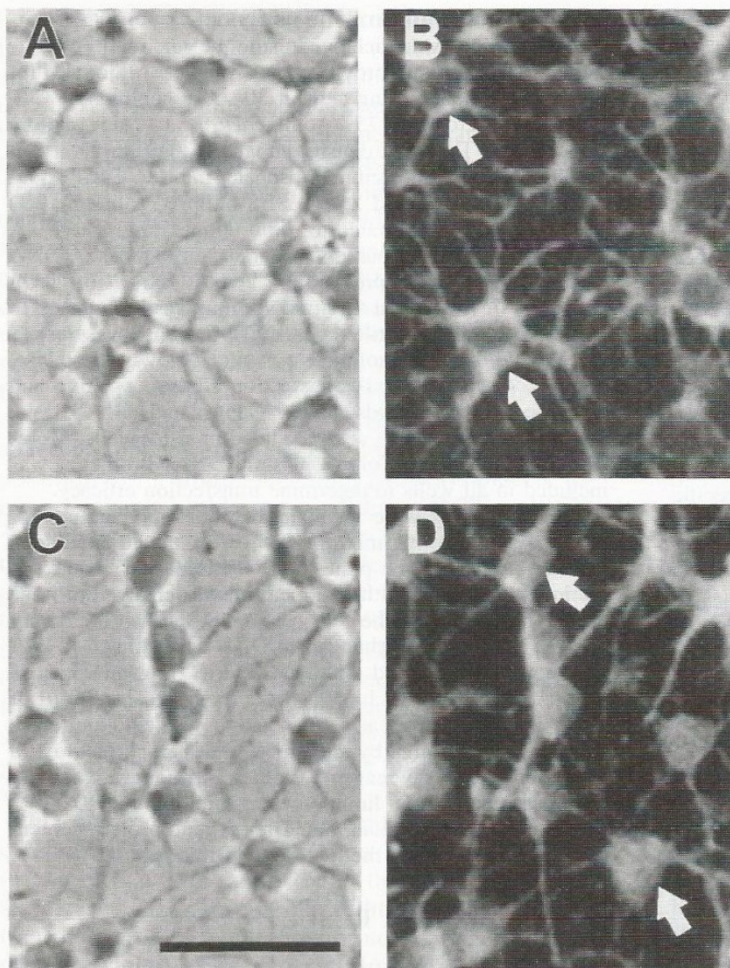

FIG. 1. CNTF-induced signal transduction in CG-4 cells. **A** and **C:** Phase-contrast microscopy revealed the cells to be in either an immature or a progenitor-like state. **B:** In the absence of CNTF, p91 was diffusely expressed in the cell cytoplasm (arrows). **D:** Addition of CNTF to the culture medium resulted in a rapid translocation of p91 to the nucleus within a 5-min interval, and nuclear localization was still observed after 30 min of CNTF exposure (arrows). The same temporal pattern of nuclear p91 expression was observed in three separate experiments. Bar = 20 μm.

regulate GFAP transcript expression (Fig. 2A). The CNTF-αR mRNA was present in CG-4 cells and was not altered by exposure to CNTF (Fig. 2B).

CG-4 cells are a heterogeneous and highly plastic O-2A progenitor cell line. Exposure to the appropriate epigenetic factors can provide large numbers of cells at various stages within the oligodendrocyte lineage. CG-4 cells were allowed to proliferate in the growth medium until 50% confluency and then transferred into differentiation medium containing 2% FCS. After 24 h, the cells were induced with 20 ng/ml CNTF or 15% FCS and fixed 2 days later. In the presence of CNTF, ~40–50% of the cells maintain a mature oligodendrocyte phenotype and express myelin basic protein (Fig. 3A and B). Moreover, ~20–30% convert to a type-2 astrocyte phenotype and express GFAP (Fig. 3A and B). Cell populations that do not express either of these markers appear O-2A-like or immature based on morphological criteria. In the presence of high-level serum, ~60–75% of the cells are flat and protoplasmic and colocalize for GFAP and A2B5 (Fig. 3C and D). Under these high-level serum conditions, <15% of the cells express mature oligodendrocyte markers such as myelin basic protein (data not shown).

To characterize further the CNTF- and serum-induced GFAP response, CG-4 cells were transiently transfected with serial-deleted GFAP promoter constructs. A series of GFAP upstream promoters were made containing different 5′ regions of the GFAP promoter located upstream from a luciferase gene (Fig. 4). At least three separate transfection assays were performed per construct. The specificity of our promoter construct was inferred by comparing the luciferase activity of CG-4 cells maintained in differentiation medium that were transfected with G1, pGL-2-Control, or pGL-2-Basic. The relative luciferase activities for these differentially driven promoters following 6 h in the differentiation medium were as follows: G1 (6.1×10^5), G1 plus CNTF (1.8×10^6), pGL2-Control (2.4×10^3), and pGL-2-Basic (1.5×10^2).

To determine optimal time points to monitor CNTF- or serum-induced GFAP activation, the whole 1.9-kb promoter region (G1; Fig. 4) was transfected into CG-4 cells for a time course analysis. Protein assays were

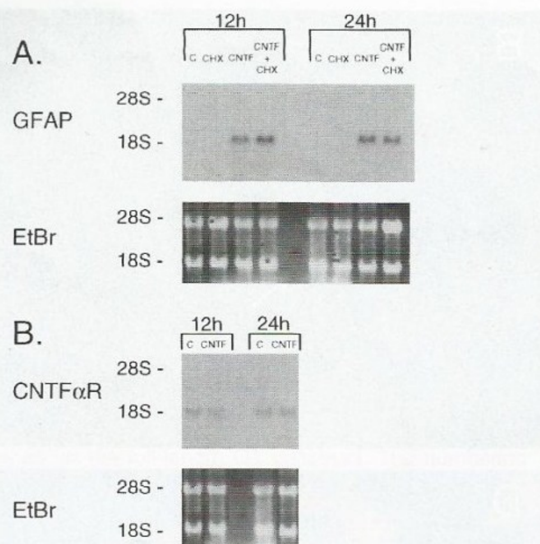

FIG. 2. Northern blots of RNA derived from CG-4 cells exposed to CNTF and/or CHX, except the control lane (C), which received no treatment. **A:** GFAP mRNA was absent in CG-4 cells for control and CHX treatments, whereas mRNA was strongly up-regulated following the addition of CNTF. CHX did not alter the induction of GFAP mRNA by CNTF. **B:** The CNTF-α receptor (CNTF-αR) was expressed by CG-4 cells and was not altered by exposure to CNTF. A similar profile for GFAP and CNTF-αR mRNA was observed for both 12- and 24-h time points in duplicate experiments. **Lower panels:** Equal loading based on ethidium bromide (EtBr) staining of the 28S and 18S bands.

conducted to normalize for differences in plating densities, and the resulting activity was expressed in relative luciferase units per nanogram of protein. Although CNTF-induced luciferase activity was significantly greater than control levels at all time points examined ($p < 0.05$ by Student's t test with $df = 4$), maximal luciferase activity was observed at 6 h with an approximately three-to fourfold increase over control levels (Fig. 5). In contrast, serum did not significantly induce luciferase activity above that of control levels until 12 h postinduction. Serum-induced luciferase activity was significantly greater than control levels for all time points examined thereafter ($p < 0.01$ by Student's t test with $df = 4$).

To identify regions within the GFAP promoter that were necessary for CNTF- or serum-induced transcriptional activation, serial-deleted constructs (Fig. 4) were transfected into CG-4 cells. Luciferase activity was determined in the presence and absence of either CNTF or high-level serum after treatment for 6 (Fig. 6A) or 24 h (Fig. 6B). Analysis of the 5′-deleted constructs revealed two regions within the GFAP promoter that are important for CNTF-induced transcriptional activation. G1 consistently maintained the highest level of CNTF-induced luciferase activity, a level of expression that was approximately three times that of control levels. Luciferase activity was abolished in the smaller G2 and G3 constructs. Activity was reestablished, however, in cells transfected with the G4 construct, which resulted in a three- and fourfold increase compared with control values following exposure to 20 ng/ml CNTF at 6 and 24 h, respectively. These data suggest that *cis*-elements found between −384 and −106 bp are important for CNTF-induced activation (compare G3 vs. G4; Fig. 4). This region, however, was not enough to regain full activity, as was observed in cells transfected with the whole G1 construct. Transfections using G5–G7 constructs did not significantly affect the promoter activity when compared with cells transfected with G4. These data suggest that *cis*-elements within the GFAP promoter that are delineated by these constructs are not important for CNTF regulation of the GFAP gene. A moderate increase in luciferase activity was observed for the G8 construct, albeit this increase was found not to be statistically different from the values for G4–G7 constructs. Transfections using the whole G1 construct reestablished full activity, which was not observed in G8; therefore, we can conclude that additional regions between −1,857 and −1,546 bp are important for CNTF activation of the GFAP gene (compare G1 vs. G8; Fig. 4).

Comparison of these data with results obtained from cells exposed to high-level serum (20% FCS) revealed a different pattern but suggested the involvement of a common regulatory region for CNTF- and serum-induced activation of the GFAP gene. In contrast to CNTF, serum-induced promoter activity was not significantly greater than control levels at the 6-h time point (Fig. 6A). By 24 h, however, a significant increase in luciferase activity in some of the serial-deleted constructs was observed (Fig. 6B). Similar to CNTF-treated cells, the G4 construct did reestablish serum-induced luciferase activity that was lost in G2 and G3, suggesting that this region of the GFAP promoter is important for serum-induced activation. The level of luciferase activity, however, was significantly less than that observed for cells treated with CNTF. Serum-induced CG-4 cells transfected with the G4 construct experienced only a twofold increase over control values for the 24-h interval, whereas CNTF-treated cells experienced a fourfold induction. It is possible that this moderate increase in serum-induced activation of the promoter is due to the presence of a small amount of serum (2% FCS) in the medium to enhance cell survival. This might lead to the regions on the gene that are normally activated by serum to be partially saturated. Constructs G5–G8 did not significantly increase luciferase activity beyond that observed for G4. The G1 construct was essential to confer maximal serum-induced luciferase activity. From these data, proximal (−384 to −106 bp) and distal (−1,857 to −1,546 bp) regions similar to those observed for CNTF are also necessary for maximal serum-induced activation of the GFAP promoter.

Analysis of the distal and proximal CNTF- and serum-responsive regions (Fig. 6) revealed putative transcriptional control elements that may be important for

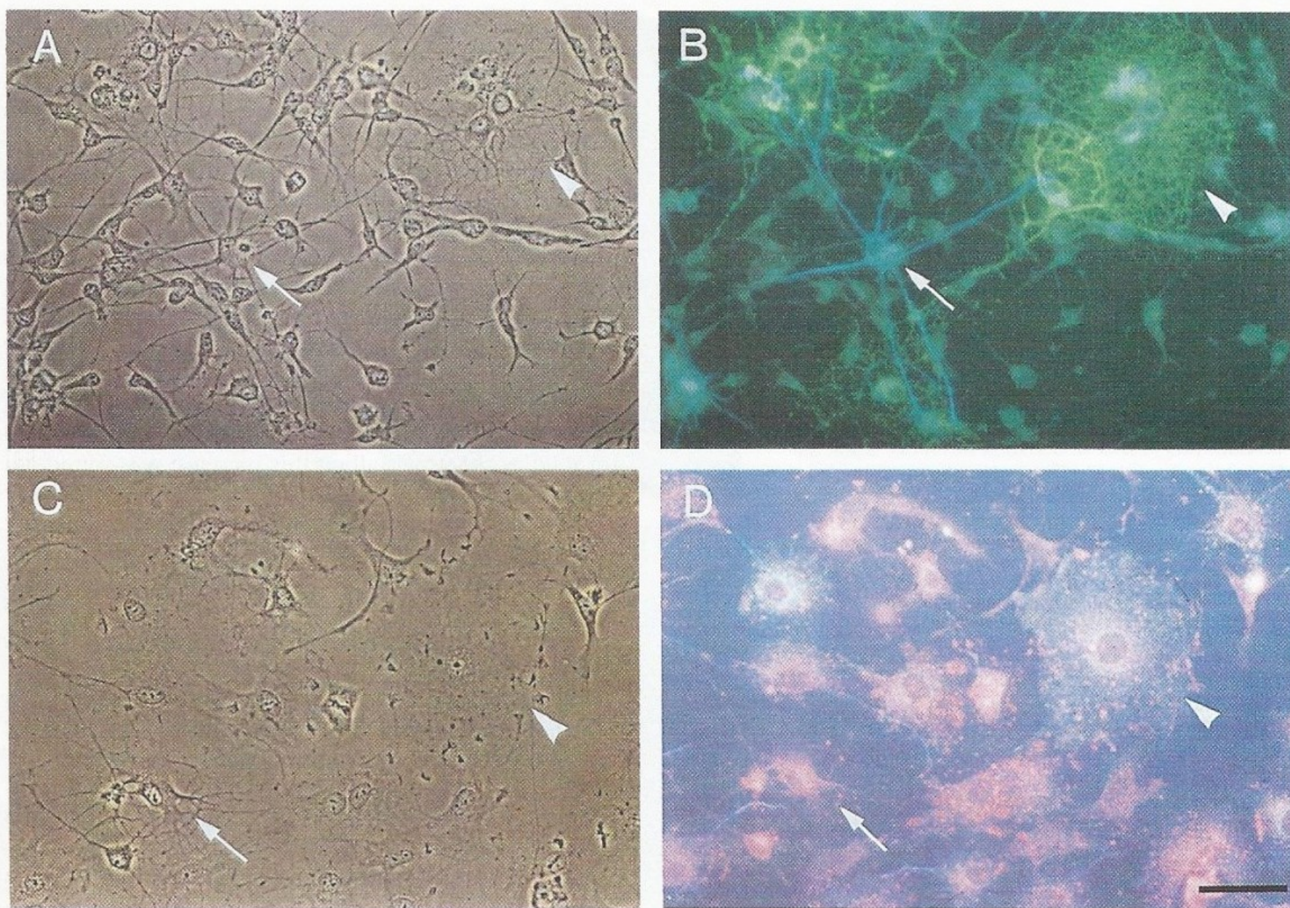

FIG. 3. CG-4 cell plasticity and gene regulation in the presence of CNTF or FCS. **A** and **B:** In the presence of CNTF, many of the cells are complex multipolar and express the mature oligodendrocyte marker, myelin basic protein (arrowhead; green). Approximately 20–30% of the cells are star-shaped in morphology and express the astrocytic marker GFAP (arrow; blue). **C** and **D:** Approximately 60–75% of the cells cultured in the presence of high-level serum are large and protoplasmic in morphology (arrowhead); few cells possess a fibrous morphology (arrow). All of the cells in this field colocalize for GFAP (blue) and display a punctate distribution pattern for A2B5 (red). A similar protein expression profile was exhibited in three separate experiments. Three different fields per experiment were counted with each field containing a minimum of 30 cells. Cells were counted and phenotyped using immunofluorescence microscopy. Bar = 20 μm.

GFAP gene activation. Figure 7 is a schematic representation of putative regulatory regions that we believe may be important players in CNTF-induced GFAP activation. Close attention was given to *cis*-elements that have been deemed important for GFAP regulation by other ligands and to *cis*-elements that are known to be activated by other members of the IL-6 family in different systems. The distal (−1,857 to −1,546 bp) and proximal (−384 to −106 bp) regions that confer CNTF responsiveness showed high evolutionary conservation among species. The *cis*-elements within the GFAP promoter were identified based on a >67% homology to known consensus sequences using the IUPAC code string analysis and following the rat GFAP promoter sequence determined by Kaneko and Sueoka (1993). Examination of a putative CNTF-RE (TTCCNNNNAA) (Bonni et al., 1993) revealed several existing *cis*-elements; two appeared in the distal region of the GFAP gene at −1,855 (TTCCNNNgg) and −1,731 bp (gTCCNNNNtA), whereas five were identified in the proximal region at −340 (TTCCNNNcc), −254 (TTCCNNNgc), −230 (TTaCNNNAA), −215 (TTCCNNNgA), and −183 bp (TTCCNNNgA). In addition to IUPAC confirmation of these putative CNTF-REs, MatInspector analysis was also used (Quandt et al., 1995). Through this method, all seven putative CNTF-REs were found, therefore suggesting a stronger possibility of biological functionality. It is interesting that in the GFAP gene, two of the novel CNTF-REs were flanked by putative sequences to activator protein-1 (AP-1) (TGA[G/C]TCA) (Angel et al., 1987; Lee et al., 1987) found at −1,719 (gGACTCA) and −166 bp (TGACTCt). An additional putative AP-1 site was also found to exist in the proximal region at −238 bp (TGACcCA). We observed a putative sequence to glucocorticoid-responsive element (GRE; RDDACRNNNHGTYCY; consensus sequence 1) (Laping et al., 1994) flanking the putative distal

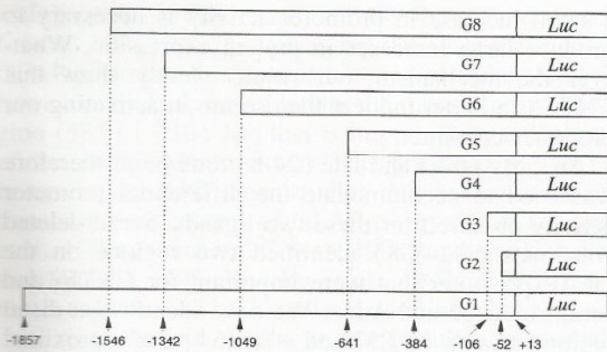

FIG. 4. Serial-deleted GFAP promoter constructs containing different 5′ regions linked to a luciferase (Luc) reporter gene. Constructs were generated by endonuclease cutting at specific restriction sites on the full-length G1 construct. The numbering corresponds to the transcription start site of +1.

CNTF-RE and AP-1 sites at −1,731 bp (GGGACA-GGGAGaGCC). This close proximity of responsive elements suggests the possibility of a complex between the AP-1 CNTF-RE and/or GRE sites. A similar putative GRE sequence, however, was not found in the proximal region. Three putative sequences to serum-responsive element (SRE; CC[6A/T]GG) (Treisman, 1992) were observed: One appeared in the distal region at −1,615 bp (tC[6A/T]GG), and two appeared in the proximal region at −274 (Cg[6A/T]GG) and −227 bp (CCAgAAATGG). A putative cyclic AMP-responsive element (CRE) sequence (T[G/T]A[C/T]GT[A/C][A/G]−[C/G]AG[A/C]) (Roesler et al., 1988) was also found in the proximal region at −153 bp (TGACcTtG−GAGA); the dash represents a series of nucleotides creating a break in the sequence.

DISCUSSION

Considering the strong evidence suggesting that CNTF is an injury factor involved in the astrogliotic response in the CNS, we have explored the molecular mechanisms governing the early signal transduction events as well as identified a distal and a proximal region within the GFAP promoter that are necessary to confer CNTF-induced GFAP activation. CNTF signaling to the nucleus requires the phosphorylation of tyrosine residues on proteins, termed signal transducers and activators of transcription (Stats), that are found within the cell cytoplasm (Bonni et al., 1993). Examination of CNTF-induced CG-4 cells revealed that Stat1α/p91 is activated within 5 min as determined by its translocation from the cell cytoplasm to the nucleus. Nuclear p91 was still apparent after 30 min but was absent after a 1-h interval. These data strongly suggest the involvement of a transiently activated p91 in CNTF-induced gene activation.

Examination of the mRNA expression pattern for CNTF-induced GFAP revealed an increase in mRNA level within 12 h. Similar levels were also observed for a 24-h interval. CHX did not alter CNTF-induced GFAP mRNA, suggesting the involvement of preexisting proteins in gene activation. Our current results suggest that members of the JAK/Stat family are likely to be involved. Moreover, this initial signal transduction cascade probably results in the activation of early response genes. Indeed, a previous study revealed the transient up-regulation of several early response gene mRNAs, including *c-fos, NGFIB, Tis11,* and *junB* in CNTF-induced CG-4 cells (Kahn and de Vellis, 1994). To confer CNTF responsiveness, cell populations must possess the CNTF-αR. Examination of CG-4 cells revealed that these cells express receptor transcript and that the level of CNTF-αR mRNA is not affected by its ligand.

In addition to CNTF, factors in serum are known to up-regulate GFAP. Although the mRNA patterns for CNTF- and serum-induced GFAP appear remarkably similar for both the 12- and 24-h intervals (data not shown), protein levels are substantially different. Through immunocytochemistry, CNTF induced GFAP expression in ~20–30% of the CG-4 cell population; serum, on the other hand, up-regulated GFAP in 60–75% of the cells examined. Moreover, the expression of GFAP was stable in cells exposed to serum, whereas CNTF-induced GFAP was transiently expressed as observed through both immunocytochemistry and western analysis (data not shown).

Further characterization of the CNTF- and serum-induced GFAP response was obtained through our transient transfection studies, whereby more similarities and differences between these GFAP inducers became apparent. Optimal time points for maximal luciferase activity were obtained using the complete 1.9-kb GFAP promoter, termed G1, linked to a luciferase reporter gene. CNTF-induced luciferase activity significantly increased within 1 h of treatment, and maximal levels were observed at a 6-h time point. In con-

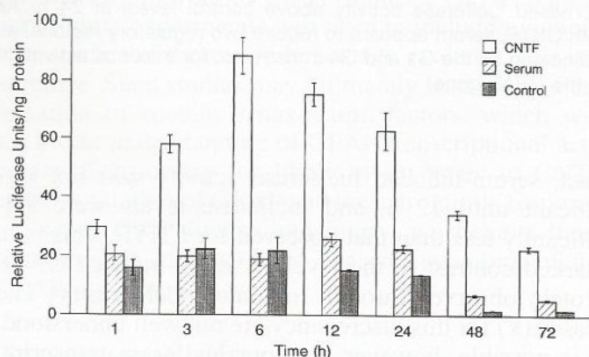

FIG. 5. Temporal expression pattern for CNTF- and serum-induced luciferase activity in CG-4 cells transiently transfected with the 1.9-kb rat GFAP gene promoter construct (G1; Fig. 4). CNTF-induced luciferase activity was significantly greater than control levels at all time points examined. Maximal CNTF-induced luciferase activity was observed following a 6-h induction, whereas serum-induced luciferase activity was not significantly different from control levels until 12 h.

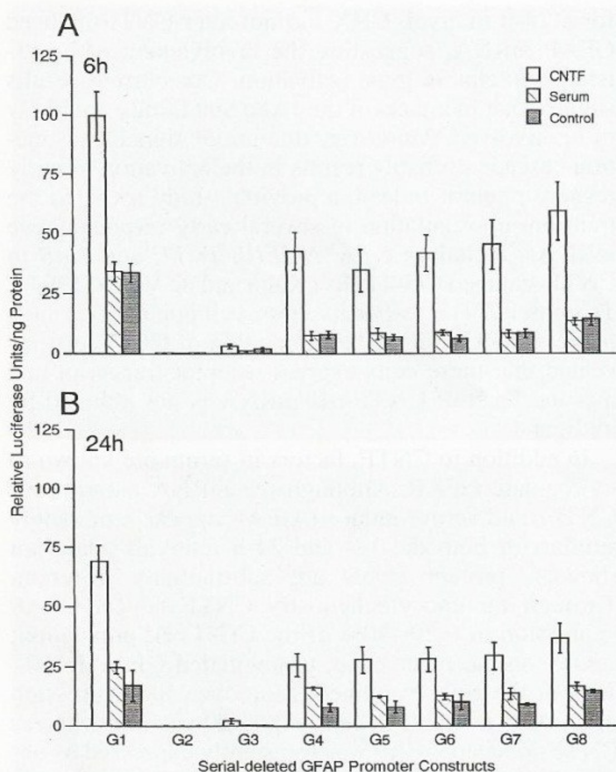

FIG. 6. CNTF- and serum-induced luciferase activity at a time point of (**A**) 6 h and (**B**) 24 h in CG-4 cells following transient transfections with different 5′-serial-deleted constructs of the rat GFAP promoter (Fig. 4). A: CNTF-induced luciferase activity was approximately three times that of control levels in cells transfected with the G1 construct. This activity was abolished in cells transfected with the G2 and G3 constructs; however, a significant increase in activity was reestablished in cells transfected with the G4 construct. No significant differences were observed using the G5–G7 constructs when compared with G4. G8 revealed a modest, albeit not significant, increase in luciferase activity. Serum-induced CG-4 cells showed no significant difference from control cells at this interval. B: A similar pattern to the 6-h time point was observed for CG-4 cells exposed to CNTF for 24 h. Unlike the 6-h interval, however, serum significantly increased luciferase activity above control levels at 24 h. As with CNTF, serum appears to require two regulatory regions, as delineated by the G1 and G4 constructs, for maximal activation of the GFAP gene.

trast, serum-induced luciferase activity was not significant until 12 h, and luciferase levels were significantly less than that observed for CNTF. This is in marked contrast to the level of serum-induced GFAP protein observed through immunocytochemistry. The reason(s) for this discrepancy are not well understood. It is possible, however, that our luciferase transcript, driven by our 1.9-kb GFAP promoter, undergoes post-transcriptional processing different from that observed for the endogenous gene. Our promoter could lack essential regulatory elements found within introns and/or upstream and downstream sequences. Moreover, because factors present in serum result in the stabilization of the GFAP protein product, it is possible that only a small increase in promoter activity is necessary to produce large increases in protein expression. Whatever the mechanism, our results clearly show that CNTF is a better inducer than serum in activating our promoter construct.

An early (6-h) and late (24-h) time point therefore was used to accommodate the differential promoter activity observed for these two ligands. Serial-deleted constructs (G2–G8) identified two regions on the GFAP promoter that were important for CNTF- and serum-induced activation. We have identified a distal region between −1,857 and −1,546 bp and a proximal region between −384 and −106 bp as containing important *cis*-elements necessary for optimal GFAP promoter activity. It is interesting that comparison of the rat GFAP gene (Feinstein et al., 1992; Kaneko and Sueoka, 1993; Condorelli et al., 1994) with that of the mouse (Balcarek and Cowan, 1985; Miura et al., 1990) and human (Brenner et al., 1990; Kumanishi et al., 1992) revealed that highly homologous regions fall within our distal and proximal sites. Such evolutionary conservation across species suggests the presence of important GFAP regulatory elements existing in our identified regions. Although fundamental differences exist, others have reported similar regions to be important in the regulation of the human and mouse GFAP promoter (Miura et al., 1990; Besnard et al., 1991; Masood et al., 1993; Kaneko and Sueoka, 1993; Kaneko et al., 1994). Besnard et al. (1991) identified three regions in the GFAP promoter, A (−1,757 to −1,604 bp), B (−1,612 to −1,489 bp), and D (−132 to −57 bp), and determined that the B region, linked to the basal promoter, was the most important in activating transcription of GFAP in human U251 cells. It should be noted, however, that a combination of all three regions resulted in another large increase in gene transcription. These data correspond well with our distal region (−1,857 to −1,546 bp) that we have determined to be important in regulation of the rat GFAP promoter. Moreover, as in their combinatorial study involving a construct containing all three A, B, and D regions, we also only observed the return of complete transcriptional activation in the presence of the whole G1 construct. Other studies have determined that the region between −1,882 and −1,465 bp (Sarid, 1991)

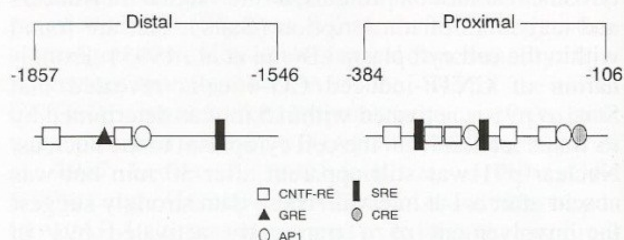

FIG. 7. Schematic representation of putative CNTF transcriptional control elements in the distal (−1,857 to −1,546 bp) and proximal (−384 to −106 bp) regions of the rat GFAP promoter as deduced by regions obtained in Fig. 6.

and between −1,981 and −1,479 bp (Sarkar and Cowan, 1991) is important in regulation of the mouse GFAP promoter. In contrast to some of these studies, however, we have also identified a more proximal region (385 to −106 bp) that is important in activation of the rat GFAP promoter. This proximal region is in agreement with other studies that have examined transcriptional control of GFAP using mouse (Miura et al., 1990; Sarkar and Cowan, 1991) and rat (Kaneko and Sueoka, 1993; Kaneko et al., 1994) GFAP promoter constructs. These studies determined that a proximal region exists, similar to our identified proximal region, that is important in activation of GFAP transcription. In general agreement with the human, mouse, and rat studies, therefore, we have identified both a distal region (as identified for the human and mouse genes) and proximal region (as identified for the mouse and rat genes) to be important in regulation of the rat GFAP promoter. Although many similarities exist between our study and those conducted by others, fundamental differences are apparent. These differences are not only restricted to analysis of promoter activity, but also are evident in studies examining cellular- and tissue-specific expression for the GFAP gene (Miura et al., 1990; Besnard et al., 1991; Sarid, 1991; Sarkar and Cowan, 1991; Kaneko and Sueoka, 1993; Brenner, 1994; Brenner et al., 1994; Kaneko et al., 1994).

In further studies using the B region of the human GFAP promoter, certain *cis*-elements were identified as playing an important role in transcriptional activation (Masood et al., 1993). An AP-1 site at −1,592 bp and a human GFAP common sequence at −1,537 bp were found to be important players in regulating transcription. (In the rat the AP-1 site homologous to the human AP-1 site is localized at −1,505 bp.) Other regions within the B region of the promoter that showed increased transcriptional activity but did not contain any identifiable *cis*-elements were not explored in this study. It is interesting that these regions fall outside of the areas delineated by our study; however, we would like to mention that an increase in serum- and CNTF-induced activity was observed for our G8 construct (−1,546 to −1,342 bp) at both the 6- and 24-h intervals. This increase, however, was not significant based on our statistical method of analysis and was definitely not on the same order of magnitude as the increases observed for the G1 and G4 constructs. There are many possible reasons for the existing discrepancies, including differential regulation of the gene expression by different species and/or by different ligands, the phenotype of the cells used for the transfection, slightly different regions of the promoter being used, and differences in experimental design. Regardless of the differences between groups, the majority of the studies are in general agreement that important distal and proximal regions exist on the GFAP promoter.

To characterize further our distal and proximal regions, we have identified putative transcriptional control elements. We believe these *cis*-elements may be important because they are known to play a role in the regulation of the GFAP gene or in the regulation of other genes by members of the IL-6 family. We consider the most important candidates to be CNTF-RE, AP-1, SRE, CRE, and GRE. Except for the CRE, which was only found in the proximal region, all other putative *cis*-elements were found in both our distal and proximal sites. Some of the potential roles of these *cis*-elements are well characterized. The activation of the protein kinase pathway has been linked to GFAP transcriptional regulation. This cascade is most likely the result of a complex series of events involving several factors including a CRE binding site (Shafit-Zagardo et al., 1988; Kaneko et al., 1994). Members of the *fos* and *jun* family of transcription factors are known to form the AP-1 complex that is thought to be involved in the transcriptional control of GFAP within human U251 cells (Masood et al., 1993). Moreover, our previous studies have shown that CNTF results in the transcriptional activation of members within the *fos* and *jun* family (Kahn and de Vellis, 1994). Modulatory effects of glucocorticoids on GFAP regulation have been identified, and it is likely that steroid action is linked to GRE sites on the gene promoter (Huang et al., 1992, 1993; Rozovsky et al., 1995). Serum is known to increase GFAP levels in many different cell types and is thought to operate through SRE (Treisman, 1992), although a functional SRE has not been identified. The CNTF-RE was recently identified using CNTF-induced human neuroblastoma cells (Bonni et al., 1993). Considering the large increase in GFAP content following exposure to CNTF, we believe that the CNTF-RE in the distal and proximal regions of the rat GFAP promoter may play a critical role in CNTF-induced gene activation.

It is possible, however, that other *cis*-elements, yet to be identified within our proximal and distal regions, may be key players in CNTF-induced GFAP regulation. Future experiments will need to include functionality studies of these *cis*-elements to evaluate their importance. Such studies may ultimately lead to the identification of specific *trans*-acting factors, which will aid in our understanding of GFAP transcriptional activation. Considering the likely involvement of CNTF in the regulation of GFAP and the astrogliotic response following CNS disease and injury, we believe these studies to be imperative to our understanding of the GFAP regulatory cascade.

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