Astrocyte-specific Expression of the $\alpha_1$-Antichymotrypsin and Glial Fibrillary Acidic Protein Genes Requires Activator Protein-1*

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An amyloid-associated serine proteinase inhibitor (serpin), $\alpha_1$-antichymotrypsin (ACT), is encoded by a gene located within the distal serpin subcluster on human chromosome 14q32.1. The expression of these distal serpin genes is determined by tissue-specific chromatin structures that allow their ubiquitous expression in hepatocytes; however, their expression is limited to a single ACT gene in astrocytes. In astrocytes and glioma cells, six specific DNase I-hypersensitive sites (DHSs) were found located exclusively in the 5' flanking region of the ACT gene. We identified two enhancers that mapped to the two DHSs at $-13$ kb and $-11.5$ kb which contain activator protein-1 (AP-1) binding sites, both of which are critical for basal astrocyte-specific expression of the ACT gene. The expression of these distal serpin genes suggests that they play a critical role in their expression in hepatic cells. However, HNF1, HNF3, HNF4, and C/EBP-$\beta$, the predominant member of the C/EBP family found in nonstimulated hepatocytes, are not expressed in brain astrocytes. Therefore, yet to be identified astrocyte-specific factors likely determine the astrocyte-specific chromatin structure of the distal serpin subcluster and the astrocyte-specific expression of the ACT gene in both astrocytes and glioma cells. Glial fibrillary acidic protein sensitive sites (DHSs) and to determine their effect(s) on astrocyte-specific ACT expression. Here we identify two AP-1-binding elements that are indispensable for the basal expression of the ACT gene in astrocytes. Our work was supported by National Institutes of Health Grant NS044118 (to T. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: serpins, serine proteinase inhibitors; ACT, $\alpha_1$-antichymotrypsin; AP-1, activator protein-1; CAT, chloramphenicol acetyltransferase; C/EBP, CAAT enhancer-binding protein; CHIP, chromatin immunoprecipitation; DHS, DNase I-hypersensitive sites; EMSA, electron mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; HNF, hepatocyte nuclear factor; IL, interleukin; NF-κB, nuclear factor κB; OSM, oncostatin M; STAT, signal transducer and activator of transcription; tk, thymidine kinase.
that AP-1 is also indispensable for the expression of GFAP, which is an astrocyte-specific marker.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human hepatoma HepG2, glioma U373-MG and A172, and cervical carcinoma HeLa cells were obtained from American Type Culture Collection (Rockville, MD). These cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, antibiotics, sodium pyruvate, and nonessential amino acids. U373 stable clones expressing c-jun(TAM67) were cultured in the presence of 10 μg/ml blasticidin, 100 μg/ml zeocin, and 2 μg/ml tetracycline (all from Invitrogen) as needed.

**Cytokines and Cell Stimulation**—Cells were stimulated with 25 ng/ml OSM and 5 ng/ml tumor necrosis factor (all from Invitrogen) as needed.

**RNA Preparation and Northern Blot Analysis**—Total RNA was prepared by phenol extraction exactly as described previously (3). The filters were prehybridized at 65 °C for 3 h in 0.5 m sodium phosphate buffer, pH 7.2, 7% SDS, and 1 mM EDTA, and hybridized in the same solution with cDNA fragments of ACT, GFAP, and GAPDH labeled by random priming (12). After the hybridization, nonspecifically bound radioactivity was removed by four washes in 40 μl phosphate buffer, 1% SDS, and 1 mM EDTA at 65 °C for 20 min each.

**Synthetic Oligonucleotides**—The following oligonucleotides were synthesized and subsequently used to generate PCR products containing the six DHSs. These PCR products were generated using Taq DNA polymerase, purified from gels using the gel purification kit (Qiagen, Valencia, CA), and subsequently used in gel retardation assays: DHS1, 5′-CTGGGAGGCTTCCTGCTA-3′ and 5′-GACAAAAACTGTGTAAGTGACACTGAC-3′; DHS2, 5′-GATGGTTATGATGAGTTACTGCTG-3′ and 5′-CAGGAACTCTGAGAGATTACATGATAC-3′; DHS3, 5′-AGCAGGATTATTTCTGCTC-3′ and 5′-ATTTCGAAGAATCTTCTGGCATTG-3′; DHS4, 5′-GATTGTATTATGAGATTTACTGG-3′ and 5′-GATTCCAAAGCCGTCTGTC-3′; DHS5, 5′-GACAGAGTCTCTCTGTG-3′ and 5′-AGACAGAGTCTCTCTGTG-3′; DHS6, 5′-CAAGGCCGATTTCCACAAAT-3′ and 5′-GGAGAAGGCTTAACACATTCCACAGC-3′.

**Plasmid Construction**—Plasmids pG3CATΔEH, p’αScAT, pSS-CAT, pΔACTCAT, and pStACTCAT have been described previously (11). Plasmids pDH51-ck-CAT, pDH52-ck-CAT, pDH53-ck-CAT, pDH54-ck-CAT, pDH55-ck-CAT, and pDH56-ck-CAT were generated by insertion of the corresponding BamHI-digested PCR products into the BamHI site of the plasmid pSκCATAEH. Plasmid pmutAP-1 (DHS2)-ck-CAT with the mutated AP-1 site in the DHS2 region was constructed using the QuikChange XL Site-directed Mutagenesis kit (Strategene, La Jolla, CA) according to the manufacturer’s instructions. Plasmid pS(E-S)ACT was generated by deletion of the EcoRV-BamHI fragment from plasmid pSSCAT. Subsequently, plasmids pDIA-SACT and pAAaIIACT were generated by deletion of the AflII-SpeI or AflII-AflII fragments, respectively, from plasmid pS(E-S)ACT, whereas plasmid pS(E-S)ACT was by cloning of the 1.2-kb Sphl-EcoRV fragment from pSSCAT into the Sphl-BamHI blunt-digested pStACTCAT. Plasmids pST4ST2, pST1ST5, ppoxAP-1, pmutNF, and pppmutAP were generated by insertion of the BamHI-digested PCR products into the BamHI-BglII sites of pStACTCAT. A plasmid encoding dominant-negative c-jun, pcMVC-jun(TAM67) (13), was obtained from Dr. Zendra Zehner, VCU, Richmond, VA. The expression vector was constructed using the T-REX™ system (Invitrogen) according to the manufacturer’s instructions. The plasmid pcMVc-jun(TAM67) was digested with BamHI, and the 931-bp product containing c-jun(TAM67) was cloned into the BamHI site of pcDNA4T/O.

**Transient Transfections**—Cells were transfected using Genejuice transfection reagent (Novagen, Darmstadt, Germany), according to the supplier’s instructions. Plasmid DNA (350 ng of the CAT reporter plasmid and 50 ng of the β-galactosidase expression plasmid) and 5 μl of Genejuice diluted in 50 μl of serum-free medium were used to transfect cells growing in 1 ml of culture medium. One day after transfection, cells were stimulated, cultured another 24 h, and harvested. Protein extracts were prepared by freeze thawing (14), and protein concentration was determined by the BCA method (Sigma). Chloramphenicol acetyltransferase (CAT) and β-galactosidase assays were performed as described (15). CAT activities are normalized to the internal control β-galactosidase activity. Experiments were repeated three to five times yielding similar results, and a representative example of each experiment is shown.

**Nucleofection**—U373 cells (1 × 10⁶/6-cm dish) were trypsinized, collected by centrifugation, and resuspended in 600 μl of T Nucleofector solution™ (Amaxa, Köln, Germany). Two μg of the respective plasmids (c-jun(TAM67) or pUC19) were added to the solution, and transfection was performed using the Nucleofector device (Amaxa) with the electrical setting of T-20. One ml of warm Dulbecco’s modified Eagle’s medium was added, and cells were incubated at 37 °C for 10 min and transferred to 6-cm dishes containing 5 ml of Dulbecco’s modified Eagle’s medium. Typical nucleofection efficiency was greater than 70% with cell viability close to 100%. One day after nucleofection, cells were stimulated with 25 ng/ml OSM and 5 ng/ml tumor necrosis factor (all from Invitrogen) as needed. The cytometric analysis for the representative times with 10% dexamethasone (Sigma) was also added to enhance cytokine action.

**Stable Transfections**—U373 cells expressing c-jun(TAM67) were generated using the T-REX™ system. U373 cells were transfected in 10-cm dishes with pcDNA6/TR using Genejuice transfection reagent, according to the supplier’s instructions. Four μg of pcDNA6/TR and 20 μg of Genejuice were diluted in 650 μl of serum-free medium/dish. Two days after transfections, transformants were selected in the presence of 10 μg/ml blasticidin. Stable clones were isolated after 3 weeks by glass cylinder cloning. Subsequently these stable cells were transfected with pcDNA4/Toc-jun(TAM67) in 10-cm dishes as described above and selected in the presence of 2 μg/ml zeocin. Positive clones were isolated and maintained in the presence of both blasticidin and zeocin.
AP-1 and Astrocyte-specific Expression

Nuclear Extract Preparation and Electron Mobility Shift Assays (EMSA)—Nuclear extracts were prepared as described previously (16). Double-stranded fragments were labeled by filling the 5’-protruding ends with Klenow enzyme using 3,000 Ci/mmol [α-32P]dCTP (17). Alternatively, they were labeled by PCR in the presence of 3,000 Ci/mmol [α-32P]dCTP using Taq DNA polymerase. Gel retardation assays were performed according to published procedures using 5 μg of nuclear extracts (18, 19). Competition experiments were performed in the presence of a 100-fold concentration of the cold oligonucleotides. Polyonal anti-c-jun (sc-45), anti-junB (sc-8051), anti-junD (sc-74), anti-c-fos (sc-52), anti-frac1 (sc-605), anti-frac2 (sc-171), and anti-fosB (sc-7203) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and used for supershift experiments.

Western Blotting—U373 cells growing in 6-well plates were lysed in 200 μl of 10 mM Tris, pH 7.4, 150 mM sodium chloride, 1 mM EDTA, 0.5% Nonidet P-40, 1% Triton X-100, 1 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, and a mixture of protease inhibitors (Roche Applied Science). These 20-μl samples were subjected to SDS-PAGE and electroblotted onto nitrocellulose membranes (Schleicher & Schuell). GFAP and c-jun were detected using polyonal sc-9065 and sc-45 antibodies, respectively. Antigen-antibody complexes were visualized by enhanced chemiluminescence (Pierce).

Isolation of Nuclei and DHS Mapping—Clones were cultured in the presence of 2 μg/ml tetracycline for 5 days. Nuclei were isolated and treated with increasing concentrations of DNase I exactly as described previously (3). DNA was isolated using a DNA isolation kit from Gentra Systems (Minneapolis) according to the manufacturer’s instructions. Purified genomic DNA (10 μg/gel lane) was digested with the appropriate restriction enzyme, separated in 0.8% agarose gel, and transferred to Hybond-XL membranes (Amersham Biosciences). Membranes were hybridized to random primer-labeled SG5 and PRA7 probes (3) in 500 mM sodium phosphate, pH 7.1, 7% SDS, 1 mM EDTA, and 10 μg/ml herring DNA at 65 °C. After the hybridization nonspecifically bound radioactivity was removed by three washes in 40 mM phosphate buffer, 1% SDS, and 1 mM EDTA at 65 °C for 20 min.

Chromatin Immunoprecipitation (ChIP) Assay—Stable clones were cultured in the presence of 2 μg/ml tetracycline for 5 days, and chromatin was cross-linked by the addition of formaldehyde to 1% followed by a 10-min incubation at 37 °C. Subsequently, the cells were washed with ice-cold phosphate-buffered saline containing 25 mM glycerine and 1 mM phenylmethylsulfonyl fluoride. Chromatin was sonicated and immunoprecipitated using specific antibodies exactly as described in the ChIP protocol from Upstate Inc. (Charlottesville, VA). The following antibodies were used: anti-acyetyl histone H3 (ab2381), anti-acyetylhistone H4 (ab1758) (Abcam, Inc., Cambridge, MA), anti-dimethylhistone H3 (lysine 4) (07–030) (Charlottesville, VA), anti-c-jun (sc-45), and anti-c-fos (sc-52) both from Santa Cruz Biotechnology, Inc.

RESULTS

Activator Protein-1 (AP-1) Binds to DNase I-hypersensitive Sites DHS1 and DHS2—We have shown previously that the astrocyte-specific expression of the ACT gene is associated with the tissue-specific chromatin structures at the distal serpin subcluster (3). This subcluster is easily accessible in HepG2 cells where all three genes encoding kallistatin, protein C inhibitor, and ACT are expressed. In contrast, it is accessible only near the IL-1-responsive enhancer and the ACT gene promoter in primary human astrocytes and glioma cells. We have identified six DHSs located nearby the IL-1 enhancer and the ACT promoter in astrocytes and U373 cells (3). To identify the astrocyte-specific factors required for the ACT expression, we analyzed the binding of proteins to the DNA fragments covering these six DHSs by EMSA. Each of these fragments was assayed for binding in vitro using nuclear extracts from ACT-expressing glioma cells (U373 and A172), hepatoma cells (HepG2), and nonexpressing carcinoma (HeLa) cells. We have obtained multiple bands and hence performed competition experiments to confirm their specificity (Fig. 1).

Simultaneously, we analyzed the fragments using the Mat Inspector program (www.genomatix.de) to identify transcription factor binding sites. A number of binding sites were identified including two AP-1 elements within the DHS1 and DHS2 fragments. These elements were analyzed further for the binding of AP-1 because this factor is very abundant in astrocytes (compared with hepatoma cells (20)), and it has also been suggested to regulate the expression of the GFAP gene, which is an astrocyte-specific marker. We confirmed the binding of AP-1 to both fragments (DHS1 and DHS2) by competition with the AP-1 oligonucleotide in both glioma cell lines, as well as in HeLa cells, which contain abundant amounts of endogenous AP-1 (Fig. 1, A and B). However, AP-1 did not bind to DHS1 and DHS2 in hepatoma HepG2 cells. The same expression pattern of ACT in both A172 and U373 glioma cells suggests that a common factor should bind to the DHSs in both cell lines. The binding pattern observed with probes DHS3 and DHS4 was different among these cells (Fig. 1, C and D). Therefore, we concluded that factors binding to DHS3 and DHS4 are likely not relevant for glioma-specific expression. The DHS5 probe did not bind any protein specifically, whereas DHS6, which corresponds to the ACT core promoter, likely binds factors from the basal transcriptional machinery (Fig. 1, E and F).

To identify the components of the AP-1 complex that bind to DHS1 and DHS2, we performed supershift analysis using extracts from glioma and hepatoma cells. The supershifts (Fig. 2) indicate that c-jun is the major component of the AP-1 complex and binds to both DHS1 and DHS2 in U373 cells, whereas amounts of c-fos were much lower. Neither c-jun nor c-fos binding was detected in HepG2 cells because none of the DNA-protein complexes was supershifted. We conclude that AP-1 specifically binds to DHS1 and DHS2 in glioma cells; however, some other factor(s) bind(s) to these elements in hepatoma cells.

DHS1 and DHS2 Can Function as Enhancers in U373 Cells—To analyze whether these six DHSs have a regulatory role, we linked each of the six DHSs to the thymidine kinase (tk)-promoter driving the transcription of the CAT reporter gene and analyzed these reporter constructs in transient transfections of U373 cells. The fragments containing both DHS1 and DHS2 increased the basal activity of the reporter constructs by 4–5-fold, whereas the other DHSs had no effect (Fig. 3A). To determine whether DHS1 and DHS2 fragments can also act as IL-1-responsive elements, we stimulated U373 cells (transiently transfected as before) with IL-1. The fragments containing DHS1 and DHS2 conferred IL-1 responsiveness (1.5–2-fold) onto the tk promoter, whereas the other fragments were ineffective (Fig. 3B).

A previously identified 413-bp IL-1-responsive enhancer located 13 kb upstream from the transcription start site of the ACT gene contains two NF-κB and one AP-1 binding site (11). The 160-bp DHS1 fragment partially overlaps this IL-1-responsive enhancer including its AP-1 binding sites. It has already been shown that mutation of this AP-1 site (at 11.5 kb) leads to a 50% decrease in the IL-1 responsiveness. To determine whether the other AP-1 site located within the DHS2 fragment (at −13 kb) leads to a 50% decrease in the IL-1 responsiveness. To determine whether the other AP-1 site located within the DHS2 fragment (at −11.5 kb) is also required for full transcriptional activity of this new enhancer, we mutated this site in the corresponding reporter plasmid. This mutation resulted in a dramatic loss of basal activity of the reporter plasmid when transfected into U373 cells (Fig. 3C).
We have antecedently reported that the reporter containing the 413-bp IL-1-responsive enhancer (located at -13 kb) is less responsive to IL-1 than the reporter containing 8-kb fragment, which suggested the presence of an additional regulatory element (11). Therefore, we generated a series of deletion reporters and tested them in transfection experiments (Fig. 4). This analysis resulted in the identification of an additional IL-1-responsive fragment that contained putative NF-kB and AP-1 binding sites. Interestingly, this fragment partially overlaps with the DHS2 fragment, and the AP-1 site we identified by this deletion analysis is identical to the site we identified by EMSA. Mutation of the AP-1 element within this reporter also abolished the responsiveness to IL-1, whereas mutation of the NF-kB element had no effect. From these results we conclude that both DHS1 and DHS2 fragments containing two AP-1-binding elements are critical for the full basal transcriptional activity.

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**AP-1 and Astrocyte-specific Expression**

![Diagram](image1.png)

**FIGURE 1.** AP-1 binds to DHS1 and DHS2 in glioma cells. DHS map of the 5'-flanking region of the ACT gene. The map is drawn to scale, with position +1 defined as the transcription start site of the ACT gene. Exons are indicated as black boxes, and gray boxes represent IL-1-responsive enhancers. Arrows indicate the positions of DHSs. A–F, nuclear extracts were prepared from human glioma U373 and A172, hepatoma HepG2, and carcinoma HeLa cells. The binding was analyzed by EMSA using the 32P-labeled DHS probes as indicated. A 100-fold excess of unlabeled oligonucleotide competitors was added to the binding reactions as indicated. Arrows indicate the positions of bands competed off by an AP-1 oligonucleotide.

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**c-jun, c-fos, fosB, and fra1 Are Major Components of the AP-1 Complex Binding to the ACT Enhancer Elements in Glioma Cells**—The AP-1 family of transcription factors includes the Jun and Fos family members. c-jun can form stable homo- and heterodimers with other members of the AP-1 family, whereas members of the Fos family do not homodimerize, but form stable heterodimers with Jun proteins (21). We analyzed the composition of AP-1 complexes that bind to the newly identified AP-1 binding site at -11.5 kb (DHS2) in untreated U373 cells and cells treated with IL-1, OSM, and tumor necrosis factor. The binding of AP-1

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**FIGURE 2.** c-jun binds to DHS1 and DHS2 in glioma cells. Nuclear extracts were prepared from U373 and HepG2 cells. Five µg of nuclear extracts was incubated with anti-c-jun, anti-c-fos antibodies, or normal rabbit serum (NRS). The binding was analyzed by EMSA using DHS1 and DHS2 probes.
AP-1 and Astrocyte-specific Expression

FIGURE 3. DHS1 and DHS2 contain functional AP-1 binding sites. U373 cells were transfected with the indicated plasmids pDHS1-trCAT, pDHS2-trCAT, pDHS3-trCAT, pDHS4-trCAT, pDHS5-trCAT, and pDHS6-trCAT (A), pDHS2-mutAP-1-trCAT (C), and β-galactosidase expression vector as an internal control for transfection efficiency. One day after transfection cells were stimulated with IL-1 (B and C) or left untreated. They were cultured for another 24 h and harvested. CAT activities were normalized to β-galactosidase activities (cpm/units). A representative result of three separate experiments that produced similar results is shown.

to this oligonucleotide was competed off by cold AP-1 probes (both -13 kb and -11.5 kb AP-1 elements), but not by cold oligonucleotides containing binding sites for NF-kB, STAT, or C/EBP (Fig. 5A). The AP-1 complexes in untreated U373 cells contained c-jun and a limited amount of fra1, whereas IL-1 stimulation resulted in induction of some c-fos and fosB (Fig. 5, B and C). Hence, we conclude that c-jun homodimers are likely bound to the -11.5 kb AP-1 element in untreated cells, whereas IL-1 stimulation results in the heterodimerization of c-jun with c-fos, fosB, or fra1.

The Elements at -11.5 kb and -13 kb Are Bound by AP-1 Complexes in Vivo—Next, we analyzed the binding of AP-1 to the elements located at -13 kb (DHS1) and -11.5 kb (DHS2) in vivo using ChIP analysis in different cell lines (Fig. 6). c-jun binding was detected in both elements in untreated U373 cells, whereas the binding of c-fos was marginal. IL-1 treatment resulted in a decrease of c-jun and an increase in c-fos binding, mostly at the -11.5 kb element, indicating replacement of c-jun homodimers with c-jun/c-fos heterodimers. In contrast to glioma cells, no binding of c-jun or c-fos was detected in vivo in HeLa cells, even though they express abundant amounts of endogenous AP-1. This discrepancy can be explained because both of these enhancers (-11.5 and -13 kb elements) are localized in the inaccessible chromatin in HeLa cells (3). We conclude that c-jun is the critical AP-1 component binding to both enhancers in vivo in untreated U373 cells.

Generation of Stable U373 Cells Expressing Dominant-negative c-jun (TAM67) in a Tetracycline-inducible system—The binding of AP-1 to both the -13 and -11.5 kb elements in vivo and the dramatic decrease of basal activity after mutating the AP-1 binding sites within the reporter constructs suggested that AP-1 may be critical for the basal ACT expression in glioma cells. To test this hypothesis in vivo, we generated a stable U373 cell line that inducibly overexpresses a dominant-negative c-jun(TAM67) that lacks amino acids 3-122 within the transactivation domain. Several clones that showed inducible c-jun(TAM67) expression in the presence of tetracycline but differed in the levels of expression of an astrocytic marker GFAP were obtained (Fig. 7A). We analyzed ACT expression in two of these clones either untreated or stimulated with IL-1 or OSM in the presence or absence of tetracycline using Northern blot analysis (Fig. 7B). The basal expression of ACT was completely abolished when expression of c-jun(TAM67) was induced using tetracycline (Fig. 7B, long exposure). IL-1 and OSM were found to induce ACT expression as reported previously. However, there was a considerable drop in the ACT mRNA levels after IL-1 and OSM treatment in the presence of tetracycline (Fig. 7B, short exposure). This effect...
was specific because the GAPDH mRNA levels were unaffected. We infer that AP-1 is critical for both basal and cytokine-induced expression of the ACT gene in glioma cells.

**Figure 6. In vivo binding of c-jun to DHS1 and DHS2.** U-373 and HeLa cells were stimulated with IL-1 for 1 h, chromatin was prepared, and equal amounts of chromatin were immunoprecipitated with specific anti-c-jun or anti-c-fos antibodies. Subsequently, DNA was purified, and the DHS1 and DHS2 regions were amplified by PCR in the presence of α-32PdCTP. PCR products were separated on 12% native polyacrylamide gels, and gels were exposed to PhosphorImager screens. Input represents 2% of chromatin used for immunoprecipitation. A representative of two independent experiments is shown.

**Figure 7. Dominant-negative c-jun(TAM67) down-regulates basal and cytokine-induced ACT mRNA expression.** A, U373 stable clones expressing c-jun(TAM67) were cultured in the presence of tetracycline (tet) for 48 h (as indicated), and lysates were prepared and analyzed by Western blotting using anti-c-jun and anti-GFAP antibodies. B, U373-TAM67 cells (clones 4.4 and 4.10) were cultured in the presence of tetracycline for 48 h and then stimulated with IL-1 or OSM. RNA was isolated after 18 h and subjected to Northern blot analysis using ACT and GAPDH cDNA as probes. Both short and long exposures of the blot are shown.

**Figure 8. DHS analysis at the 5'-flanking region of the ACT gene.** U373-TAM67 cells (4.4 and 4.10) were cultured in the presence of 2 μg/ml tetracycline for 72 h. Nuclei were isolated and digested with increasing concentration of DNase I, and DNA was purified and digested with BglII (promoter) or HindIII (enhancer). DNA samples were analyzed by Southern blotting using SG5 "promoter" and PRA7 "enhancer" probes.

ACT gene associates with the tissue-specific chromatin structures (3). To evaluate whether c-jun(TAM67) expression can influence the astrocyte-specific chromatin structure near the ACT gene, we probed the chromatin structure in the c-jun(TAM67)-expressing cells using DNase I. We expected that overexpression of dominant-negative c-jun would result in either total or partial loss of the six DHSs found in parental cells. However, all of these DHSs present in parental cells were also present in U373-TAM67 cells (Fig. 8, two additional DHSs are seen on a longer exposure). We conclude that c-jun(TAM67) can drastically down-regulate ACT expression; however, it cannot by itself alter accessibility of the 5'-flanking region of the ACT gene.
both ACT expression and histone H3 lysine 4 methylation at the ACT promoter but does not influence acetylation of histone H3 and H4 at the 5'-flanking region of this gene.

AP-1 Is Indispensable for Basal Expression of the GFAP Gene—Because functional AP-1 was needed for ACT expression in glioma cells we suspected that it may be indispensable for expression of other astrocyte-, glioma-specific genes including GFAP. To test this hypothesis, we transiently transfected U373 cells with an expression vector for c-jun(TAM67) using nucleofection technology and analyzed the expression of both GFAP and ACT. Indeed, c-jun(TAM67) drastically repressed expression of GFAP and ACT (Fig. 10A). We also analyzed GFAP expression in U373-TAM67 cells and found that GFAP expression was completely lost after c-jun(TAM67) expression was induced by tetracycline (Fig. 10B). We conclude that AP-1 is required for GFAP and ACT gene expression in glioma cells.

DISCUSSION

ACT is expressed constitutively at low levels by brain astrocytes and at moderate levels by liver hepatocytes (20, 22, 23). Expression of this serpin gene in the liver is likely determined by transcription factor(s) belonging to four conserved families: HNF1, HNF3, HNF4, and C/EBP. However, the factors required for the expression of the ACT gene in astrocytes and/or glioma cells are unknown. Here we identify AP-1 as a critical transcription factor needed for the basal expression of the ACT gene in glioma cells. We conclude this from the following observations: (i) there are two DHSs present in the 5'-proximal region of the ACT gene which contain functional AP-1 binding sites; (ii) mutation of these AP-1 sites leads to the loss of enhancer activity; (iii) overexpression of the dominant-negative c-jun abolished the basal expression of the ACT gene; (iv) AP-1 is also critical for the expression of GFAP, which is an astrocyte-specific marker; and (v) resting glioma cells and astrocytes express high levels of AP-1. These data suggest the necessity for AP-1 for the astrocyte-specific expression of the ACT gene. In addition, the two DHSs containing an AP-1 binding site in the 5'-flanking region of the ACT gene can act as enhancers when linked to the reporter gene. These elements bind mainly c-jun and fra1 in untreated cells. These results suggest that c-jun homodimers, and to a lesser extent c-jun/fra1 heterodimers, mediate the basal expression of the ACT gene (Fig. 6).

However, an exchange of these dimers likely occurs in astrocytes stimulated with IL-1 because of the observations that c-jun-, c-fos-, fra1-, and fosB-containing complexes can bind to the AP-1 sites in IL-1-treated cells (Fig. 5).

We have reported previously that astrocyte- and glioma-specific expression of the ACT gene is determined by the astrocyte-specific chromatin structure of the distal serpin subcluster (3). In this study we propose that AP-1 is critical for the astrocyte-specific expression of the ACT gene. Our data raise the question as to whether AP-1 (specifically c-jun) is the key factor determining the chromatin structure at the distal serpin subcluster in astrocytes. Fig. 6 demonstrates the in vivo binding of c-jun to DHS1 and DHS2 in glioma cells. These c-jun homodimers likely recruit coactivator complexes containing histone acetyltransferases, CBP/p300, and/or chromatin remodeling factors as reported previously (24). This recruitment likely leads to the acetylation of histones and subsequent decondensation of chromatin at both the enhancer and the promoter, which facilitates the binding of the basal transcriptional apparatus.

This model is partially supported by the results obtained from U373 cells stably expressing the dominant-negative c-jun(TAM67), which lacks the transactivation domain. Expression of c-jun(TAM67) resulted in the complete inhibition of the basal ACT expression (Fig. 7). However, the up-regulation of ACT expression by IL-1 and OSM was retained in these cells, suggesting that NF-κB and STAT3 binding to the −13 kb enhancer and promoter elements, respectively, could counteract AP-1 deficiency. Similar results were obtained in transient transfection experiments of U373 cells with c-jun(TAM67) expression vector thus ruling out the clonal differences in the expression of the ACT gene (Fig. 10). Our data (Fig. 8) raise the question as to why the c-jun(TAM67) clones retained all six DHSs (suggesting a lack of alteration in chromatin structure) even though the basal expression of ACT was completely abolished. This lack of change in the chromatin structure suggests that c-jun(TAM67) retained the ability to recruit coactivator
proteins (including histone acetyltransferases and ATP-dependent chromatin remodeling factors). In support of this, the c-jun(TAM67) truncation lacks the transactivation domain (amino acids 3–122); however, it retains approximately half of the region (amino acids 96–193) that interacts with p300 (24), suggesting that c-jun(TAM67) could still recruit p300. Another alternative is that a yet to be identified factor could cooperate with c-jun and be sufficient to recruit coactivator complexes. However, both of these possibilities could result in histone acetylation as confirmed by the presence of acetylated histones H3 and H4 at both enhancers (Fig. 9). The loss of lysine 4 methylation on histone H3, at the ACT promoter, in U373-TAM67 cells correlates with the loss of basal ACT expression. This confirms that functional AP-1 is needed for ACT expression in glialoma cells. The absence of the transactivation domain in c-jun(TAM67) and the inhibition of basal ACT expression suggest that a functional enhansosome is not formed at the ACT gene.

Recently a locus control region has been described in the proximal serpin subcluster (25). Deletion of this locus control region resulted in the decrease of histone acetylation throughout the proximal subcluster (26). It is tempting to speculate that the region containing both enhancers within the distal serpin subcluster could also constitute a locus control region. In the future, the deletion of this region may determine the relevance of these enhancers and the role of c-jun in determining the astrocyte-specific chromatin structure and astrocyte-specific gene expression.

Expression of the ACT gene in hepatocytes is likely controlled by HNF1, HNF3, HNF4, and C/EBP. Because the levels of AP-1 found in the hepatocytes are low (compared with astrocytes) and the levels of HNFs and C/EBPs are high, one can invoke a model in which HNFs and C/EBPs outcompete AP-1 for closely spaced binding sites and determine the liver-specific expression pattern of these serpin genes. Because the expression levels of these transcription factors are reversed in astrocytes versus hepatocytes; (ii) the recruitment of astrocyte-specific coactivator complexes; and (iv) cooperation with the yet to be identified c-jun-dependent astrocyte-specific transcription factor.

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