

Regulation of the Urokinase-type Plasminogen Activator Receptor Gene in Different Grades of Human Glioma Cell Lines¹

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ABSTRACT

We reported previously that the production of urokinase-type plasminogen activator receptor (uPAR) protein is greater in high-grade glioblastomas than in low-grade gliomas. Transcriptional activation of the *uPAR* gene or increased stability of the uPAR mRNA that encodes this protein could cause the increased production of this protein in cell lines of different grades of gliomas. We found similar half-life of uPAR mRNA of 10–12 h in glioblastoma multiforme (UWR3) and anaplastic astrocytoma (SW1783) cells. However, the human uPAR promoter was up-regulated 6–8-fold in SW1783 cells and 11–13-fold in UWR3 cells as compared with its activity in low-grade gliomas, a finding that correlates well with previous findings of increases in uPAR mRNA and protein levels in higher-grade gliomas. uPAR mRNA level was increased 11-fold over a 24-h period in low-grade glioma cell lines after treatment with phorbol myristate acetate. The region spanning –144 to –123 bp of the human uPAR promoter that contains the Sp-1 site and a PEA-3 element and an AP-1 site at –184 plays major roles in uPAR promoter activity in glioblastoma cells. Specific antibodies used in an electrophoretic mobility shift assay identified fra-1, fra-2, Jun D, and c-Jun proteins in the nuclear protein complex that bind a 51-mer containing the AP-1 consensus sequence at –184 and its flanking sequences in the uPAR promoter. We further studied the inhibition of uPAR promoter by coexpression of a transactivation domain lacking C-Jun; a dominant-negative ERK1 and ERK2 mutant and a dominant-negative C-raf in glioblastoma cell lines showed the repressed uPAR promoter activity compared with the effect of the empty expression vector. We conclude from our findings that increased transcription is

the more likely mechanism underlying the increase in uPAR production in high-grade gliomas.

INTRODUCTION

The uPA⁴ protein and its receptor (uPAR) protein are important in the localized activation of plasmin at the extracellular surface of cells that produce uPAR. Urokinase is sequestered at the cell surface by its receptor, and the pericellular activation of circulating plasminogen, a serine protease with broad substrate specificity, enhances the proteolysis of extracellular components such as collagen, fibronectin, and laminin (1, 2). uPAR is heavily glycosylated and disulfide linked, and it binds uPA with high affinity with a K_D of ~0.5 nM (3, 4). Structural features of the M_r 55,000 uPAR protein, which consists of ~284 amino acids, including three similar repeats of 90 residues (5), the first of which interacts with the ligand and the last of which anchors uPAR to the plasma membrane by a glycosyl-phosphatidylinositol chain (6).

The *uPAR* gene is located on chromosome 19q13 (7, 8) and consists of seven exons. The uPAR transcript is 1.4 kb long, and a shorter spliced transcript that encodes the soluble receptor lacking the COOH-terminal domain has also been described (9). The upstream sequence of the human *uPAR* gene contains putative binding sites for AP-1, AP-2, NF- κ B, Sp-1, and ets transcription factors but no consensus TATA or CAAT boxes (10, 11). The AP-1 sites and the Sp-1 sites of the human uPAR promoter play important roles in the regulation of this gene in colon cancer and other cell types (12). It is known that activity and synthesis of a number of transcription factors (including those in the AP-1 and ets family) are regulated by multiple signals from the cell surface to the nucleus. In addition, the activity and synthesis of these factors have been shown recently to be regulated by JNK. The JNKs are activated by the dual-activity kinase JNK kinase (13), which in turn is stimulated by a serine threonine kinase, MEEK (14). MEEK transduces the signals from both ras-dependent (15) and ras-independent (16) growth factor cytokine cell-surface receptors.

Human malignant glial tumors concentrate the majority of primary intracranial tumors, and they are often characterized by rapid growth and invasion into surrounding normal brain tissues (17). The diffuse invasive nature of malignant gliomas can result in failure of potentially curative treatments (18). Although the specific mechanisms that underlie the invasive behavior of

Received 8/31/00; accepted 11/20/00.

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¹ This work was supported in part by National Cancer Institute Grant CA-75557 (to J. S. R.).

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⁴ The abbreviations used are: uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; PMA, phorbol myristate acetate; DRB, 5,6-dichlorobenzimidazole riboside; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; β -gal, β -galactosidase; CoA, coenzyme A; EGFR, epidermal growth factor receptor; AP, activator protein; JNK, c-Jun NH₂-terminal kinase; ERK, extracellular signal-regulated kinase; NF- κ B, nuclear factor- κ B.

malignant brain tumors remain unknown, activation of different proteases has been implicated as playing an important role (19–24).

We have shown previously that increased levels of uPA and uPAR protein correlate well with higher grades of glioma (21, 25). Furthermore, the production of uPA and uPAR at the extracellular surface of malignant brain tumors, but not in normal tissue, indicates that these proteins play an important role in the invasion of tumorigenic tissue into normal brain (21, 22, 25). On the other hand, exposure to antibodies to uPAR reduces the invasiveness of human glioblastoma cells (26). We have also observed that the invasive behavior of glioma cells in stable transfected clones of a glioblastoma cell line with 300 bp of antisense uPAR cDNA was reduced significantly, both *in vitro* and *in vivo*, as compared with the behavior of the parental cell line, vector, and sense uPAR stable transfectants (27, 28). Activation of the protein kinase C pathway by PMA has been reported to increase uPAR mRNA in other cell types (29), and elevated levels of protein kinase C isoforms in glioblastoma cell lines have been described (30–32).

We undertook the present study to determine whether the increased amount of uPAR protein in higher-grade gliomas is caused by enhanced transcription of the *uPAR* gene or by increased uPAR mRNA stability and also determine the role of JNK- and ERK-dependent signaling modules in regulating the production of uPAR in human glioblastoma cell line. Our results demonstrated clearly that increased transcription is the more likely mechanism underlying the increase in uPAR production in high-grade gliomas and also showed that uPAR production in glioblastoma cell line SNB19 is regulated by JNK- and ERK-dependent signaling modules.

MATERIALS AND METHODS

Materials. DMEM/F12 medium was obtained from Life Technologies, Inc. (Gaithersburg, MD). Tissue culture plates were purchased from Becton Dickinson and Co. (Franklin Lakes, NJ). DRB was purchased from Biotex Laboratories (Houston, TX). [α - 32 P]dCTP random prime labeling Mega Prime kit was purchased from Amersham Corp. (Arlington Heights, IL). Specific antibodies against fra-1 (Sc-605), fra-2 (Sc-604), c-fos (Sc-52), fos B (Sc-48), c-Jun (Sc-822), Jun D (Sc-74), and Jun B (Sc-46) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Tissue Culture and Cell Lines. H4 (low-grade glioma cell line) and SW1783 (an anaplastic astrocytoma cell line) were purchased from American Type Culture Collection (Rockville, MD). UWR3 (a glioblastoma cell line) was a generous gift from Dr. Francis Ali-Osman (M. D. Anderson Cancer Center). H4 and SW1783 cells were maintained in DMEM/F12 high-glucose medium with 10% FCS, 100 μ g/ml streptomycin, and 100 units of penicillin. UWR3 cells were maintained in DMEM low-glucose medium with 15% FCS. All cell lines were maintained in a humidified atmosphere of 5% CO₂ at 37°C and were subcultured every 3–5 days.

DRB Treatment and Northern Blot Analysis. Twenty μ g/ml of DRB was added to cells plated on 100-mm dishes. Total RNA was isolated at 0, 1, 2, 4, 8, and 24 h after the addition of DRB using the Ultraspec RNA isolation reagent

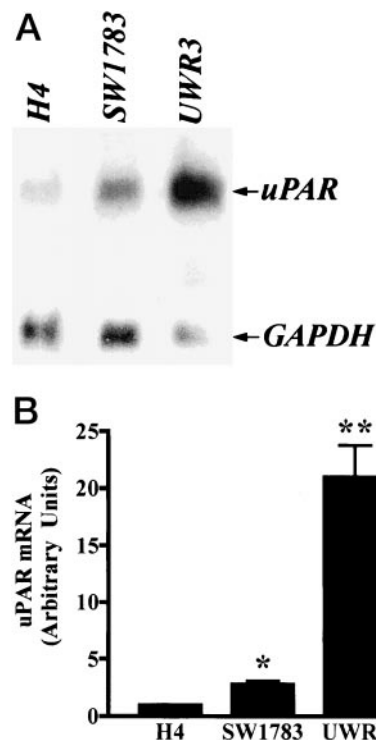
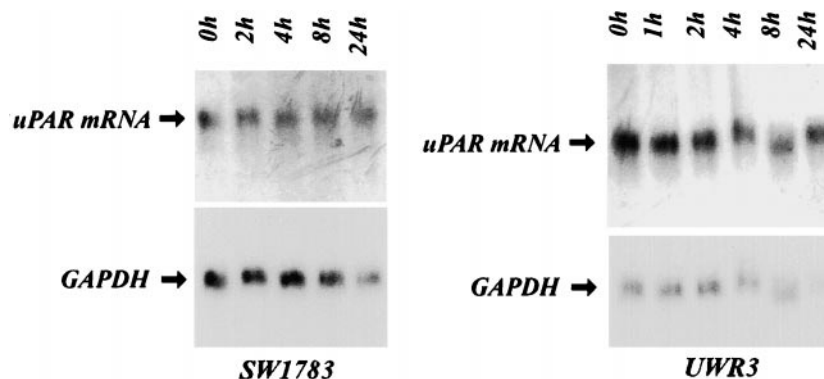


Fig. 1 Relative levels of uPAR mRNA in low-grade glioma (H4), anaplastic astrocytoma (SW1783), and glioblastoma (UWR3) cell lines. Twenty μ g of total RNA isolated from H4, SW1783, and UWR3 cells, shown in the upper panel, was electrophoresed in a 1.2% agarose formaldehyde gel and then transferred to Hybond N⁺ membrane. The membrane was then hybridized with a radiolabeled cDNA probe specific for uPAR mRNA. The same blot was stripped and hybridized with radiolabeled GAPDH cDNA to check for equality of loading. uPAR mRNA levels were measured by scanning autoradiograms with a laser densitometer, and relative hybridization signals were calculated by assigning an arbitrary value of 1 to the least intense signal seen by Northern blot analysis corrected for mRNA loading inequalities. In each group, the uPAR mRNA band was scanned in three positions at different exposures by laser densitometry, and the peak areas were averaged to give the values presented. Columns, the means for samples from five different experiments in each cell line; bars, SD. *, $P < 0.001$; **, $P < 0.0001$.

(Biotex Laboratories). Twenty μ g of RNA was separated on formaldehyde/agarose gels, transferred to Hybond N⁺ (Manufacturer) membrane, and hybridized to radiolabeled uPAR cDNA or GAPDH cDNA. Radiolabeling was done using [32 P]dCTP using a random prime labeling Mega Prime kit (Amersham). Specific RNA was quantitated by scanning autoradiograms using a personal densitometer (Molecular Dynamics, Sunnyvale, CA), and the stability of uPAR mRNA was estimated after correcting for loading inequalities with the GAPDH signal, which has a reported half-life of 8 h (33).

Transient Transfection and CAT Activity. Cells were plated to 80% confluence and transiently transfected with 4 μ g of uPAR CAT reporter plasmid for 16 h using the standard calcium-phosphate transfection procedure, with a 25% glycerol shock for 1 min. Cells were harvested after 24 h. Each plate was also transfected with 1.5 μ g of CMV- β -gal plasmid as an

Fig. 2 The half-life of uPAR mRNA in SW1783 and UWR3 cells. Northern blot of uPAR mRNA in 20 μg of total RNA isolated from SW1783 and UWR3 cells isolated at 0, 2, 4, 8, and 24 h of exposure to 20 $\mu\text{g}/\text{ml}$ DRB, using the same procedure described in Fig. 1, is shown. After stripping of the uPAR signal, the blots were rehybridized with radiolabeled GAPDH cDNA, as shown in the lower panel of each set.



internal control for transfection efficiency. After the cells were harvested, 4% of the cytoplasmic extract was used for protein estimation using the Bradford method (34), and 20% of the extract was used for β -gal assays performed according to published procedures (35). Cytoplasmic extracts showing equivalent β -gal activity were used to determine CAT activity (12). PMA was added at a concentration of 200 nM to cells plated in 1% serum for 2 h, after which the medium was changed to PMA-free medium. The cells were harvested 24 h later. For specific experiments, cells were left in PMA-containing medium until they were harvested. CAT activity was measured by incubating cell lysates (normalized for transfection efficiency) at 37°C for 6 h with 4 μM [^{14}C]chloramphenicol and 80 μg (20 μl of a 4 mg/ml solution) of acetyl CoA. After 3 h, acetyl CoA was replenished, and at 6 h, the mixture was extracted with ethyl acetate. The acetylated products were separated from the unreacted substrate by TLC using chloroform:methanol (95:5) as a mobile phase. The amount of acetylated [^{14}C]chloramphenicol was determined using a 603 Betascope (Betagen, Cambridge, MA; Ref. 35).

β -gal Assay. Twenty % of cellular extract was used to determine β -gal activity, as described previously (35). Briefly, 20 μl of cell extract was mixed with 0.1 M sodium phosphate, 10 μM MgCl_2 , 45 mM β -mercaptoethanol, and 800 mg/ml *o*-nitrophenyl- β -D-galactopyranoside and the reactions were incubated at 37°C for 30 min to 1 h until a faint yellow color developed. The reactions were stopped by adding Na_2CO_3 to each reaction to a final concentration of 625 mM, after which the absorbance of the reactions was read at a wavelength of 420 nm.

Nuclear Extract Preparation and Mobility Shift Assays. Nuclear extracts were prepared from cells plated on 100-mm dishes, as described previously (35). Briefly, cells were rinsed with PBS; pelleted in a microcentrifuge at 2000 rpm; resuspended in 400 μl of 20 mM HEPES (pH 7.9), 10 mM KCl, 0.2 mM EDTA, 1 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride and incubated on ice for 15 min. Then, 25 μl of 10% NP40 was added, cells were vortexed and centrifuged for 10 s, and the pellet was resuspended in 50 μl of 20 mM HEPES, 0.4 M NaCl, 1.0 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride and 1 mM DTT. The pellet was rocked vigorously at 4°C for 15 min and centrifuged for 5 min at 13,000 rpm, and the supernatant was used as a nuclear extract. Ten μg of nuclear

extract protein was used for incubation with 10,000 cpm (radioactively end-labeled using T4 polynucleotide kinase) of a 53-bp double-stranded oligomer containing the uPAR AP-1 consensus site at room temperature for 15 min. Additional incubations with specific antibodies against fra-1 (Sc-605), fra-2 (Sc-604), c-fos (Sc-52), Fos B (Sc-48), c-Jun (Sc-822), Jun D (Sc-74), and Jun B (Sc-46) were carried out for an additional 10 min, after which the protein/DNA complexes were separated on a 6% polyacrylamide gel containing 5% glycerol in $0.5\times$ TBE buffer at 120 V for 4.5 h. The gel was then rinsed, dried, and subjected to autoradiography.

RESULTS

Up-Regulation of uPAR mRNA Level in Glioblastomas.

To estimate the relative increase in uPAR mRNA in high-grade glioblastoma cell lines as compared with low-grade glioma cell lines by Northern blot analysis, total RNA was extracted from various grades of glioma cell lines (H4, SW1783, and UWR3). Fig. 1A shows that the intensity of the uPAR band was much higher in UWR3 cells than in SW1783 and H4 cell lines. Scanning autoradiograms of the hybridization signals with a laser densitometer and normalization with the GAPDH signal showed a 2.8-fold increase in uPAR mRNA in SW1783 cells and a 20-fold increase in UWR3 cells ($P < 0.001$) as compared with the uPAR mRNA levels in H4 cells (Fig. 1B).

Stability of uPAR mRNA in Various Glioma Cell Lines.

Transcriptional up-regulation of the *uPAR* gene or mRNA stabilization could account for the increased levels of uPAR mRNA and protein (21) seen in higher glioma grades of human tissue samples. We determined the relative half-life of uPAR mRNA in SW1783 and UWR3 cells by placing them in DRB for different times. Fig. 2A shows that the uPAR mRNA was present in both SW1783 and UWR3 cells at all times. After normalization with GAPDH, uPAR mRNA levels were much higher in glioblastoma UWR3 cells than in anaplastic astrocytoma SW1783 cells. The published half-life for GAPDH is 8 h (33), and this was used to normalize the uPAR signal. As seen in Fig. 2, the half-life of uPAR mRNA was ~ 10 h in SW1783 cells and ~ 12 h in UWR3 cells. The initial level of uPAR mRNA in H4 cells was very low; hence, the half-life could not be estimated.

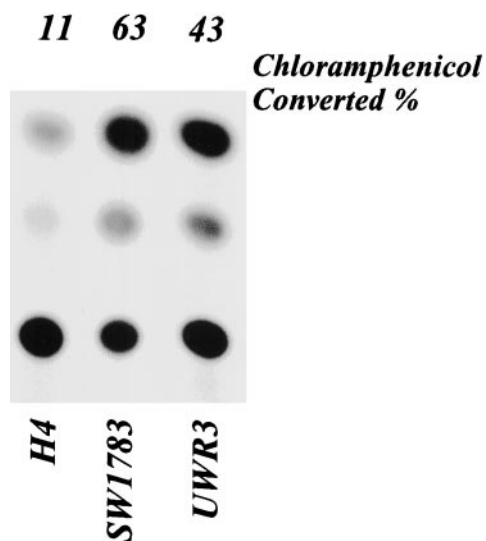


Fig. 3 Relative transcriptional activity of the human uPAR promoter in different grades of human gliomas. Four μg of a recombinant construct containing ~ 400 bp of upstream sequences of the human uPAR gene cloned upstream of the CAT reporter gene was transiently transfected into H4, SW1783, and UWR3 cells. Cotransfection with 1 μg of CMV- β -gal plasmid was used to control for transfection efficiency. Equivalent extracts were used from H4 and SW1783 cells, and to stay within the linear range of the CAT assay, only one-third of the equivalent UWR3 extract measured by β -gal activity was used. CAT activity was measured by incubating cell lysate (after normalization) at 37°C for 3 h with [^{14}C]chloramphenicol. The mixture was extracted with ethyl acetate, and acetylated products were subjected to TLC. The amount of acetylated [^{14}C]chloramphenicol was determined with a 603 Betagen Betascope. The data represent the results from five different experiments, the range of which did not exceed 10%.

Up-Regulation of Transcriptional Activity of the uPAR Promoter in the Glioblastoma Cell Line. The calculated half-life of uPAR mRNA was similar if not shorter in the UWR3 cells than in the SW1783 cells (8 and 12 h, respectively), which suggests that increased stability of the uPAR mRNA is not a primary mechanism. We determined the relative transcriptional activity of the uPAR promoter in these different grades of human glioma cell lines. Four hundred bases of the human uPAR promoter in a CAT vector was transiently transfected into H4, SW1783, and UWR3 cell lines (Fig. 3). Because of low CAT activity in the low-grade glioma cells and anaplastic astrocytoma cells, we used equivalent extracts containing 3-fold more β -gal activity to estimate reporter CAT activity in these cells compared with CAT activity in glioblastoma cells (UWR3). The uPAR promoter was the least active in low-grade gliomas (H4), with a 11% conversion of substrate. A 6–8-fold increase in uPAR promoter activity over that in low-grade gliomas was seen in anaplastic astrocytoma cells. uPAR promoter activity was 11–13-fold higher in UWR3 cells than in low-grade gliomas (H4). These results suggest that increased transcription of the uPAR gene accounts for the higher levels of uPAR mRNA in anaplastic astrocytoma and glioblastoma cell lines than in the low-grade glioma cell line.

The Role of AP-1 Consensus Elements in Up-Regulation of the uPAR Promoter in Glioblastomas. We studied a number of human uPAR promoter deletion constructs and pro-

motor constructs containing specific point mutations to identify *cis* elements that play a regulatory role in uPAR gene transcription in glioma cells and thereby further understand the mechanisms responsible for the promoter in glioblastoma cells. Transient transfection of the constructs shown in Fig. 4 was performed in UWR3 and H4 cell lines, and the relative CAT activity of the transfected clones was measured by a Betagen Betascope. Individual clones containing a deletion of the region between -98 and -78 or -144 and -123 or mutation of the NF- κ B site at -43 were tested in UWR3 cells. Removal of the region between -98 and -78 (72%) and mutation of the NF- κ B site (88%) did not alter the activity of the uPAR promoter (81% conversion) dramatically; however, deletion of the region between -144 and -123 reduced promoter activity 6–7-fold (13%) compared with wild-type promoter activity (Fig. 4A). Mutation of the AP-1 site at -69 did not alter the activity of the uPAR promoter in the H4 cell line (Fig. 4B). However, deletion of the region between -144 and -123 decreased promoter activity 4-fold when compared with the activity of the wild-type promoter. On the contrary, uPAR promoter activity was decreased by 3–4-fold and 2-fold compared with wild-type promoter activity in constructs containing mutations of the AP-1 sites at -184 and -69 , respectively, in the glioblastoma cell line UWR3 (Fig. 4A).

Up-Regulation of uPAR mRNA in Low-Grade Gliomas in Response to PMA. To determine the degree of up-regulation of uPAR mRNA in low-grade glioma cells, H4 cells were treated with PMA at 0, 2, 4, 8, and 24 h. Fig. 5A shows the level of uPAR mRNA in samples with and without PMA and in particular shows the increase in uPAR mRNA in PMA-treated samples compared with the levels in controls. Fig. 5B shows that there was an 8–10-fold increase in uPAR mRNA levels over basal levels at 8 h and a 13-fold increase at 24 h in response to PMA treatment after normalization of the signal to the internal GAPDH control (shown in the lower panel).

Response of uPAR Promoter Activity to PMA. Therefore, to determine which regions of the uPAR promoter confer PMA inducibility, we transfected promoter constructs containing mutations in the AP-1 site at -184 or in the NF- κ B binding site at -43 or the wild-type promoter into UWR3 cells. Duplicate sets of cells transfected with these constructs were treated with 200 nM PMA for 2 h. Fig. 6A shows that the wild-type promoter was up-regulated 5–6-fold and 3-fold in cells treated with PMA for 2 and 24 h, respectively. In addition, the construct containing a mutation in the NF- κ B binding site was active in these cells, demonstrating that the basal activity of the uPAR promoter in the glioma cell line H4 was not activated through the NF- κ B site (Fig. 6B). This construct was also induced in response to PMA treatment, indicating that the PMA-responsive element of the uPAR promoter lies in a region outside the NF- κ B binding site. However, the construct containing a point mutation in the AP-1 site at -184 had decreased promoter activity compared with the activity of the full-length promoter in H4 cells, and the addition of PMA to H4 cells transfected with this construct also did not increase the activity of uPAR promoter. These two findings indicate that this specific response element plays a major role in the response of the uPAR promoter to PMA in these cells.

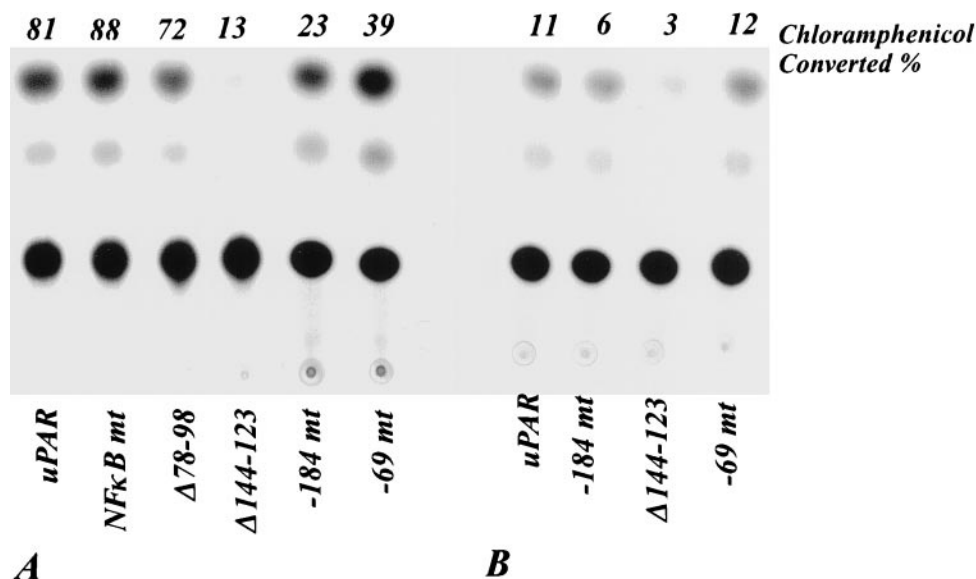


Fig. 4 Analysis of specific elements in the uPAR promoter that are active in high-grade UWR3 (A) and low-grade H4 (B) cells. A set of promoter constructs containing deletions (-98 to -78 and -144 to -123) or point mutations [NF- κ B mt; $-(1)84$, -69 , AP-1 mts] in the context of 400 bp of uPAR promoter construct were analyzed in UWR3 and H4 cells by a transient cotransfection experiment using 4 μ g of the uPAR promoter construct and 1 μ g of CMV- β -gal plasmid. All panels show the results of TLC separation of the reaction products from the CAT activity assay, as described in Fig. 3. A representative experiment from a similar study in H4 cells using a subset of the uPAR promoter constructs is shown. Equivalent extracts relevant to this experiment were used for comparison. The numbers above each lane are the percentage conversions of the substrate determined after quantitation of the signals by the Betagen. The data represent the results of three different experiments, the range of which did not exceed 10%.

Components of the AP-1 Transcriptional Complexes in Glioma Cell Lines. In view of the requirement for the AP-1 binding site at -184 to ensure optimal promoter activity in UWR3 cells and considering the importance of this motif in regulating the expression of several inducible genes (33, 34), we performed EMSA to identify the proteins that bind at this site. In this experiment, a 51-mer oligonucleotide spanning the uPAR promoter from -201 to -150 was synthesized (Genosys, The Woodlands, TX), radiolabeled, and incubated with nuclear extracts prepared from UWR3 cells in the presence and absence of a 100-fold excess of unlabeled wild-type competitor (wild-type oligonucleotide) or competitor with a mutated AP-1 motif in the wild-type sequence. A specific complex was present (Fig. 7A, arrow) after incubation of the radioactive oligonucleotide with the nuclear extract, but this band was absent in the oligonucleotide not incubated with the nuclear extract. In addition, the binding of the factors to the oligonucleotide was specific because a 100-fold excess of wild-type oligonucleotide, but not the oligonucleotide with the mutated AP-1 motif, competed for the binding proteins to the radiolabeled oligonucleotide (Fig. 7A). Furthermore, using specific antibodies to detect AP-1 proteins in UWR3 nuclear extracts that recognized the AP-1 motif at -184 of the uPAR promoter, we detected the fra-1, fra-2, c-Jun, and Jun D proteins but not the c-fos protein. Fig. 7B shows that the intensity of the specific complete band was much less than that in UWR3 cells when compared with low-grade glioma and the anaplastic astrocytoma cell line, which produce much less uPAR protein and mRNA compared which glioblastoma cell line (UWR3).

Repression of uPAR Promoter Activity by Coexpression of a Transactivation Domain Lacking c-Jun. We used a dominant-negative c-Jun expression vector (TAM-67) to determine the role of AP-1 binding in the transcriptional regulation of uPAR production in UWR3 cells. This construct encodes a mutant c-Jun that lacks the entire transactivation domain of this transcription cofactor and that inhibits the AP-1 process by inhibiting the function of endogenous Jun proteins. UWR3 cells were transiently transfected with a CAT reporter driven by 398 nucleotides of a 5' regulatory sequence and the TAM-67 expression vector. Promoter activity decreased from 60 to 12% with increasing amounts of TAM-67 (Fig. 8). In contrast, cotransfection of the uPAR construct with equivalent amounts of the empty vector did not reduce uPAR promoter activity.

Inhibition of uPAR Promoter Activity by a Dominant-negative ERK-1 Mutant. Because our data indicated that uPAR promoter activity in UWR3 cells was regulated in part by an AP-1-dependent mechanism, we undertook experiments to determine the role of the ERKs in uPAR promoter activity. To determine when this uPAR promoter activity was controlled by the constitutively activated ERK1, we used expression vectors encoding dominant-negative ERK1 and ERK2. uPAR promoter-driven CAT reporter activity was decreased in a dose-dependent manner by the cotransfection of the dominant-negative ERK1 expression vectors (Fig. 9). Specifically, chloramphenicol acetylation was reduced from 62 to 15% and 58 to 22% in the presence of 2 μ g of the ERK1 or ERK2 mutant expression vectors. By contrast, the uPAR promoter was not inhibited in UWR3 cells cotransfected with the empty expression vectors.

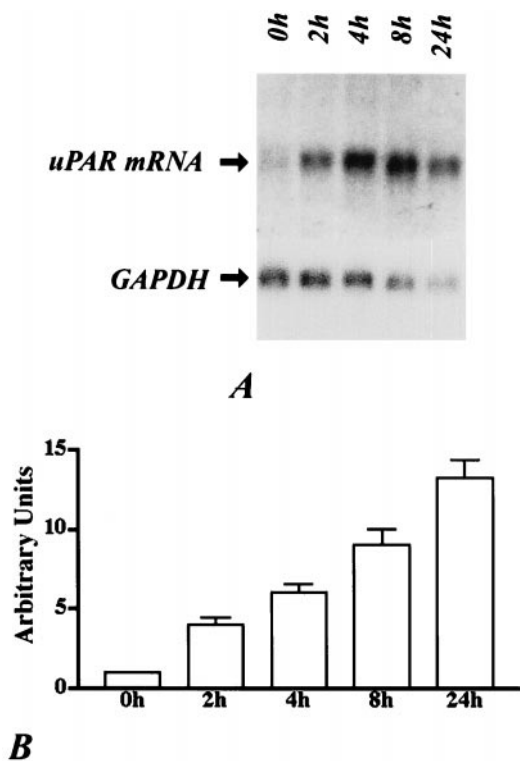


Fig. 5 Induction of uPAR mRNA in response to PMA in low-grade H4 cells. A, 20 μ g of total mRNA isolated from H4 cells treated for 0, 2, 4, 8, and 24 h with 200 nM PMA was used in a Northern blot analysis for uPAR mRNA expression (upper panel), as described in Fig. 2. The same blot was stripped and was hybridized to radiolabeled GAPDH cDNA (lower panel). B, quantitation of uPAR mRNA in PMA-treated samples at different times as described in Fig. 2, after correcting for loading equality, relative to the GAPDH signal using a scanning densitometer. Columns, the means for samples from five different experiments ($P < 0.001$); bars, SD.

Expression of Dominant-negative c-raf Blocks Constitutive uPAR Expression. Because the ERKs lie downstream from the c-raf serine-threonine kinase, we undertook experiments to determine whether uPAR promoter activity could be repressed by the coexpression of a dominant-negative c-raf. UWR3 cells were transiently cotransfected with a uPAR promoter-driven CAT reporter and increasing amounts of an expression vector encoding a kinase-inactive c-raf (raf C4). The encoded molecule contains the NH₂-terminal 257 amino acids of c-raf and thus lacks the kinase domain of the serine-threonine kinase. Cotransfection of the raf C4 mutant into UWR3 cells led to a dose-dependent reduction in the activity of the uPAR promoter-driven CAT (Fig. 10). By contrast, the expression vectors lacking the raf C4 coding sequence (vectors) failed to repress the activity of the uPAR promoter-driven CAT reporter.

DISCUSSION

In this study, we established that one of the mechanisms that leads to 15–20-fold higher levels of uPAR mRNA and protein in higher-grade gliomas is increased transcription. We also identified that an AP-1 site and the region containing

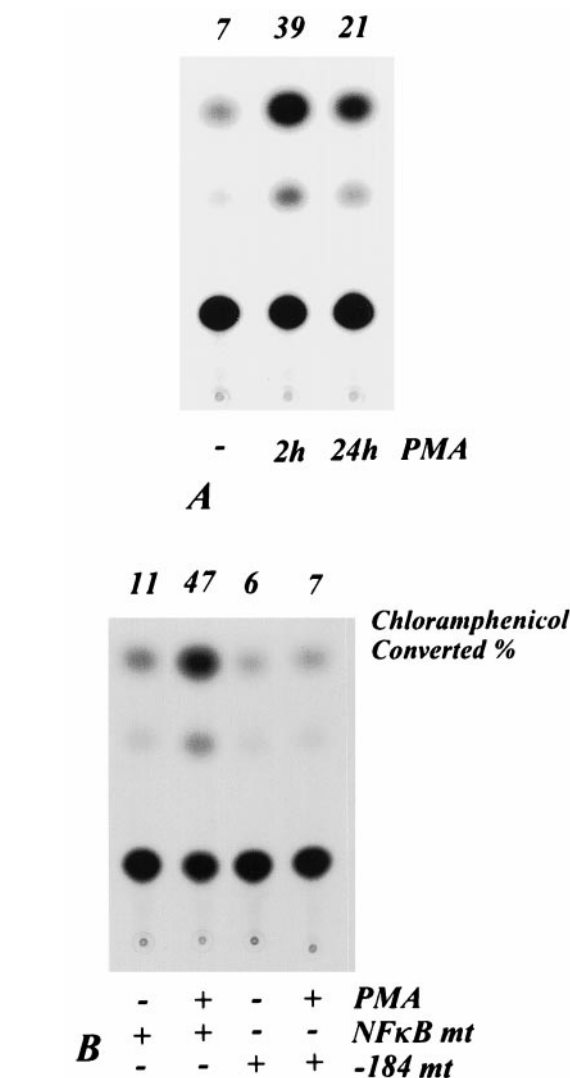
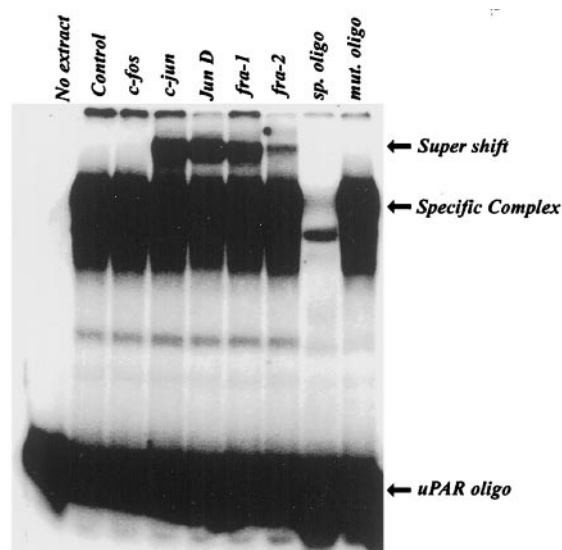
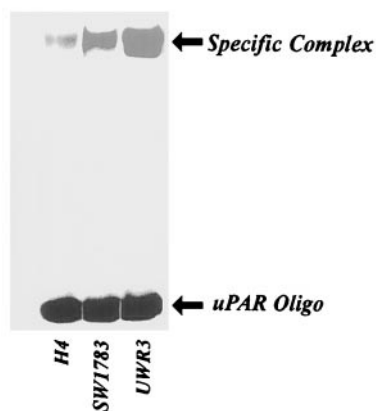


Fig. 6 Induction of uPAR promoter activity in response to 2- or 24-h treatment with PMA. A, H4 cells were placed in a low dilution of serum (1% versus 15%) for 24 h before transient transfection of 4 μ g of the 400-bp wild-type human uPAR CAT promoter plasmid and 1 μ g of CMV- β -gal plasmid. PMA was added at a concentration of 200 nM for 2 h (replaced by fresh medium containing 1% serum) or 24 h, after which the cells were harvested. Equivalent extracts were used in the CAT assay, and reactants were separated by TLC, as described in Fig. 3. The numbers at the top of the figure are percentage conversion values calculated after Betagen Betascope analysis of the TLC results. The data represent the results from four experiments, the range of which did not exceed 15%. B, the AP-1 element at -184 is required, at least in part, and the NF- κ B element is not required for PMA induction of the uPAR promoter in H4 cells. This panel shows a separate experiment in H4 cells, in which two of the constructs shown in Fig. 4 (NF- κ B mt or -184 AP-1 mt) were used in an experiment similar to the one shown in A, in which the cells were exposed to PMA for only 2 h. The data represent the results from five experiments, the range of which did not exceed 10%.

the Sp-1 and ets motif are important in the up-regulation of the uPAR promoter in human glioma cell lines. We also determined that the AP-1 site, and not the NF- κ B site, is important for the up-regulation of transcription of the uPAR



A



B

Fig. 7 Specific AP-1 proteins that recognize the AP-1 element at -184 of the uPAR promoter as determined by EMSA. **A**, specific AP-1 proteins that interact with the AP-1 element in UWR3 cells. Eight μg of UWR3 nuclear extract was incubated with a radiolabeled, double-stranded 51-bp oligomer spanning -151 to -201 of the human uPAR promoter in the presence of 100-fold excess cold mutant oligonucleotide or specific oligonucleotide or with antibodies against c-fos, c-Jun, Jun D, fra-1, and fra-2. The autoradiogram shown here is the polyacrylamide gel separation of the protein-bound radiolabeled oligonucleotide or the antibody-bound complex from free radiolabeled oligonucleotide. The first lane is a control for a radiolabeled oligonucleotide subjected to similar reaction conditions in the absence of nuclear extract. The data are representative of triplicate experiments. **B**, EMSAs were performed by incubating 4 μg of nuclear extracts from different glioma cell lines with radiolabeled, 51-bp double-stranded oligomers containing the AP-1 site at -184 of the uPAR promoter.

gene in response to PMA. We further studied the inhibition of uPAR promoter by coexpression of a transactivation domain lacking c-Jun; a dominant-negative ERK1 and ERK2 mutant and a dominant-negative c-raf in glioblastoma cell line

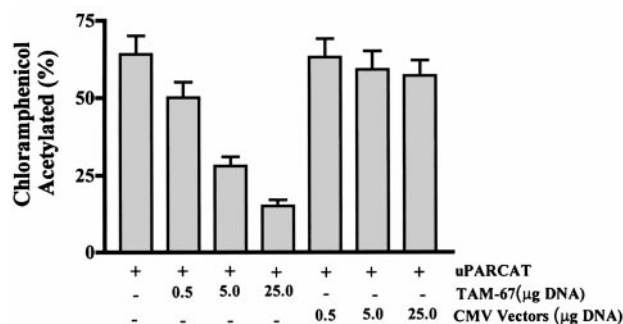


Fig. 8 Repression of uPAR promoter activity by coexpression of a transactivation domain lacking c-Jun (TAM-67). UWR3 cells were transiently transfected with 10 μg of CAT reporter driven by the wild-type uPAR promoter (uPAR CAT) and the indicated amounts of either an expression vector encoding a transactivation domain lacking c-Jun protein (TAM-67) or an empty vector (CMV Vectors). An equal amount of cell lysate protein was incubated for 6 h with [^{14}C]chloramphenicol and extracted with ethyl acetate, after which the acetylated products were resolved by TLC. Each value is the mean of the result from five different experiments; bars, SD.

showed the repressed uPAR promoter activity compared with the effect of empty expression vector.

Tumor cell invasiveness is a complex, multistep process that involves cell attachment, the proteolysis of matrix components, and the migration of cells through the disrupted matrix (36). In malignant tumors, most uPAR protein is concentrated at invasive foci (37); it accelerates plasmin formation at the cell surface and has been implicated in tissue remodeling in a number of physiological and pathological processes. However, the mechanism by which the expression of uPAR is regulated in human gliomas is poorly understood. We found a relatively similar half-life of uPAR mRNA of 8–10 h in anaplastic astrocytomas (SW1783) and glioblastomas (UWR3) but considerably higher levels of the uPAR promoter in UWR3 cells (11–13-fold) and SW 1783 cells (6–8-fold) when compared with the low-grade glioma cell line H4. However, because we were unable to determine the half-life of uPAR mRNA in low-grade glioma cell line H4, it is still possible that increased stability of uPAR mRNA in glioblastomas contributes to their more malignant behavior. The observation of uPAR mRNA half-life of 45 min to 10 h in different cell lines does suggest a role for increased stability of uPAR mRNA (10, 37). It has also been reported that agents such as PMA and cycloheximide can enhance uPAR mRNA stability in human mesothelial cells (38). In addition, the uPAR mRNA molecule contains regions rich in A+U sequences, motifs that are associated with relatively less stable mRNA (38). It is conjectured that PMA and cycloheximide achieve this by preventing the production of labile proteins that may be involved in the degradation of uPAR mRNA (29). Thus, the overall 11-fold increase in uPAR mRNA levels in response to PMA in low-grade gliomas seen in our study results from a combination of increased mRNA stability and increased transcriptional activation of the uPAR gene.

PMA is also known to increase the levels of c-fos and c-Jun mRNA and proteins. We and others (12) found that the AP-1 element at -184 is important in the regulation of uPAR pro-

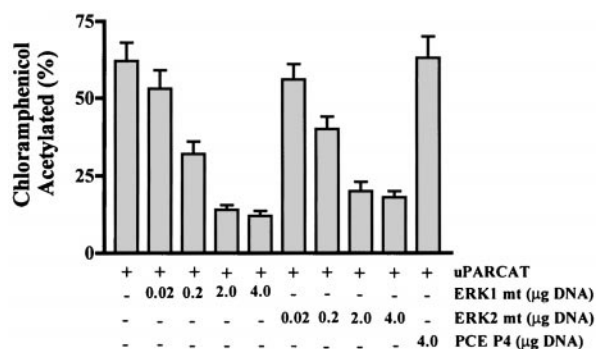


Fig. 9 Inhibition of uPAR promoter activity by dominant-negative ERK1 and ERK2 mutants. UWR3 cells were cotransfected with 1 μg of a CAT reporter driven by the wild-type uPAR promoter and increasing amounts of a dominant-negative ERK1 mt (ERK1 mutant) or ERK2 mt (ERK2 mutant) or an equal amount of the empty expression vector (PCE P4, pCEP4) equivalent to 4 μg of the ERK1 mutant. An equal amount of cell lysate protein was incubated for 6 h with [¹⁴C]chloramphenicol extracted with ethyl acetate, and the acetylated products were resolved by TLC. Each value is the mean of the result from five different experiments; bars, SD.

motor activity, and we also observed increased amounts of AP-1-specific protein complexes binding to this motif in nuclear extracts of cells treated with PMA. In addition, we have detected a phosphorylated form of c-Jun (at serine 63) in nuclear extracts of both unstimulated and PMA-stimulated cells. Furthermore, we observed the induction of c-fos protein into this AP-1 protein complex after a 1-h stimulation with PMA, which could result in higher transcriptional activation of the *uPAR* gene. Jun D homodimers (39); Jun D heterodimerization with fra-1, c-fos, and Jun D (40); c-fos and c-Jun heterodimers; and c-Jun homodimers (39, 40) have all been described as efficient activators of AP-1 *cis* element-mediated transcription. Similarly, it has been found in PC12 cells that the orphan receptor gene *nur77* is transcriptionally activated by Jun D through an AP-1-binding element that was bound only with this transcription factor (41). In our studies, we identified fra-1, fra-2, c-Jun, and Jun D proteins in complexes bound to the AP-1 site at position -184 of the uPAR promoter in nuclear extracts of both unstimulated and PMA-stimulated cells. Therefore, it is possible on the basis of these findings that transcription of the *uPAR* gene in glioblastomas is increased by the induction of specific member of the c-fos and c-Jun family of proteins, *i.e.*, c-fos.

We found that a CAT reporter driven by a 400-bp uPAR promoter was strongly activated in the UWR3 glioblastoma cell line compared with low-grade gliomas. The observation that UWR3 promoter activity is reduced substantially by the coexpression of a construct (TAM-67) which interferes with the ability of endogenous Fos and Jun proteins indeed suggests that AP-1 binding transcription factors are required for the expression of the uPA promoter in glioma cell lines. The synthesis of c-fos is regulated by both the JNK-dependent (42) and ERK-dependent (43) signaling modules via the phosphorylation of p62^{tef}^{E1k}. It has been shown that the serum response element in the fos promoter represents a point of convergence of the JNK- and ERK-dependent signaling modules (42). Increased c-fos modulation drives the forma-

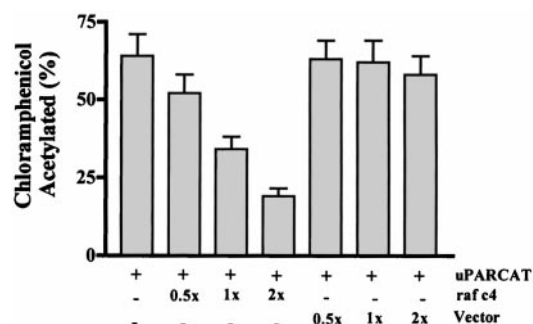


Fig. 10 Repression of uPAR promoter activity by the expression of a kinase-inactive c-raf. UWR3 cells were cotransfected with a CAT reporter-driven, full-length uPAR promoter in the presence or absence of an equimolar amount of the mutant c-raf expression vector (raf C4) or the empty expression vector. Reporter activity was determined by incubation of the cell extract, adjusted for variation in transfection efficiency. After this, the cell lysate was extracted with ethyl acetate, and the acetylated products were resolved by TLC. Each value is the mean of the result from five different experiments; bars, SD.

tion of Jun-Fos heterodimers, which are more stable than the pre-existing jun-jun homodimers. Such increased dimer stability results in higher levels of AP-1 DNA-binding activity. JNK/stress-activated protein kinases are involved in c-Jun induction through the phosphorylation of c-Jun and ATF2, which forms a heterodimer and increases their transcriptional activity, leading to enhancing c-Jun transcription and hence c-Jun synthesis (44, 45). The newly synthesized c-Jun may combine with newly synthesized c-fos or other proteins such as ATF2 or form homodimers, all of which can contribute to increased AP-1 activity. In addition to stimulating c-Jun synthesis, the JNK/stress-activated protein kinases contribute to elevating AP-1 activity by phosphorylating the activation domain of c-Jun, thereby enhancing its transcriptional activity. Our studies do not, however, rule out the possibility that the regulation of uPAR by ERK and c-raf signaling pathways requires the binding of transcription factors to the non-AP-1 motifs necessary for optimal promoter activation in glioblastoma cells. Nevertheless, the observations that uPAR promoter activity was reduced substantially either by coexpression of a construct (TAM-67) which interferes with the ability of endogenous FOS and Jun proteins to transactivate AP-1 controlled genes.

Induction of *uPAR* gene transcription in response to epidermal growth factor and transforming growth factor (46), hepatocyte growth factor (47), vascular endothelial growth factor (48), and phorbol esters (3) and an increase in mRNA stability in response to PMA and cycloheximide have been reported (38). More than 40% of glioblastomas have a rearrangement in the *EGFR* gene, which has been reported to produce a constitutively activated receptor (49). Amplification of the EGFR in glioblastomas has also been reported (50). Conversely, inhibition of EGFR in glioblastoma spheroids with genistein or tyrphostin, an EGFR tyrosine kinase-specific inhibitor, dramatically abolished invasion into fetal rat brain aggregates (51). On the basis of these findings, it is therefore possible that constitutive activation of the EGFR could be partly responsible for our observation of

a 20-fold increase in uPAR mRNA levels in glioblastomas over the levels in low-grade glioma. This study is the first to characterize the role of uPAR promoter activity in glioma cell lines. Because increased transcriptional activity of the endogenous promoter plays a major role in increases in uPAR mRNA and subsequently in the levels of uPAR protein in glioblastomas, the suppression of uPAR transcription could be used as a means of clinical intervention in this disease.

ACKNOWLEDGMENTS

We thank Lydia Soto for preparing the manuscript and Beth Notzon for manuscript review.

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Regulation of the Urokinase-type Plasminogen Activator Receptor Gene in Different Grades of Human Glioma Cell Lines

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Clin Cancer Res 2001;7:267-276.

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