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Promoter regions involved in density-dependent regulation of basic fibroblast growth factor gene expression in human astrocytic cells

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ABSTRACT **Expression of mitogenic basic fibroblast growth factor (bFGF) in the central nervous system is inhibited by direct cell contact and is implicated in reactive and neoplastic transformation of astrocytes. The molecular mechanisms controlling expression of bFGF were examined in cultures of human astrocytes. Cell-density-dependent depletion of bFGF mRNA levels parallels changes in bFGF protein. Regulation of transcription of a bFGF luciferase reporter gene containing an upstream region (bp −1800 to +314) of the bFGF gene promoter mimicks the density-dependent regulation of the endogenous bFGF gene in transfected astrocytes. Deletion analysis has identified a fragment (bp −650 to −513) and sequences further downstream (bp −274 to +314) as the regions required for the regulation of bFGF gene activity by cell density. Unlike in astrocytes, changing the cell density of glioma cell cultures does not affect the levels of bFGF protein and mRNA. bFGF luciferase constructs were expressed at the same level in high- or low-density cultures of glioma cells, indicating altered regulation of the bFGF gene promoter. Electrophoretic mobility shift assays showed binding of nuclear proteins to a fragment of bFGF gene promoter from bp −650 to −453. This binding was abolished by a deletion of the upstream cell-density-responsive region (bp −650 to −512). Binding was observed with nuclear extracts from subconfluent astrocytes but was reduced in extracts from confluent astrocytes. Our results indicate that induction of bFGF in astrocytes upon reduction of cell density is mediated transcriptionally by positive trans-acting factors interacting with bFGF promoter. In contrast, nuclear proteins from glioma cells bind to the promoter region from bp −650 to −453 independent of cell density. Thus, the constitutive binding of trans-acting factor(s) to the region of the bFGF promoter from bp −650 to −453 may be responsible for the continuous expression of bFGF that leads to the uncontrolled growth of glioma cells.**

Basic fibroblast growth factor (bFGF) is a pleiotropic protein that can stimulate growth, proliferation, motility, and differentiation of cells of astrocytic and neuronal lineages (1). In the normal mature brain, only a few astrocytes express low levels of bFGF (2–4). FGF content increases during the reversible transition of astrocytes from a quiescent to a reactive proliferating state in response to injury or neuronal degeneration. bFGF levels remain elevated until the space left by degenerated tissue is filled and astrocytes become quiescent (5, 6). bFGF has also been implicated in the abnormal growth of tumors, including human gliomas (7, 8). Expression of bFGF in glioma cells correlates with the grade of the tumor and the extent of anaplasia (9, 10). Glioma cells, similar to astrocytes, bear FGF receptors and are stimulated to proliferate by bFGF (11). In addition to promoting proliferation, bFGF may increase the resistance of neoplastic cells to radiation-induced apoptosis and promote neovascularization of tumorigenic tissue (12).

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With the many fundamental cellular events regulated by bFGF, its expression must be under stringent control. In contrast to numerous studies describing the pleiotropic effects of bFGF, little is known about the mechanisms that regulate bFGF synthesis, its restricted cellular expression, and how it exerts biological functions. In human glioma cells, bFGF is regulated by serum and activators of protein kinase C (13, 14). Also, cell density was shown to affect bFGF protein and mRNA content in the glioma cells (14). The mechanisms that control expression of bFGF in nontransformed human astrocytes have not been examined. To identify the mechanisms of bFGF induction in reactive astrocytes and in glioma cells, we developed cultures of human astrocytes and utilized glioma cell lines (15). Astrocytes maintained in high-density cultures formed a confluent monolayer of nonproliferating cells similar to stellate astrocytes in the brain tissue. After cell density is reduced, events parallel the *in vivo* transition from quiescent to reactive astrocytes. Cell hypertrophy and proliferation last until a new confluent state was achieved. Our studies indicate that inhibition of bFGF expression in confluent astrocytes is evoked by direct cell–cell interaction. Accordingly, release from cell contact inhibition due to increased intercellular space may underlie the induction of bFGF in reactive astrocytes in the brain. Our results indicate that transient induction of bFGF promotes reversible mitotic activation of quiescent astrocytes, whereas density-independent expression of the bFGF gene may contribute to uncontrolled growth of glioma cells.

In this study we show that cell-density-dependent regulation of bFGF content in astrocytes occurs at the transcriptional level and is mediated by the bFGF gene promoter. In contrast, in tumorigenic glioma cells, the expression of bFGF protein and mRNA shows little response to changes in cell density. The loss of cell contact inhibition of bFGF gene expression is due, at least in part, to altered trans-regulation of bFGF promoter in glioma cells. The results of DNA–protein binding analysis are consistent with positive trans-acting factor(s) binding to the bFGF gene promoter and stimulating transcription after the transition from the confluent to the subconfluent state. In glioma cells, these factors bind to the bFGF promoter independent of cell density and result in constitutive expression of the bFGF gene.

MATERIALS AND METHODS

Materials. Culture media were purchased from Life Technologies (GIBCO/BRL), bFGF was from Boehringer Mannheim, bFGF monoclonal antibody was from Upstate Biotechnology (catalogue no. 05-118, Lake Placid, NY), the reagent for protein measurement was from Bio-Rad, luciferase re-

Abbreviations: bFGF, basic fibroblastic growth factor; STAT, signal transducers and activators of transcription; RT–PCR, reverse transcriptase-coupled PCR; RSV, Rous sarcoma virus.

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porter vector pGL_{2basic} was from Promega, [^{32}P]dNTPs were from DuPont/NEN, and poly(dIdC) was from Pharmacia/LKB Biotechnology. Reverse transcription of RNA was performed with a StrataScript kit (Stratagene). A GeneAmp kit (Perkin–Elmer/Cetus) was used for PCR. All other chemicals were from Sigma.

Astrocytic and Glioma Cell Cultures. Normal astrocyte cultures were obtained from dissociation of brain tissue taken from trauma patients (16). QG cultures were from the frontal lobe of a 7-year-old male. Results obtained with cultures from different individuals were essentially the same. Cells were maintained in Waymouth 87/3 medium supplemented with 20% (vol/vol) fetal bovine serum. Experiments were done with cultures at passages 12–20. They were identified as pure astrocytic cultures by expression of glial fibrillary acidic protein and by the lack of expression of galactocerebroside, an oligodendrocytic marker (data not shown). Established glioma cell line U251MG (17) expresses glial fibrillary acidic protein.

Immunohistochemical Staining. Cultured cells were permeabilized with 1.0% Triton X-100, fixed, and incubated with primary bFGF monoclonal antibody (13). Immune complexes were detected with biotinylated secondary antibody, followed by avidin-linked peroxidase and diaminobenzidine/hydrogen peroxide solution (18, 19). The specificity of bFGF immunostaining has been demonstrated (18, 19).

Quantitative Reverse Transcriptase-Coupled PCR (RT–PCR). Cells were trypsinized and counted with hemocytometer after which RNA was extracted (20). Five micrograms of total RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase using random hexamers as primers. PCR was performed by using a Perkin–Elmer/Cetus GeneAmp kit, 1.5 mM $MgCl_2$, 2.5 units of *Taq* polymerase, and 5 μl of the RT reaction products. The sequences of the 25-nt bFGF primers were taken from refs. 8 and 11. The length of amplification products for human bFGF mRNA was consistent with the predicted length of 179 nt. A pair of primers complementary to the mRNA of ubiquitously expressed human histone gene H3.3 (21) was used as a control. PCR was performed as follows: denaturation for 1 min at 94°C, annealing for 1 min at 60°C, elongation for 2 min at 72°C, for the indicated number of cycles. The products were electrophoresed on 2% agarose gels, stained with ethidium bromide, and photographed. The relationship between the concentration of input RNA and the amount of PCR product was linear (see Fig. 2). The identity of RT–PCR-generated bFGF and H3.3 DNA products was further confirmed by hybridization to ^{32}P-labeled human bFGF and histone cDNAs (data not shown) using standard methods (22).

Construction of bFGFLuc Reporter Plasmids and Transfection Assays. Plasmid (−1800/+314)bFGFLuc and its deletion mutants were constructed in our laboratory (18) by using a fragment of the human bFGF gene from bp −1800 to +314 (23) and a promoterless pGl_{2basic}. RSVLuc expresses constitutively high levels of luciferase from the Rous sarcoma virus (RSV) promoter (24). Plasmid bFGFLuc, RSVLuc, or pGl_{2basic} was transfected into human astrocytes or U251MG glioma cells by electroporation (18). Transfected cells were plated into 12-well dishes. Two days later, cells were lysed and an aliquot of extract (10–50 μg of protein) was used to determine luciferase activity. Efficiency of transfection of individual plasmids and culture dishes was normalized by measuring the content of transfected plasmid DNA by dot-blot hybridization in lysates used for luciferase assay (18, 19). Luciferase activity is expressed relative to the content of intracellular plasmid DNA and cellular proteins (25).

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay. Fragments of bFGF gene promoter (bp −650 to −453 and bp −512 to −453) were end-labeled with [α-^{32}P]dNTPs by using the Klenow fragment of DNA polymerase I. Nuclear extracts were prepared as described (26) except that buffer A contained 0.75 mM spermidine and 0.15 mM spermine. The electrophoretic mobility shift assay was performed as described by Sawadogo *et al.* (27). Briefly, nuclear extracts (1–5 μg of protein) were preincubated for 10 min at room temperature in 20 μl of 5 mM Hepes, pH 7.8/50 mM KCl/1 mM EDTA/5 mM $MgCl_2$/10% (vol/vol) glycerol/2 mM dithiothreitol/2 mM phenylmethylsulfonyl fluoride/2 μg of bovine serum albumin/2 μg of poly(dIdC). Labeled DNA probe (5 fmol, 2000–5000 cpm) was then added, and the reaction was allowed to progress for an additional 20 min at room temperature. Products from the binding reactions were resolved in 5% nondenaturing gels in electrode buffer (pH 7.8) containing 10 mM Tris base, 0.275 mM EDTA, and 9.25 mM sodium acetate/acetic acid.

RESULTS

Cell-Density-Dependent Regulation of bFGF Immunoreactivity in Human Astrocytes. In gliotic brain tissue, bFGF protein levels are increased and bFGF accumulates in the nuclei of reactive astrocytes accompanying neuronal degeneration (15). A similar induction of bFGF is observed in the cultured adult human astrocytes when cell density is reduced. Fig. 1 compares intracellular bFGF immunoreactivity (bFGF-IR) in sub- and confluent astrocytic cultures. In astrocytes growing at low cell density, bFGF-IR is found in the cell with intense staining in the nuclei and nucleoli. In cells maintained at the confluent state, little or no bFGF staining is observed. In contrast, in glioma cells U251MG, bFGF-IR is independent of cell density. Confluent glioma cells express bFGF in both the cytoplasm and nucleus at similar levels as subconfluent cells. These results were confirmed by Western blot analysis of bFGF content in the cytoplasmic and nuclear fractions (M.K.S., R.F., and J.M., unpublished data).

bFGF mRNA Levels Are Regulated by Cell Density in Astrocytes but Not in Glioma U251MG Cells. The differences in the expression and regulation of bFGF protein in astrocytes and glioma cells are reflected in the steady-state levels of bFGF mRNA estimated by RT–PCR. Total RNA was isolated from cultures of astrocytes or glioma cells plated at different cell densities. To quantitate the levels of bFGF mRNA, we determined the amount of input RNA in the reverse transcription reaction that produced a linear increase in bFGF PCR products (Fig. 2*A*). We also determined the cycle number that was in the linear range of the PCR (Fig. 2*B*). The mRNA levels of control histone H3.3 gene are not affected by density of cells in the culture (Fig. 3). High-density astrocytic cultures show >95% reduction of bFGF mRNA levels compared to the low-density cultures (Fig. 3*A*). In contrast to astrocytes,

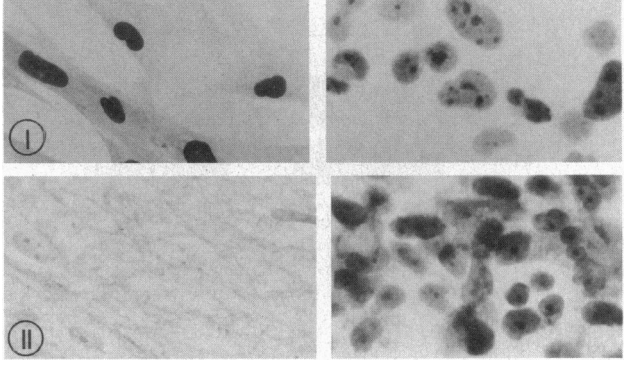

FIG. 1. Expression and localization of bFGF in human astrocytes and glioma cells as a function of cell density. Normal human astrocytes (*Left*) or glioma cells (*Right*) were cultured at low (*Upper*) or high (*Lower*) densities, and the expression of bFGF was followed by using anti-bFGF antibody.

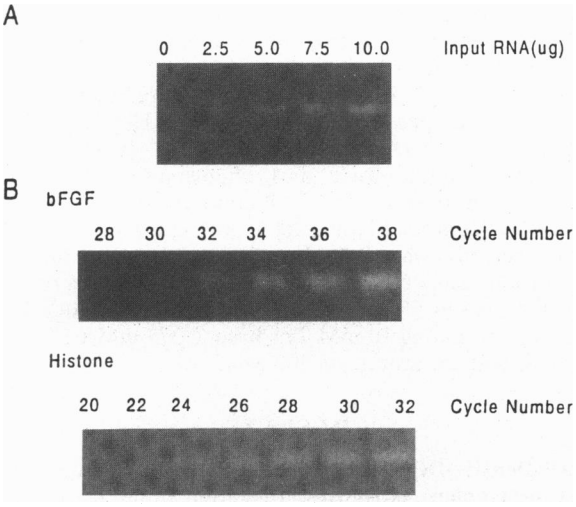

FIG. 2. Dependence of bFGF and histone H3.3 PCR products on the amount of input RNA and cycle number. (*A*) Increasing amounts of total RNA were added to the RT reaction. Five microliters of the RT product was used for PCR and the reaction was carried out for 36 cycles. The PCR products were resolved on 2% agarose gels, stained with ethidium bromide, and photographed. (*B*) Five micrograms of total RNA from normal human astrocytes was reverse-transcribed. PCR used primers for bFGF or histone H3.3 mRNAs. Aliquots from the PCR mixture were removed and the PCR was terminated at the indicated cycle number. The sample was electrophoresed through 2% agarose gels, stained with ethidium bromide, and photographed.

U251MG bFGF mRNA levels show no reduction with increasing cell density (Fig. 3*B*). The reduction in bFGF protein levels in high-density cultures of astrocytes found earlier (27) is, therefore, paralleled by changes in the levels of bFGF mRNA. The lack of regulation of bFGF protein levels in glioma cells is also reflected in the steady-state levels of bFGF mRNA.

The Effect of Cell Density on Expression of a bFGF Luciferase Reporter Plasmid. The bFGF core promoter maps to the region from bp −1800 to +314 relative to the transcriptional start site of the bFGF gene (18). The upstream region of the human bFGF gene from bp −1800 to +314 contains the necessary cis elements to mimic regulation of the endogenous bFGF gene by neurotransmitter, hormone, and growth factor receptors and by second messenger pathways (18, 28). To determine whether the density of cells in culture affects transcription of the bFGF gene, cells were transfected with chimeric bFGF promoter–luciferase constructs or control plasmids, RSVLuc, or promoterless pGL_{2basic} and plated at different densities. As cell density increased in astrocyte cultures, expression of the (−1800/+314)bFGFLuc transgene decreased (Fig. 4). Luciferase activity in 70% confluent astrocytes was inhibited by 91%. In confluent cells, the inhibition was >95%. Analysis of variance (ANOVA) followed by Neuman–Keuls post hoc test show a statistically significant effect of cell density on (−1800/+314)bFGFLuc expression ($P < 0.0005$). Expression of luciferase from the RSV promoter was also reduced. However, changes in the RSV promoter activity are significantly smaller than the changes in bFGF promoter activity ($P < 0.005$, ANOVA). In confluent astrocytic cultures, the mean inhibition of (−1800/+314)bFGFLuc was 23.8-fold ($n = 10$) and is significantly greater than the 1.8-fold ($n = 4$) inhibition of the RSVLuc ($P < 0.01$).

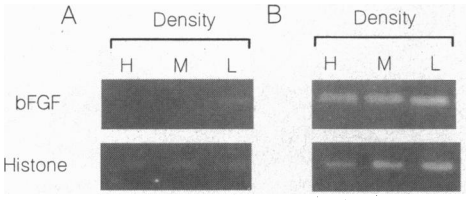

FIG. 3. Density-dependent regulation of bFGF mRNA levels in human astrocytes and deregulation in U251MG glioma cells. RT–PCR was performed by using primers for bFGF and for control histone H3.3 mRNA. Total RNA was isolated from cells cultured at three densities: (*A*) Human astrocytes at high (lane H; 3.5×10^4 cells per cm^2), medium (lane M; 8.8×10^3 cells per cm^2), or low (lane L; 1.2×10^3 cells per cm^2) density. (*B*) Glioma U251MG cells at high (lane H; 1.6×10^5 cells per cm^2), medium (lane M; 5.0×10^4 cells per cm^2), or low (lane L; 1.6×10^4 cells per cm^2) density. Under these conditions, the high-, medium-, and low-density cultures were 100%, 70%, and 35% confluent, respectively. For bFGF, PCR was stopped at cycle 34. For histone, the reaction was stopped at cycle 26.

In U251MG cells transfected with (−1800/+314)bFGFLuc or RSVLuc, increased cell density reduces luciferase expression only minimally (Fig. 4 and Table 1). However, this reduction does not reach a statistically significant level and there is no significant difference between (−1800/+314)bFGFLuc and RSVLuc, indicating a lack of bFGF promoter-specific inhibition. The effects of cell density on expression of (−1800/+314)bFGFLuc in astrocytes are significantly different than in glioma cells ($P < 0.000001$, ANOVA).

These experiments show that density-dependent inhibition of bFGF gene expression in astrocytes is mediated at least partially by regulation at the transcriptional level, and this regulation is lost in U251MG glioma cells.

Identification of Promoter Regions Mediating Cell-Density-Dependent Regulation of bFGF Gene Expression. We produced a progressive series of deletions of the bFGF promoter ligated to a luciferase reporter gene to map the sequences responsible for density-dependent regulation of the bFGF gene. Astrocytes transfected with (−650/+314)bFGFLuc exhibited basal promoter activity and inhibition in confluent astrocytes similar to the parental −1800/+314bFGFLuc plasmid (Fig. 5). The deletion to a promoter fragment from bp −512 to +314 dramatically decreased promoter activity ($P < 0.0005$ in subconfluent astrocytes), while further deletion of bp −512 to −274 had no additional effect. Although plasmids (−512/+314)bFGFLuc and (−274/+314)bFGFLuc show significantly reduced inhibition in confluent cells, cell-density inhibition was still 7- to 10-fold in astrocytic cultures transfected with either plasmid (Fig. 5). The inhibition of (−512/+314)bFGFLuc was significantly stronger than that of RSVLuc. Consequently, the promoter region between bp −650 and −512 and the sequences downstream from bp −274 may be

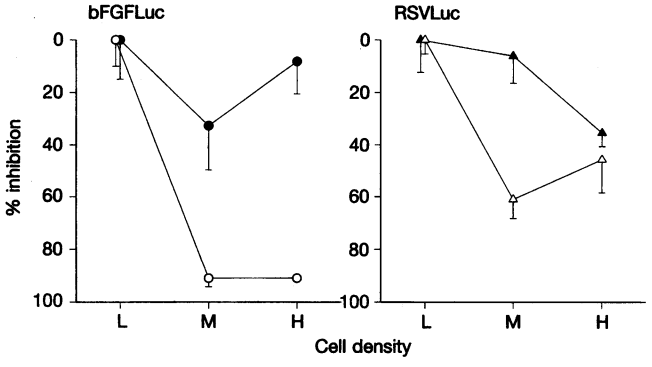

FIG. 4. Cell-density-dependent expression of the (−1800/+314)bFGFLuc reporter gene in cultures of human astrocytes and glioma cells. Approximately 1×10^7 astrocytes (QG strain) or U251MG glioma cells were electroporated with 150 μg of (−1800/+314)bFGFLuc or RSVLuc. Cells were seeded into 12-well dishes at the indicated densities yielding 100%, 70%, and 35% confluent cultures (see Fig. 3). Cells transfected with bFGFLuc expressed luciferase activity above background. Each point is the mean ± SEM of 4–10 samples. The results of statistical analysis are discussed in text. Astrocytes: ○, (−1800/+314)bFGFLuc; △, RSVLuc. U251MG cells: ●, (−1800/+314)bFGFLuc; ▲, RSVLuc.

Table 1. Glioma cells can induce cell-density-dependent inhibition of bFGFLuc(−1800/+314) expression in astrocytes

Transfected cells	Cells (density)	Luciferase activity (fold inhibition)* bFGFLuc†	RSVLuc
Astrocytes	Astrocytes (low)	1.0 ± 0.13 (—)	1.0 ± 0.15 (—)
	Astrocytes (high)	0.04 ± 0.01 (25)	0.5 ± 0.14 (2)
	Astrocytes + glioma (high)‡	0.04 ± 0.01 (25)	0.7 ± 0.04 (1.4)
U251MG glioma	Glioma (low)	1.0 ± 0.10 (—)	17.0 ± 0.20 (—)
	Glioma (high)	0.74 ± 0.2 (1.4)	15.0 ± 0.13 (1.1)
	Glioma + astrocytes (high)‡	0.40 ± 0.1 (2.5)	6.0 ± 2.2 (2.8)

Astrocytes or U251MG glioma cells were electroporated with (−1800/+314)bFGFLuc. After electroporation, cells were seeded into 12-well tissue culture plates. After 48 hr of incubation, cells were lysed and luciferase activity (Light units per microgram of protein and picogram of DNA) was determined. Low-density (35% confluency) and high-density (100% confluency) cell counts are given in Fig. 3.

*Fold inhibition of promoter activity was calculated by dividing the luciferase activity found at low cell density by the activity found at high cell density.

†For bFGFLuc, the bFGF luciferase construct used contained the promoter sequences from bp −1800 to +314.

‡High density conditions for QG-4 and U251MG maintained in mixed cultures in which the two morphologically distinct cell types were evenly distributed as determined by microscopic examination.

responsible for inhibition of bFGF gene expression in high-density astrocytic cultures.

The Effect of Cell Density on Binding of Nuclear Proteins to the Region of the bFGF Promoter from bp −650 to −512. To identify mechanisms by which the region from bp −650 to −512 confers cell-density-dependent changes in promoter activity in astrocytes, we examined binding of nuclear proteins to the bFGF promoter by using electrophoretic mobility shift assays. When the ^{32}P-labeled bFGF promoter fragment from bp −650 to −453 is incubated with nuclear proteins from low-density astrocytic cultures and resolved on a 5% polyacrylamide gel, two shifted bands form (Fig. 6*A*). Competition with an excess of unlabeled target DNA prevent formation of these complexes (Fig. 6*B*). A fragment mapping from bp −512 to −453 gave no protein binding, indicating the dependence of complex formation on promoter sequences between bp −650 and −512 (Fig. 6*C*). Extracts made from high-density astrocytic cultures show no protein binding to the target DNA from bp −650 to −453 (Fig. 6*A*). Thus, changes in promoter activity correlated with the *in vitro* binding of nuclear proteins to the involved promoter region. Nuclear proteins extracted from U251MG glioma cells and extracts from subconfluent astrocytes produce similar complexes. Unlike in astrocytes, formation of these complexes was independent of cell density (Fig. 7). This finding suggests that induction of the bFGF gene in astrocytes during the transition from the confluent to the subconfluent state is brought about in part by positive trans-acting factors interacting with the promoter region from bp −650 to −512. In glioma cells the lack of regulation of bFGF gene expression may reflect binding of positive trans-acting factors independent of cell density.

Glioma U251MG Can Induce Density-Dependent Inhibition of bFGFLuc in Astrocytes in Mixed Glioma/Astrocyte Cultures. Next we determined whether the lack of cell-density-dependent regulation of bFGF promoter activity in U251MG cells could reflect the inability of glioma cells to generate a cell-contact-dependent inhibitory signal(s) or to process these signals internally. We first examined whether glioma cells could induce contact-dependent inhibition of the bFGF promoter in transfected astrocytes. Astrocytes transfected with the (−1800/+314)bFGFLuc reporter gene were cultured at low or high cell density. In some astrocytic cultures plated at low density, a high cell density condition was produced by coculturing with U251MG cells. Glioma cells produced a 25-fold inhibition of luciferase expression in astrocytes much as was observed in high-density cultures that contain only astrocytes (Table 1).

Glioma cells transfected with (−1800/+314)bFGFLuc followed by coculture with astrocytes show a small (2.5-fold) inhibition in luciferase expression compared to low-density

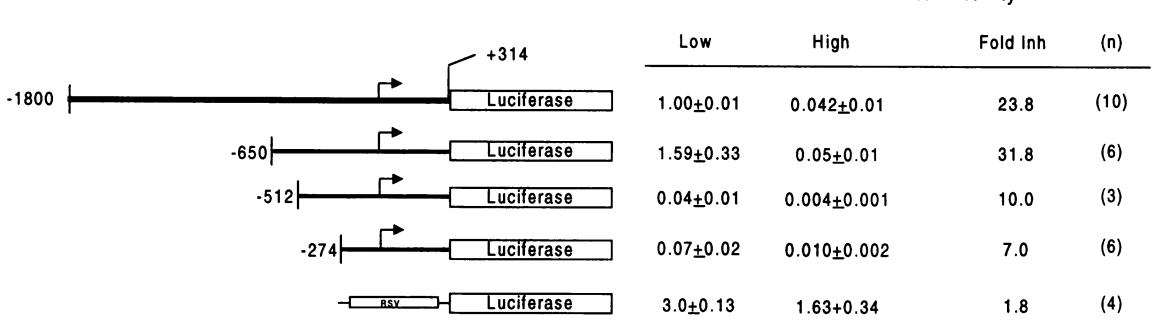

FIG. 5. Deletion analysis of bFGF promoter in human astrocytes. Astrocytes (QG) were transfected. The numbers given for DNA constructs indicate the included region of the bFGF gene. Luciferase activity was measured and normalized to the amount of transfected plasmid DNA. Luciferase activity is shown relative to the levels of (−1800/+314)bFGFLuc activity under low-density condition. The terms low and high refer to the density of the cultures at the time of harvest for luciferase assays. Fold Inh, the fold inhibition, was calculated by dividing the luciferase activity at low density by the value obtained at high density for each individual plasmid. The overall effects of cell density, promoter deletions, and the interaction between these two variables were statistically significant ($P < 0.00001$; 2-way ANOVA). Effects of individual deletions on basal promoter activity were assessed by Neuman–Kuels post hoc test (see text). To compare the effects of cell density between individual plasmids, the results for each plasmid were normalized to luciferase activity in high-density culture. The inhibition of (−512/+314)bFGFLuc or (−274/+314)bFGFLuc in high-density cultures was reduced compared to (−1800/+314)bFGFLuc ($P = 0.056$ and $P < 0.05$, respectively; 1 way ANOVA). The inhibition of (−1800/+314)bFGFLuc, (−650/+314)bFGFLuc, or (−520/+314)bFGFLuc was significantly greater than that of the RSVLuc ($P < 0.01$, $P < 0.05$, and $P < 0.001$, respectively).

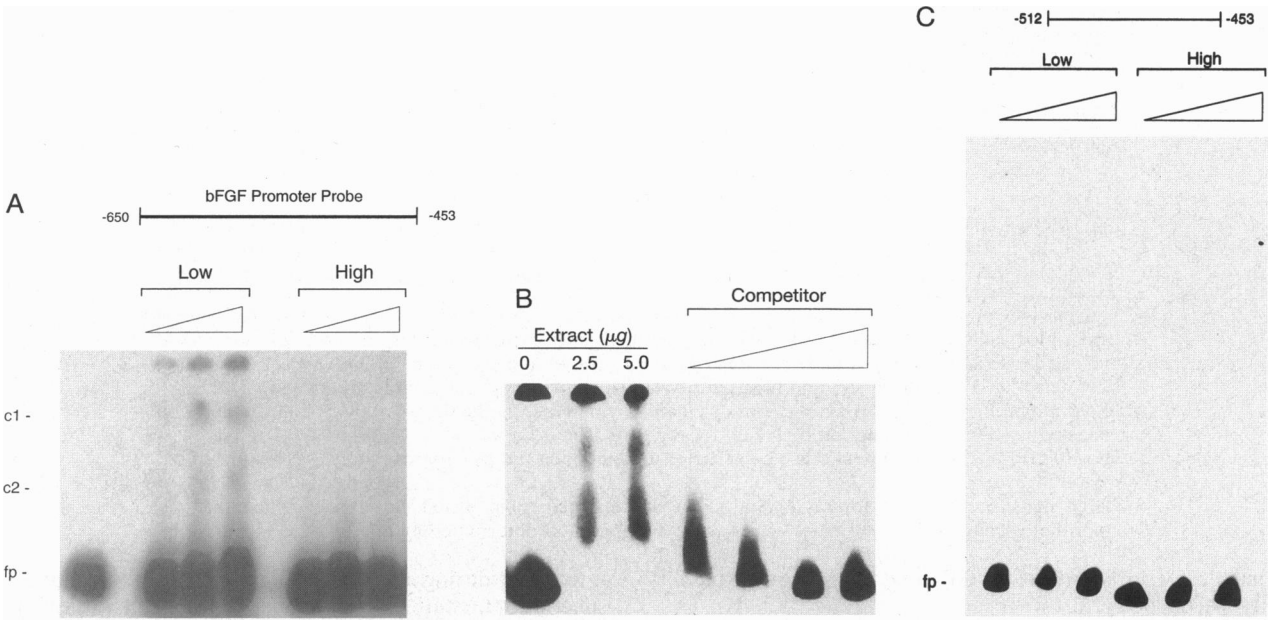

FIG. 6. Nuclear extracts from astrocytes bind to the region of the bFGF promoter from bp −650 to −453 in a cell-density-dependent manner. (*A*) Extracts from astrocytes (1, 2.5, and 5.0 μg) from low- and high-density cultures were incubated with ^{32}P-labeled fragment for 10 min. The two protein–DNA complexes formed are labeled c1 and c2 (fp, free probe). (*B Left*) Increasing amounts of extract from low-density astrocytes were added to ^{32}P-labeled probe (bp −650 to −453). (*B Right*) Five micrograms of nuclear extract was incubated with 25, 50, 100, or 250 ng of unlabeled competitor promoter fragment from bp −650 to −453. (*C*) Labeled probe (bp −512 to −453) was incubated with 1, 2.5, and 5.0 μg of protein from nuclear extracts isolated from low- and high-density cultures.

glioma cultures. However, the same inhibition is also found with the RSV promoter enhancer. These experiments suggest that U251MG glioma cells have lost the signaling mechanism(s) that mediate cell-density-dependent regulation of bFGF promoter activity.

DISCUSSION

This study confirms earlier observations that expression of bFGF in human astrocytes is inhibited by cell contact and that tumorigenic glioma cells that are no longer susceptible to contact inhibition of growth express bFGF constitutively (28). The changes in bFGF expression are paralleled by changes in bFGF mRNA levels. Cell-density-dependent regulation is mediated transcriptionally and involves regulatory sequences in the bFGF promoter.

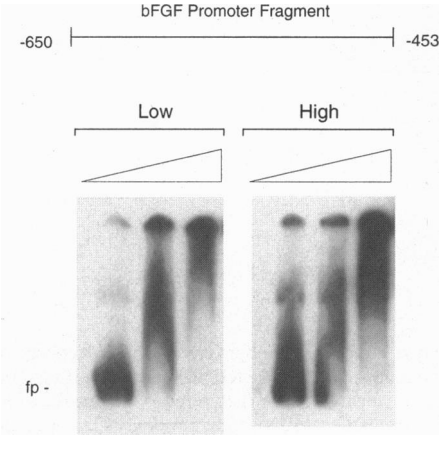

FIG. 7. Nuclear extracts from glioma U251MG bind to the bFGF gene promoter in a density-independent manner. Labeled promoter fragment (bp −650 to −453) was incubated with 1, 2.5, or 5.0 μg of nuclear proteins extracted from U251MG cells grown at low or high density.

The high level of bFGF gene promoter activity in subconfluent astrocytes is supported by the promoter region located between bp −650 and −512. Downstream sequences (bp −511 to +314), including the core promoter described earlier (23), can maintain only low basal promoter activity. The same upstream region (bp −650 to −512) is involved in the cell-density-dependent regulation of bFGF promoter activity in astrocytes. Its deletion reduces promoter inhibition in confluent astrocytes 3- to 4-fold. Since it does not completely abolish density-dependent inhibition (see Fig. 5), downstream promoter sequences in addition to bp −650 to −512 may also be involved in cell-density-dependent regulation. Recently, Ueba *et al.* (29) reported both negative and positive regulation of bFGF promoter activity by the wild-type and mutant p53 (respectively) through a short fragment of the bFGF gene promoter (bp −20 to +50). Whether p53 is involved in the cell-contact inactivation of bFGF gene in astrocytes and whether the mutation of p53 found in U251MG disrupts bFGF inactivation remain to be determined.

The promoter region from bp −650 to −513 also mediates activation of the bFGF promoter by bFGF and other growth factors and by neurotransmitters, hormones, cAMP, and protein kinase C (18, 28). Thus, the upstream (bp −650 to −513) promoter region serves as a center for converging regulatory effects of soluble extracellular agents and their signaling pathways, as well as for the contact-dependent inhibition.

The promoter region that mediates cell-density-dependent regulation contains a number of putative regulatory sequences. One site is a dyad symmetry element (DSE) mapping at bp −597 to −564. A similar site has been implicated in negative regulation of c-*myc* promoter activity (30). DSEs are known to bind dimeric transcriptional factors and to extrude cruciform-like structures by using energy from the negative DNA supercoiling (31). The capacity of the DSE in the bFGF gene promoter to extrude a non-B-DNA structure has been confirmed in our laboratory (E. Kim and M.K.S., unpublished observations). In addition, there are several A/T-rich regions containing sequences similar to STAT (signal transducers and activators of transcription) protein binding sites [AA(N)$_n$TT] (32–34). In known STAT sites, the core spacing is 4–6 bp (35).

The bFGF promoter fragment from bp −650 to −512 has several STAT-like sequences with 8-bp spacing as well as a c-*fos*-like STAT site with a 5-bp spacing (36). Perhaps novel STAT proteins are involved in the regulation of bFGF promoter activity by cell-contact interaction.

Induction of the bFGF gene during transition from the confluent to subconfluent state could be mediated by positive transcriptional factor(s) or by inactivating a repressor. That the promoter deletion from bp −650 to −512 reduced luciferase activity in subconfluent astrocytes (Table 1) is consistent with regulation by positive transcriptional factors. That the nuclear extracts from subconfluent astrocytes contain proteins capable of binding to the bFGF promoter and that this binding is reduced in extracts from confluent astrocytes also support this hypothesis. We have identified two protein–DNA complexes within the promoter region from bp −650 to −453. Their binding requires the presence of cell-density-responsive sequences (bp −650 to −512). Since their binding is inhibited in confluent astrocytes, these complexes may represent proteins that activate the bFGF promoter in astrocytes transitioning from confluent to subconfluent state. The levels of proteins forming these complexes may be reduced in the nuclei of confluent astrocytes or may be unable to interact with the bFGF gene promoter.

The present study shows a near complete loss of density-dependent regulation of bFGF gene expression in U251MG cells. Murphy *et al.* (14) showed that in glioma U87MG cells bFGF mRNA was decreased by 70% in high-density cultures. Thus, the degree to which cell-density-dependent control of bFGF expression is impaired may differ in glioma cells. In U251MG cells, the expression of the bFGFLuc constructs, similar to the endogenous bFGF gene, changes little when glioma cells are maintained at different densities. The inhibition is similar to that observed with the control RSVLuc construct, indicating a lack of specific bFGF promoter inactivation. Thus, altered trans-regulation of the bFGF gene promoter rather than its cis mutation may be responsible for the constitutive expression of bFGF gene in glioma cells. Consistent with this mechanism, we observed that nuclear extracts from glioma cells form complexes with the promoter region from bp −650 to −453 independent of cell density.

The control of bFGF gene activity by cell contact could be interrupted at several levels: from signaling molecules in the cell membrane through their receptors or second messengers signaling to nuclear regulatory proteins. Glioma cells retain the ability to generate signals that inhibit bFGF promoter activity in astrocytes. Therefore, the lack of cell-contact inhibition in glioma cells could reflect an absence of cell-contact receptors or disruption of their signals that restrict nuclear proteins from interacting with the bFGF promoter region from bp −650 to −453.

In conclusion, the results suggest that induction of the bFGF gene in astrocytes released from cell-contact inhibition is activated by the binding of activator proteins to the bFGF gene promoter. In glioma cells, the binding of these proteins is unresponsive to changes in cell density and causes constitutive expression of the bFGF gene. Given the mitogenic effects of bFGF, the loss of cell-density-dependent regulation of these trans-activating factors may be one of the steps in the progression from normal to neoplastic astrocytes. Identification of these factors may shed further light on the mechanisms controlling reactive and neoplastic transformation of human astrocytes.

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