Expression of Human Basic Fibroblast Growth Factor cDNA in Baby Hamster Kidney-derived Cells Results in Autonomous Cell Growth

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Abstract. Growth factor over-production by responsive cells might contribute to their autonomous proliferation as well as their acquisition of a transformed phenotype in culture. Basic fibroblast growth factor (bFGF) has been shown to induce transient changes in cell behavior that resemble those encountered in transformed cells. In addition, several types of human tumor cells have been shown to produce bFGF. To determine directly the role that bFGF might play in the induction of the transformed phenotype, we have introduced a human bFGF cDNA expression vector into baby hamster kidney-derived (BHK-21) fibroblasts. One of the BHK transfectants, termed clone 19, expresses the bFGF mRNA and produces biologically active bFGF that accumulates to a high concentration inside the cells. These properties correlate with the ability of the cells to grow in serum-free medium without the addition of exogenous bFGF. Clone 19 cells also proliferated in soft agar, indicating that constitutive expression of the bFGF gene results in a loss of anchorage-dependent growth.

Basal fibroblast growth factor (bFGF) is a potent broad spectrum mitogen that in bovine and human cells is encoded by 3.7- and 7.0-kb mRNA species (1, 6). This mitogen appears to be synthesized initially as a 155 residue protein, which can be further processed through proteolytic cleavage of its amino-terminal region into two shorter forms (146 and 131 residues), which are as active as the native form (II). bFGF is structurally related to acidic fibroblast growth factor (aFGF) (7), and both growth factors interact with the same cell surface receptors (29). Both growth factors trigger a similar range of biological effects, but differential activities are apparent. For example, bFGF induces the proliferation and differentiation of responsive cells with a much stronger potency (14). In vivo, as well as in vitro, both peptides act as potent mitogens and morphogens for a similar wide variety of neuroectodermal- and mesenchymal-derived cells (14), including vascular endothelial cells (13, 16, 17). Recent studies indicate that in vivo, bFGF mimics the effect of the vegetalizing factor, which is responsible in early embryos (Xenopus laevis, stage 8 blastulae) for the formation of mesenchyme from the animal pole (42). It therefore appears that in vivo, FGF can instruct cells that are destined to differentiate into ectoderm to form mesenchymal tissue instead.

Early studies have also shown that in addition to its proliferative and differentiative effect on normal diploid cells, bFGF could also induce phenotypic changes resembling those encountered in transformed cells. It induces density-inhibited monolayers of BALB/c 3T3 cells grown to confluence in the presence of serum to resume proliferation and to form multiple cell layers (12). It also stimulates the growth in soft agar of some established cell lines (12). It also stimulates the growth in soft agar of some established cell lines (34) and potentiates the effect of transforming growth factor beta (TGFβ) (35). Recent studies have indicated that various cells derived from solid human tumors, such as rhabdomyosarcomas, which are derived from bFGF-responsive myoblasts, express high bFGF levels (40). The significance of high concentrations of bFGF in tumor cells is presently unknown, but could relate to the in vivo angiogenic activity of this factor, which would result in increased tumor vascularization; additionally, bFGF could contribute to the growth of tumor cells by stimulating their proliferation.

The concept of autocrine stimulation of cell proliferation postulates that normal diploid cells can gain growth autonomy by acquiring the ability to produce, secrete, and respond to a given growth factor (45, 48). Verification of the autocrine hypothesis, in the case of bFGF, requires demonstration that expression of an introduced bFGF gene in cells that do not express this gene, but have functional bFGF cell.
surface receptors, results in autonomous cell growth and possibly, in the expression of a transformed phenotype.

To determine whether bFGF could act as an autocrine growth factor, we have introduced a plasmid that directs constitutive bFGF synthesis into baby hamster kidney cell line clone 21 (BHK-21) cells. Expression of bFGF resulted in autonomous cell growth and a loss of anchorage dependence for growth, as indicated by the ability of the transfected cells to grow in serum-free medium and to form colonies in soft agar.

**Materials and Methods**

**Materials**

Tissue culture media and Genetin (G-418 sululate) were obtained from Gibco (Grand Island, NY). Saline containing 0.05% trypsin, 0.01 M sodium phosphate (pH 7.4), and 0.02% EDTA (STV) was obtained from Difco Laboratories Inc. (Detroit, MI). Calf serum (CS) was purchased from HyClone Laboratories (Logan, UT). Gentamicin was from Schering (Kenilworth, NJ) and Fungizone was from E. R. Squibb (Princeton, NJ). Tissue culture dishes (96-, 48-, and 24-well clusters, 35-mm, 5-cm, and 10-cm dishes) were obtained from Falcon Labware (Oxnard, CA). 530-cm² dishes were from Nunc (Assembled Scientific, San Francisco, CA). Nitrocellulose filters were purchased from Schleicher & Schuell, Inc. (Keene, NH) and molecular size markers for Northern blots from Bethesda Research Laboratories (Gaithersburg, MD). Polycl-lysine, dimethyldihexylp-tetranzoium bromide (MTT), Ponceau red, transferrin, and gelatin were obtained from Sigma Chemical Co. (St. Louis, MO). Heparin-Sepharose was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Agar (ISO mesh) was purchased from Calbiochem-Behring Corp. (La Jolla, CA).

Bovine pituitary-derived bFGF and brain-derived aFGF were isolated as previously described (11, 18), and the homogeneity of both factors was determined by SDS-PAGE, amino acid composition, and NH₂-terminal sequence analysis of the molecules (18). 125I-bFGF was prepared as described (28). Rabbit anti-bFGF polyclonal antibodies were prepared as previously described (39). Avidin-alkaline phosphatase and biotinylated goat anti-rabbit IgG were obtained from Vector Laboratories, Inc. (Burlingame, CA), and 5-bromo-4-chloro-3-indol phosphate and nitro-blue-tetrazolium from Promega (Madison, WI). 125I-bFGF was prepared as described (28). Rabbit anti-bFGP and polyclonal antibodies were prepared as previously described (39). Avidin-alkaline phosphatase and biotinylated goat anti-rabbit IgG were obtained from Vector Laboratories, Inc. (Burlingame, CA), and 5-bromo-4-chloro-3-indol phosphate and nitro-blue-tetrazolium from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). Human high-density lipoproteins (HDL, 1.07 < d < 1.21 g/cm³) were obtained from human plasma by differential ultracentrifugal flotation as previously described (80). The plasmid pSV2neo was obtained from Plasmic Laboratories, Inc. (Mountain View, CA).

**Construction of a bFGF Expression Plasmid (pBGF)**

For the expression of basic FGF in the BHK cells, a 503-bp fragment encoding the proposed 155-residue primary translation product of human basic FGF (2) was ligated to the human metallothionein II₆ promoter, essentially as has been described previously for the expression of other proteins (8, 19).

**Transfection of BHK-21 cells**

Subconfluent BHK-21 cells (1 × 10⁶ cells) grown in 10-cm dishes containing 10 ml of a 1:1 (vol/vol) mixture of DME and Ham's F-12 media supplemented with 50 µg/ml Gentamicin, 0.25 µg/ml Fungizone, and 5% CS (DF medium + 5% CS), were co-transfected with 100 µg of pBGF and 20 µg of pUC9-MT18, and 5 µg of pSV2neo (44) as a calcium phosphate precipitate (51). Control cultures were similarly co-transfected with 20 µg of pUC9-MT18 and 5 µg of pSV2neo. Cells were incubated for 6 h at 37°C and shocked with glycerol (51). The medium was then changed and cells were grown for 24 h in DF medium supplemented with 5% CS. The cells were then incubated in DF medium supplemented with 5% CS and 570 µg/ml Gentamicin (G-418). Antibiotic resistant clones were visible after 2 wk in selective medium. The clones from the pBGF transfection (80 clones) or the control transfection (48 clones) were trypanospined, pooled, and grown to confluence. The pooled clones from pBGF-transfected or control-transfected cultures were then tested for their ability to grow in serum-free medium. At this stage, only pBGF transfected cells were able to proliferate under serum-free conditions.

Geneticin-resistant cells from pBGF transfected cultures were then seeded at limiting dilution into 96-well plates in DF medium supplemented with 5% CS. After 10 d 30% of the wells contained colonies. Cells from 20 wells were transfected, the cell suspension was spun down, and the cell pellet resuspended in DF medium supplemented with 5 µg/ml transferrin, 20 µM ZnSO₄, and 250 µg/ml HDL (SF medium) (31). Cells from each of the clones (2 × 10⁵ cells/well) were then seeded in 24-well gelatin-coated tissue culture clusters. After 6 d in culture, actively proliferating clones were amplified in SF medium.

**Cell Growth Assays**

Stock cultures of bovine adrenal cortex-derived capillary endothelial cells (ACE cells) were cultured as previously described (6) in DME supplemented with 50 µg/ml Gentamicin, 0.25 µg/ml Fungizone, and 10% CS. The cultures received 1 ng/ml bFGF until they became confluent. For passaging, confluent cultures were washed once with PBS and then exposed (2-3 min, 24°C) to STV. Rounded cells were then suspended in DME supplemented with 10% CS. Cell density was determined by counting an aliquot in a Coulter Counter (Coulter Electronic, Inc., Hialeah, FL). Cells were then seeded at the various densities indicated in the figure legends.

Stock cultures of BHK-21 cells and BHK-21 transfecants were grown in DF medium supplemented with 5% CS. For passaging, confluent cultures were trypsinized as described above, and cells were resuspended in either DF medium supplemented with 5% CS, or, in the case of BHK-21 cells grown in serum-free medium, DF medium supplemented with 2 mg/ml BSA. In the latter case, the cells were then spun down and the cell pellet was resuspended in DF medium supplemented with 5 µg/ml transferrin, 20 µM ZnSO₄, and 250 µg/ml HDL (31). Aliquots containing the appropriate cell number were seeded onto 35-mm gelatinized tissue culture dishes.

For cell growth assays, cells were seeded into gelatin-coated or noncoated 35-mm dishes containing the appropriate medium as described in the figure legends. Cultures were maintained in a humidified incubator (37°C) in a 5% CO₂/95% air environment for BHK-21 cells and 10% CO₂/90% air environment for ACE cells. To determine plating efficiency, duplicate cultures were trypsinized 6 h later and cells counted. Putative-derived bFGF or samples to be tested were added every other day. After various time intervals, cultures were trypsinized and cell densities were determined with a Coulter Counter.

**FGF Purification from Crude Cell Extracts**

For large scale culture of BHK-21 cells, 6 × 10⁶ cells were seeded in 530-cm² tissue culture plates containing 120 ml of DF medium supplemented with 5% CS. Once cultures were confluent, cells were trypsinized and resuspended in STV. After the addition of calf serum (5%) to neutralize trypsin, the cell suspension was centrifuged (300 g, 5 min). The cell pellet was then resuspended in PBS and centrifuged (300 g, 5 min). The cell pellet was either extracted immediately or stored in liquid nitrogen. To extract the cells, the pellets were resuspended in 0.5 M Triton X-100 in water (2 ml per cm² of cell pellet). The suspension was agitated for 3-3.5 ml and then repeatedly forced through a 25-gauge needle until only nuclei were present. An aliquot of 10 µl Tris/HCl, pH 7.0 containing 3 M NaCl was added to obtain a final concentration of 0.3 M NaCl. The crude extract was then centrifuged (50,000 g, 30 min, 4°C) and the supernatant was applied to a heparin-Sepharose (HS) column (bed volume, 0.5 ml) which had been pre-equilibrated at room temperature with 10 mM Tris/HCl, pH 7.0, and 0.6 M NaCl. The column was extensively washed with 10 mM Tris pH 7.0, 0.6 M NaCl, and sequentially eluted with the same buffer containing 1 M or 3 M NaCl, respectively (40). The flow rate was 21 ml/h. Aliquots of the fractions eluted were diluted with 0.2% gelatin in calcium and magnesium-free PBS, and tested for their abilities to stimulate cell proliferation (16, 39).

**Western Blot Analysis and Radioreceptor Assays**

HS-purified cell extract, putative-derived bFGF, or molecular weight size markers were electrophoresed on 12.5% SDS-PAGE slab gels and trans-
ferred electrophoretically overnight to nitrocellulose filters (BA-85) using a current of 0.5 mAmp (49). The filters were stained with Ponceau Red and the positions of the size markers marked. The stain was removed by exhaustive washes with 50 mM Tris/HCl pH 7.0, 0.5 M NaCl (TBS). The filters were incubated (1 h, 21°C) with 50 mM Tris/HCl pH 7.0, 0.5 M NaCl, and 0.1% Tween 20 (TBST), that contained rabbit anti-bFGF polyclonal antibodies (1 µg/ml) directed against bFGF (39). Controls were not exposed to the antibodies. The filters were washed three times with TBST buffer and probed with biotinylated anti-rabbit IgG. The filters were then washed three times with TBST and incubated with an alkaline phosphatase-avidin conjugate according to the instructions of the vendor (Vector Laboratories, Inc.). The filters were then washed twice with TBS and the alkaline phosphatase activity visualized using 5-bromo-4-chloro-3-indol phosphate and nitroblue-tetrazolium according to the instructions of the vendor (Kirkegaard & Perry Laboratories, Inc.).

Radioisotope assays were done using BHK-21 derived cell membranes as previously described (28) except that HT-200 filters (Gelman Sciences, Inc., Ann Arbor, MI) were used instead of GA-8 filters.

**DNA Extraction and Southern Hybridization**

DNA from BHK-21 parental and clone 19 cells was extracted essentially as described (3). The DNAs were then cut, in separate reactions, with three different restriction endonucleases—Hind III, Eco RI, or Bam HI. 12 µg of cut DNA from each reaction was then loaded onto a 0.8% agarose gel, and electrophoresed. The DNA was then transferred to a nitrocellulose filter (43) and baked for 2 h at 80°C in vacuo. The filter was prehybridized overnight at 42°C in 50 mM sodium phosphate (pH 6.5), 40% formamide, 5× Denhardt's, 5× SSC, and 100 µg/ml boiled herring sperm DNA. The prehybridization mix was then removed, and 15 ml of fresh prehybridization solution containing 10% dextran sulfate was added. The filters were hybridized overnight at 42°C with a bovine bFGF probe (nick-translated, 3 x 10^7 cpm), consisting of a purified fragment containing 612 bp from the bovine bFGF cDNA (extending from an Eco RI linker 103 bp upstream from the proposed initiating ATG codon, to an artificial Hind III site created 34 bp downstream from the translation termination codon) (1). After the hybridization, the filter was washed three times at room temperature in 1× SSC, 0.1% SDS, 10 min for each wash. The filter was then washed twice (20 min per wash) at 50°C in 1× SSC, 0.1% SDS. The filter was dried and exposed to x-ray film at –80°C with an intensifying screen.

**RNA Extraction and Northern Hybridization**

Total cellular RNA was isolated from BHK-21 clone 19 cells and from parental BHK-21 cells by the method of Chirgwin et al. (4), and analyzed by electrophoresis of 20 µg of total RNA through 1% agarose formaldehyde gels (26), followed by Northern blot transfer to nitrocellulose (47) and hybridization to radiolabeled probes. The probe for bFGF sequences was the 612 bp Eco RI-to-Hind III sequence of the bovine bFGF cDNA described above for Southern hybridization. The probe for metallothionein was an 800-bp fragment extending from a Hind III site upstream of the promoter to an artificial Bam HI site located about 30 bp downstream from the transcription start site (added after BAL31 digestion of the metallothionein gene) (23). Both probe fragments were labeled to high specific activity using [α-32P]-dCTP (800 Ci/mmol; New England Nuclear, Boston, MA) by nick-translation (33). Nitrocellulose filters were baked after transfer of the RNA, and then prehybridized and hybridized in 50% formamide with 10% dextran sulfate, according to published procedures (27).

**Soft Agar Colony Assay**

DF medium (1.5 ml) supplemented with 5% CS and containing 0.6% agar was added to 35-mm dishes and allowed to solidify. Cells were trypsinized, counted, and suspended in the same medium containing 0.3% agar at a final cell density of 3,000 cells/ml. 1 ml per 35-mm dish was then added. After the agar solidified 1 ml of DF medium containing 5% CS, 40 µM ZnSO4 with or without bFGF (final concentration 500 pg/ml) was added to the dishes. The cultures were maintained in humidified incubators (37°C) in a 5% CO2/95% air environment for 3-9 d and bFGF (500 pg/ml) was added where indicated every other day. Viable colonies were stained by the addition of 10 µl per dish of MTT stain (10 mg/ml) followed by incubation overnight at 37°C. Stained colonies with a diameter exceeding 100 µm were counted using a Bausch and Lomb stereo dissecting microscope at a 10-fold magnification, and photographed using a Nikon Diaphot inverted microscope at a 100-fold magnification.

**Results**

**Expression of a Human bFGF cDNA in BHK-21 Cells**

An expression vector (pBFGF) was constructed in which the human metallothionein IIa gene promoter (23) was placed between the SV-40 enhancer and a cDNA sequence encoding the proposed 155 amino acid precursor form of human bFGF (2). A short, growth hormone gene-derived polyadenylation signal was then attached at the 3' end of the bFGF coding sequence, and the resulting construct was ligated into pUC8 to create the plasmid pBFGF (Fig. 1). This plasmid was introduced by the calcium phosphate transfection method (51) into BHK-21 cells along with two other plasmids: pSV2neo, which contains the sequences coding for aminoglycosyl-phosphotransferase II; and pUC9-MT18, which contains the human metallothionein IIa gene. The pUC9-MT18 plasmid was used to enable an eventual cadmium selection for high producers (i.e., cells expressing the products of the transfected DNA sequences at high levels), if necessary. Control cultures were transfected with plasmids pSV2neo and pUC9-MT18 only. Clones resistant to 570 µg/ml of the antibiotic Geneticin (G-418) were selected and expanded as described in Materials and Methods.

Control cells transfected only with pSV2neo and pUC9-MT18, as well as cells transfected additionally with pBFGF, proliferated actively in serum-supplemented medium. In contrast, when tested under serum-free conditions, only cells transfected with pBFGF could proliferate. Those cells were then further cloned by seeding at limiting dilution into 96-well cluster plates in the presence of serum-supplemented DF medium. After 10 d in culture, 30% of the wells contained actively proliferating clones, which were then trypsinized and further selected for their ability to grow on gelatinized dishes containing DF medium supplemented
with transferrin, and HDL instead of serum. The medium also contained 20 μM ZnSO₄, to induce the metallothionein II₆ promoter. Under these conditions, neither parental cells nor control transfected cells can proliferate. After 6 d in culture, 45% of the gelatinized wells contained actively proliferating cells. The conditioned medium from all of these clones was mitogenic when tested on ACE cells, and all cell lysates contained immunoreactive bFGF-like material (data not shown). One of the proliferating clones (clone 19) was selected for further detailed studies of bFGF expression.

When the genomic DNA from BHK-21 clone 19 cells was analyzed by Southern blot, at least two copies of bFGF cDNA appeared to be integrated in the genome (Fig. 2 A, lanes 2 and 4). These copies were distinct from the endogenous hamster bFGF gene, which was easily detected in parental genomic DNA as three separate bands that presumably correspond to the three bFGF exons (Fig. 2 A, lanes 1 and 3) (2).

Northern analysis reveals the presence of two RNA transcripts of ~0.6 and 4.3 kb, respectively, in BHK-21 clone 19 cells (Fig. 2 B, lanes 2 and 4). These copies were distinct from the endogenous hamster bFGF gene, which was easily detected in parental genomic DNA as three separate bands that presumably correspond to the three bFGF exons (Fig. 2 A, lanes 1 and 3) (2).

Transfected BHK-21 Cells Contain Biologically Active bFGF

To assess whether pbFGF transfected cells produce bioactive bFGF, cell extracts from 2 × 10⁶ BHK-21 clone 19 cells were partially purified by HS affinity chromatography. The eluted fractions were then examined for their ability to stimulate the proliferation of adrenal cortex-derived endothelial cells, a cell type that specifically responds to FGF (Fig. 3 A). The origin of the 4.3-kb mRNA was predicted from the structure of the pbFGF plasmid (Fig. 1). The origin of the 4.3-kb transcript has not been determined, but could represent transcription initiating from an endogenous promoter located close to one of the copies of the pbFGF sequences, or readthrough of the growth hormone gene-derived polyadenylation signal. The two transcripts seen differ in size from the endogenous bFGF mRNAs (7 and 3.7 kb, respectively), which can readily be detected in bovine, mouse, or human cell types that express the endogenous bFGF gene (1, 32, 38, 39, 40). The two RNA transcripts also hybridized to the metallothionein probe (Fig. 2 B, lane 4), indicating that they were derived from the pbFGF DNA which was introduced into the cells. The RNA co-migrating with the 28S ribosomal marker in Fig. 2 B, lanes 3 and 4 presumably represents the mRNA of an endogenous hamster metallothionein gene. In contrast, parental cells (Fig. 2 B, lane 1), or control cells transfected with pSV2neo and pUC9-MT18 (not shown), did not contain detectable levels of bFGF mRNA transcripts.
the 0.6 M NaCl wash. The unabsorbed material had little if any biological activity. Elution of the columns with 1 M NaCl, however, resulted in the release of bioactive material. This was unexpected since at this salt concentration only aFGF but not bFGF is reported to elute from HS (11). Subsequent elution with 3 M NaCl yielded a major bioactive peak, which contained most of the total bioactivity present in crude extracts.

The concentration of bFGF-like material present in the 3 M NaCl peak fractions, as determined by bioassay, was 12 μg/ml (Fig. 3 B). This was confirmed using a radioreceptor assay (Fig. 3 C), which gave similar bFGF concentrations (13 μg/ml) in the 3 M NaCl peak fractions. Total bFGF recovery from 2 x 10^8 cells was 15 μg.

The bFGF molecular species present in the 1 and 3 M NaCl eluates were further analyzed by Western blot (Fig. 4). In the 1 M NaCl fractions, only one molecular species with an apparent Mₐ of 18 kD reacted with the anti-bFGF antibody (Fig. 4, lane 2). This immunoreactive bFGF species therefore has an apparent molecular weight that corresponds closely to that of the 155 amino acid bFGF form encoded by the bFGF cDNA. In contrast, two immunoreactive molecular weight species were present in the 3 M NaCl fractions (Fig. 4, lane 1). One species had an apparent Mₐ of 18 kD and was therefore similar in size to that found in the 1 M NaCl fractions stepwise with increasing concentrations of NaCl, as indicated. Aliquots of the fractions eluted were then examined for the presence of bFGF-like material. For determination of bFGF-like bioactivity, aliquots of the fractions were diluted 10-fold in PBS/0.2% gelatin and 10 μl of the dilutions were added every other day to ACE cells that had been seeded at a density of 1 x 10⁵ cells per 35-mm dish. Additional dishes received either 0.2% gelatin only (no additions) or pituitary-derived bFGF (1 ng/ml). The cells were counted after 5 d. Cell numbers represent the means of triplicate determinations. Variation between replicates was <5%. (B) Effect of increasing concentrations of HS purified BHK-21 clone 19 cell extract on the proliferation of ACE cells. Increasing concentrations of bFGF-like material purified from BHK-21 clone 19 cells (fraction 27 of A) (○) or of pituitary-derived bFGF (●) were added every other day to ACE cells that had been seeded at a density of 1 x 10⁴ cells per 35-mm dish. Each dish contained 2 ml of medium. The cells were counted in a Coulter Counter after 5 d in culture. Values represent the means of duplicate determinations. Variation between replicates was <5%. (C) Examination of HS purified clone 19 cell extracts by radioreceptor assay. ¹²⁵I-bFGF (0.3 ng/tube, 2 x 10⁵ cpm/ng) and BHK-21 cell membranes (16 μg protein/tube) were incubated with increasing concentrations of HS purified BHK-21 clone 19 cell extract (A, fraction 27) (○) or of pituitary-derived bFGF (●) (28). ¹²⁵I-bFGF binding was then determined as described in Materials and Methods. In the presence of ¹²⁵I-bFGF alone, 1.3 x 10⁴ cpm were bound to the membranes. Nonspecific binding in the presence of 20 ng/tube pituitary bFGF was 2.6 x 10³ cpm, and was subtracted from the experimental values. Binding is expressed as percent of specific binding in the absence of binding inhibitors (control). Values are the means of triplicate determinations. Variation between replicates was <10%.

![Figure 3](image_url)

**Figure 3.** (A) Heparin-Sepharose affinity chromatography of extracts prepared from BHK-21 clone 19 cells. Extracts from 2 x 10⁸ cells were prepared as described in Materials and Methods, and applied to an HS affinity column. Material bound to the column was eluted stepwise with increasing concentrations of NaCl, as indicated. Aliquots of the fractions eluted were then examined for the presence of bFGF-like material. For determination of bFGF-like bioactivity, aliquots of the fractions were diluted 10-fold in PBS/0.2% gelatin and 10 μl of the dilutions were added every other day to ACE cells that had been seeded at a density of 1 x 10⁵ cells per 35-mm dish. Additional dishes received either 0.2% gelatin only (no additions) or pituitary-derived bFGF (1 ng/ml). The cells were counted after 5 d. Cell numbers represent the means of triplicate determinations. Variation between replicates was <5%. (B) Effect of increasing concentrations of HS purified BHK-21 clone 19 cell extract on the proliferation of ACE cells. Increasing concentrations of bFGF-like material purified from BHK-21 clone 19 cells (fraction 27 of A) (○) or of pituitary-derived bFGF (●) were added every other day to ACE cells that had been seeded at a density of 1 x 10⁴ cells per 35-mm dish. Each dish contained 2 ml of medium. The cells were counted in a Coulter Counter after 5 d in culture. Values represent the means of duplicate determinations. Variation between replicates was <5%. (C) Examination of HS purified clone 19 cell extracts by radioreceptor assay. ¹²⁵I-bFGF (0.3 ng/tube, 2 x 10⁵ cpm/ng) and BHK-21 cell membranes (16 μg protein/tube) were incubated with increasing concentrations of HS purified BHK-21 clone 19 cell extract (A, fraction 27) (○) or of pituitary-derived bFGF (●) (28). ¹²⁵I-bFGF binding was then determined as described in Materials and Methods. In the presence of ¹²⁵I-bFGF alone, 1.3 x 10⁴ cpm were bound to the membranes. Nonspecific binding in the presence of 20 ng/tube pituitary bFGF was 2.6 x 10³ cpm, and was subtracted from the experimental values. Binding is expressed as percent of specific binding in the absence of binding inhibitors (control). Values are the means of triplicate determinations. Variation between replicates was <10%.

![Figure 4](image_url)

**Figure 4.** Western blot analysis of purified BHK-21 clone 19 cell extract. 30 μl of fraction 27 (Fig. 3 A) (diluted 10-fold) (lane I), 30 μl of purified cell extract from fraction 23 (Fig. 3 A) (lane 2), or 15 ng of pituitary derived bFGF (lane 3) were subjected to electrophoresis on an SDS-PAGE gel, transferred to nitrocellulose paper, and incubated with an anti-bFGF polyclonal antibody (39), as described in Materials and Methods. All lanes were subsequently exposed to goat anti-rabbit biotinylated antibody. Bound antibody was then visualized using an avidin-alkaline phosphatase conjugate as described in Materials and Methods. The protein size markers used were: phosphorylase b (92,000); BSA (66,000); ovalbumin (45,000); carbonic anhydrase (31,000); soybean trypsin inhibitor (21,500); and lysozyme (14,400).
pbFGF-transfected Cells Are Capable of Anchorage-independent Growth, and of Autonomous Cell Proliferation at a Rate Similar to Parental Cells Treated with bFGF

Cells from BHK-21 clone 19, in contrast to parental BHK-21 cells, proliferate when maintained on solid substrate and exposed to DF medium supplemented with transferrin, ZnSO4, and HDL alone. The average doubling time (18 h) during the logarithmic growth phase of transfected cells was similar to that of parental cells exposed to DF medium supplemented with transferrin, HDL, and optimal concentrations of bFGF (10 ng/ml) (Fig. 5).

Parental and transfected BHK-21 cells were analyzed for their ability to grow in soft agar. Parental BHK-21 cells (Figs. 6 and 7 A) or BHK-21 cells transfected with pSV2neo and pUC9-MT18 (not shown) did not form colonies when seeded in soft agar and exposed to serum-supplemented medium even when EGF (10 ng/ml) or TGFα (10 ng/ml) were added. In contrast, in the presence of exogenous bFGF, parental BHK-21 cells formed colonies, and the colony-forming efficiency was a function of bFGF concentration (Fig. 6). bFGF was active at concentrations as low as 10 pg/ml, with an ED50 of 75 pg/ml and saturation at 200 pg/ml (Figs. 6 and 7 B). At saturating concentrations colony-forming efficiency for the parental BHK cells was 67%. When BHK-21 clone 19 cells were analyzed for their ability to form colonies under similar conditions, even in the absence of exogenous bFGF, the colony-forming efficiency was 44% (Figs. 6 and 7 C), and at saturating bFGF concentrations, it increased to 70% (Figs. 6 and 7 D).

bFGF Is Present in Media Conditioned by BHK-21 Clone 19 Cells

The possible presence of bFGF in media conditioned by BHK-21 clone 19 cells was suggested by its ability to stimulate the proliferation of ACE cells (Fig. 8 A), while media conditioned by control transfected cells did not have any activity. 1 ml of conditioned medium from BHK-21 clone 19 cells contained ~0.4 ng of bFGF (see Fig. 8). All the mitogenic activity in the conditioned medium could be inhibited by anti-bFGF directed antibodies (39), and is therefore due to bFGF (Fig. 8 B). The amount of bioactive bFGF present in conditioned media was ~1% of that present in the cell layer. The identity of the mitogen present in the conditioned medium was further explored by HS affinity chromatography. When the biological activity present in the conditioned medium from BHK-21 clone 19 cells was analyzed by HS affinity chromatography, a peak of mitogenic activity eluted at 3 M NaCl, a salt concentration at which bFGF is expected to elute (not shown).

Protamine Sulfate Inhibits the Autonomous Proliferation of BHK-21 Clone 19 Cells

Protamine sulfate has been shown in previous studies to be anti-angiogenic in vivo (46), and to prevent the biological effect of bFGF by interacting directly with its receptor (30). Protamine also inhibits the mitogenic effects of the extracellular matrix produced by bovine corneal endothelial cells (30). In contrast, the mitogenic activity of epidermal growth factor is stimulated by protamine sulfate (30). As shown in Fig. 9 A, protamine concentrations that are not cytotoxic for BHK-21 cells (42), inhibited in a concentration-dependent manner the proliferation of BHK-21 clone 19 cells exposed to transferrin, ZnSO4, and HDL-supplemented DF medium. The inhibitory effect was maximal at 10 µg/ml protamine sulfate. This result is consistent with the autonomous proliferation of BHK-21 clone 19 cells being mediated by FGF receptors.

![Figure 6. Formation of colonies in soft agar by BHK-21 cells is dependent upon bFGF even in the presence of serum. 3 × 10^4 parental BHK-21 cells (●) or BHK-21 clone 19 cells (○) were seeded in 35-mm dishes containing DF medium supplemented with 5% CS, antibiotics, ZnSO4 (11 µM) and agar, as described in Materials and Methods. The indicated bFGF concentrations were added every other day to the cultures. After 8 d in culture, 10 µl of 10 mg/ml MTT was added, and the cultures incubated overnight. The number of colonies was determined as described in Materials and Methods. The values represent means of triplicate determinations. The bars represent the standard deviation from the mean.](image-url)
Is Secretion of bFGF into the Medium Important for the Autonomous Proliferation of BHK-21 Clone 19 Cells?

Since the biological actions of bFGF are mediated by membrane receptors, it is possible that the bFGF found in the conditioned medium of pbFGF transfected cells (see Fig. 8) contributes significantly to their proliferation, while the bFGF found associated with the cells contributes little to the mitogenic effect. To investigate the relative contribution of cell-associated bFGF versus released bFGF in supporting the proliferation of BHK-21 clone 19 cells, we used two experimental designs. First, we have made use of neutralizing anti-bFGF polyclonal antibodies, which in previous studies have been shown to neutralize the activity of exogenously added bFGF (39). Second, we have analyzed the proliferative effect of conditioned medium from control or clone 19 cells on the proliferation of ACE cells. BHK-21 clone 19 or control cells (transfected with pSV2-neo and pUC9-MT18 only) were seeded at 1 x 10⁵ cells/dish in 5-cm dishes and grown to confluence (5 x 10⁶ cells/dish) in serum-supplemented DF medium. Conditioned medium was then collected and centrifuged in a table top centrifuge to remove floating cells and debris. Increasing volumes of conditioned medium were added every other day to ACE cells (1.5 x 10⁴ cells per 35-mm dish). Cell numbers were determined using a Coulter Counter after 5 d in culture. Values represent the means of triplicate determinations. Variation between replicates was <5%.

(A) Effect of conditioned medium from control or clone 19 cells on the proliferation of ACE cells. BHK-21 clone 19 or control cells (transfected with pSV2-neo and pUC9-MT18 only) were seeded at 1 x 10⁵ cells/dish in 5-cm dishes and grown to confluence (5 x 10⁶ cells/dish) in serum-supplemented DF medium. Conditioned medium was then collected and centrifuged in a table top centrifuge to remove floating cells and debris. Increasing volumes of conditioned medium were added every other day to ACE cells (1.5 x 10⁴ cells per 35-mm dish). Cell numbers were determined using a Coulter Counter after 5 d in culture. Values represent the means of triplicate determinations. Variation between replicates was <5%.

(B) Anti-bFGF polyclonal antibodies that inhibit the mitogenic effect of bFGF, inhibit the mitogenic activity of medium conditioned by BHK-21 clone 19 cells. ACE cells (5 x 10⁴ cells/well), were seeded in 0.5 ml of DME supplemented with 10% CS in 48-well clusters as described in Materials and Methods. Some cultures did not receive any additions (−) and some received bFGF (+bFGF) (1 ng/ml) in the presence (+Ab) or absence of neutralizing anti-bFGF rabbit polyclonal antibodies (10 μg/ml) (39). Other cultures received 75 μl of serum-supplemented DF medium that was conditioned by BHK-21 clone 19 cells as described under Fig. 8 A (conditioned medium). The effect of the conditioned medium was analyzed in the presence (+Ab) or absence of neutralizing anti-bFGF rabbit polyclonal antibodies (10 μg/ml) (39). Conditioned medium, anti-bFGF antibodies, and bFGF were added every other day. The cells were trypsinized and counted after 5 d as described in Materials and Methods. Values represent the means of triplicate determinations. Variation between replicates was <10%.
Figure 9. (A) The proliferation of clone 19 cells is inhibited by protamine. BHK-21 Clone 19 cells (4 x 10^4 cells per gelatinized 35-mm dish) were seeded in DF medium supplemented with HDL, transferrin, and ZnSO₄ as described in Materials and Methods. Increasing concentrations of protamine sulfate were added to cultures every other day as previously described (30). Cell numbers were determined using a Coulter Counter after 4 d in culture. Values are means of triplicate determinations. Variation between replicates was <10%. (B) Anti-bFGF polyclonal antibodies that inhibit the mitogenic activity of bFGF do not affect the proliferation of BHK-21 clone 19 cells. Parental BHK-21 or BHK-21 clone 19 cells (5 x 10^3 cells/gelatinized well), were seeded in 0.5 ml DF medium supplemented by HDL, transferrin, and ZnSO₄ in 48-well clusters as described in Materials and Methods. Parental BHK-21 cells were grown without (-) or with exogenous bFGF (1 ng/ml). BHK-21 clone 19 cells did not receive bFGF. Some clusters received in addition 10 µg/ml of neutralizing anti-bFGF rabbit polyclonal antibodies (+Ab) (39). Anti-bFGF antibodies and/or bFGF were added every other day. The cultures were trypsinized and cell number determined as described in Materials and Methods. Values represent the means of triplicate determinations. Variation between replicates was <10%. (C) BHK-21 clone 19 cells proliferate at a maximal rate even when seeded at very low density in serum-free medium. The indicated numbers of BHK-21 clone 19 cells were seeded as described under Materials and Methods in gelatinized 35-mm dishes in DF medium supplemented with HDL, transferrin, and ZnSO₄ in the presence (●) or absence (○) of 5 ng/ml bFGF. The cultures were trypsinized and counted in a Coulter Counter after 9 d in culture. Values are the means of triplicate determinations. Variation between replicates was <5%.

tion of BHK-21 clone 19 cells seeded at densities ranging from 0.2 to 10 cells/mm², with or without exogenously added bFGF.

The results of these two experiments are shown in Fig. 9. The addition of neutralizing anti-bFGF polyclonal antibodies did not result in inhibition of BHK-21 clone 19 cell growth, even though the mitogenic activity of exogenously added bFGF was completely inhibited by the antibody (Fig. 9 B). When the proliferation of BHK-21 clone 19 cells was analyzed as a function of cell density, regardless of the density at which cells were plated and regardless of whether or not they were exposed to exogenous bFGF, cells proliferated at the same rate (Fig. 9 C). It therefore seems that the bFGF found in the conditioned medium is not required in order for BHK-21 clone 19 cells to proliferate.

Discussion

Uncontrolled growth factor production by responsive cells has been shown in several cases to result in phenotypic transformation and other changes in cellular behavior. Examples include the expression of an introduced TGFβ gene in Rat-1 fibroblasts (37), expression of the gene coding for granulocyte-macrophage colony stimulating factor (GM-CSF) in hematopoietic cells (25), and expression of either the platelet-derived growth factor (PDGF) B chain gene (9) or of its acquired viral counterpart, the p28v gene of Simian Sarcoma Virus (20), in NIH 3T3 cells.

Here the hypothesis that inappropriate expression of bFGF could also lead to autonomous cell proliferation and the induction of the transformed phenotype, has been tested by introducing into BHK-21 cells a plasmid that directs the high level expression of human bFGF. BHK-21 cells were chosen because they do not express the endogenous bFGF gene, and in previous studies, they have been shown to be totally dependent on exogenous bFGF to proliferate when maintained under serum-free conditions (31). Finally, exogenous bFGF induces anchorage-independent soft agar growth of BHK-21. This effect, however, is transient, and cells revert to their normal phenotype once the mitogen is removed (data not shown). High level bFGF expression in BHK-21 cells might therefore be expected to lead to permanent anchorage-independent soft agar growth of BHK-21 cells.

Southern blot analysis demonstrates that in BHK-21 clone 19 cells, after transfection, copies of the transfected bFGF gene have been incorporated into the genome of the cells (see Fig. 2 A). That the integrated foreign bFGF genes are actively expressed is indicated by the detection through Northern blot analysis of 0.6- and 4.3-kb RNA transcripts (Fig. 2 B), which were not seen in the parental BHK-21 cell RNA, and which differed in size from those found in bovine, mouse, or human cells expressing the endogenous bFGF.
gene (3.7 and 7.0 kb, respectively; see references 1, 39, and 40). In addition, BHK-21 clone 19 cells contained $2 \times 10^6$ bFGF molecules per cell, while no detectable bioactive FGF was found in the parental BHK-21 cells. Two distinct bFGF molecular species could be detected by Western blot analysis: one bFGF molecular species was probably the 155 amino acid form bFGF, which is proposed to be the primary translation product encoded by the bFGF gene (1, 2), or a form missing one amino terminal residue (50), while the other was equivalent in size to the 146 amino acid, shorter form found in bovine pituitaries (6). This shorter form has been shown in previous studies to have an intrinsic biological activity similar to that of the larger form (II). Interestingly enough, the two forms could be partially separated by HS affinity chromatography, with a portion of the longer form having a lesser affinity for heparin than the shorter form (Fig. 3). This is in agreement with a recent report that showed that a portion of the longer form of bFGF isolated from SK-HEP1 cells has a lower affinity for heparin than does the shorter form (see Fig. 5 of reference 24).

The drastic effect of high level expression of bFGF on the proliferation of cells that do not normally express the bFGF gene is evident from the results presented here. BHK-21 cells which, on solid substrate, have an absolute requirement for exogenous FGF (or other added growth factor) in order to proliferate in serum-free medium, proliferated actively in the absence of exogenous growth factor once transfected with plasmids carrying the human bFGF coding sequence under the control of the SV-40 enhancer and human metallothionein II promoters. Likewise, in soft agar, transfected cells formed colonies, while parental cells required the addition of exogenous bFGF in order to do so. Since serum stimulates BHK-21 cell proliferation on solid substrate, but not in soft agar, these results indicate that serum-derived growth factors such as PDGF cannot support the anchorage-independent proliferation of BHK-21 cells.

Neither acidic nor basic FGF appears to be synthesized with a conventional signal peptide (1, 21). Some normal diploid cells that express the bFGF gene and have been shown to contain bFGF protein are still dependent on exogenous bFGF to proliferate actively in vitro (15), suggesting that cell-associated bFGF cannot stimulate growth of normal diploid cells in which it is contained. Together, these results have suggested that bFGF is processed through the cells in a manner different from normal secreted proteins, and therefore through a different cellular compartment than its receptor. Since cell types sensitive to bFGF have been shown to bear specific receptors, on the cell surface, the serum-free independent proliferation of the transfected cells could be in part mediated by bFGF that is somehow released from these cells. This possibility is supported by the observation that medium conditioned by transfected BHK-21 cells does contain mitogenic activity for bFGF-responsive cells. This activity, when analyzed by HS affinity chromatography, behaves in a manner similar to bFGF. Further evidence demonstrating that bFGF-like molecules are present in conditioned medium can be derived from the ability of anti-bFGF antibodies to neutralize the mitogenic activity in the medium. One should, however, observe that the total bioactivity present in conditioned media was far less (1%) than that present within the cells. In addition, neutralizing anti-bFGF polyclonal antibodies could not prevent the proliferation of BHK-21 clone 19 cells even though they inhibited the growth of parental BHK-21 cells exposed to a saturating concentration of bFGF (Fig. 9 B). Also arguing against a requirement for bFGF to be released in order to trigger cell proliferation, is the fact that BHK-21 clone 19 cells seeded at clonal density, in the absence of exogenous bFGF, proliferate as well as high density cultures seeded in the absence of bFGF. If cells needed to condition their medium with bFGF in order to respond to it, one would have predicted that clonal density cultures, in contrast to high density cultures would have had a lower growth rate, and that the growth rate would have been improved by the addition of exogenous bFGF. This was clearly not the case (Fig. 9 C).

Although one could construe from the results discussed above that bFGF acts intracellularly, the ability of protamine to inhibit the proliferation of transfected cells (Fig. 9 A) could reflect its ability to interact directly with the FGF receptor itself, thereby displacing bound bFGF (30). In the case of the results obtained with bFGF antibodies, which in previous studies have been shown to neutralize the bioactivity of soluble bFGF (32, 38, 39), their lack of neutralizing effect may reflect their inability to recognize bFGF once it is bound to its receptor. This could occur should bFGF associate rapidly with cell surface receptors once released, or be transported to the cell surface already associated with bFGF receptors. Likewise, the results obtained in regard to the growth rate of clonal cultures and their lack of exogenous bFGF requirement could also reflect either the rapid association of bFGF, once released, to their cell surface receptor, or that bFGF receptor complexes are expressed to the cell surface.

We cannot, therefore, exclude the possibility that bFGF could have part of its mitogenic activity mediated through events taking place within the cells or on the cell surface, as has been proposed for SSV-transformed cells. In that latter case, the fact that only a small fraction of the PDGF produced by SSV-transformed cells is secreted (36), combined with very limited growth inhibition of these cells by antisera to PDGF has led to the proposal that the growth factor may be able to interact and activate its receptor in an intracellular compartment. Similarly, nontumorigenic hemopoietic cells can be converted into transformed leukemic cells after expression of an introduced human GM-CSF gene. The inability of anti-GM-CSF antiserum to prevent transformation implies that at least part of the GM-CSF receptor interaction is intracellular (25).

In conclusion, the present study demonstrates that whether bFGF interacts with its receptor within the cells, or at a surface location, it is capable of triggering the autonomous, anchorage-independent growth of a single cell in which it is synthesized in high amounts.

Received for publication 24 July 1987, and in revised form 11 November 1987.

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