Regulation of bFGF Gene Expression and Subcellular Distribution of bFGF Protein in Adrenal Medullary Cells

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Abstract. Basic fibroblast growth factor (bFGF), a potent mitogenic/neurotrophic factor, controls the development and plasticity of many types of neural cells. In adrenal chromaffin cells, the appearance of bFGF protein coincided with the establishment of functional innervation, suggesting induction by trans-synaptic signals. In cultured bovine adrenal medullary cells Western blot analysis revealed 18-, 23-, and 24-kD bFGF isoforms in the cytosolic and nuclear fractions. Stimulation of acetylcholine nicotinic receptors or hormonal angiotensin II receptors or the direct stimulation of adenylate cyclase with forskolin or protein kinase C (PKC) with PMA increased the content of all bFGF isoforms. Increases in the levels of intracellular bFGF did not result in detectable presence of bFGF proteins in culture medium. Instead, bFGF proteins accumulated in the cytoplasm or the nucleus depending on whether PKC or cAMP pathways were activated.

The long-term nuclear forskolin-induced accumulation of bFGF was prevented by cycloheximide or by antisense bFGF oligonucleotide and was also accompanied by an increase in bFGF mRNA. We used luciferase reporter plasmids containing the human bFGF promoter to show that the induction of bFGF resulted from transcriptional activation of the bFGF gene and was mediated by regulatory sequences located upstream from its transcription start site. Stimulation of bFGF gene expression by forskolin and PMA was synergistic and was mediated through different promoter regions. The results suggest that stimulation by cAMP and PKC is mediated through novel cis elements. The regulation of bFGF protein content also involves post-transcriptional mechanisms since changes in the levels of individual bFGF isoforms were different depending on whether cells were treated with carbachol or angiotensin II, forskolin, or PMA.

The present study indicates that bFGF is an intracrine cytoplasmic–nuclear factor, whose expression is regulated by trans-synaptic and hormonal stimuli and which may act as a direct mediator of genomic responses to afferent stimulation.

GENESIS of cells in the developing nervous system, their differentiation, and survival are controlled by a variety of protein growth factors (Yamamori, 1992). One such protein is basic fibroblast growth factor (bFGF)[1], a member of the family of heparin binding growth/neurotrophic factors (Wagner, 1991). bFGF is expressed in developing nervous tissue (Ernfors et al., 1990; Grothe and Unsicker, 1990; Grothe et al., 1991). It stimulates mitosis of neuroblasts (Gensburger et al., 1987; Mayer et al., 1993), affects their differentiation (Stemple et al., 1988; Vescovi et al., 1993), ontogenic death (Dreyer et al., 1989), and stimulates neurite outgrowth (Hatten et al., 1988; Gurney et al., 1992) and synapse formation (Peng et al., 1991). bFGF also affects mitosis and differentiation of glial precursor cells (Engele and Bohn, 1992; Vescovi et al., 1993) and stimulates proliferation of vascular endothelial and smooth muscle cells (Hayek et al., 1987).

In some regions of the nervous system bFGF is expressed during adulthood (Woodward et al., 1992; Grothe et al., 1991). Functions of bFGF in the mature nervous tissue are still contested. bFGF may participate in the response of nervous tissue to injury; it stimulates reactive transformation of astrocytes (Kniss and Burry, 1988) and enhances regeneration of the damaged neuronal pathways (Otto and Unsicker, 1990). bFGF may also act as a neuroprotective agent, increasing survival of neurons during trans-synaptic stimula-

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1. *Abbreviations used in this paper*: ANOVA, analysis of variance; as, antisense; BAMC, bovine adrenal medullary cells; bFGF, basic fibroblast growth factor; CRE, cyclic AMP responsive element; DSE, dyad symmetry element; PKA, protein kinase A; PKC, protein kinase C; s, sense; IR, immunoreactivity; TH, tyrosine hydroxylase.

tion or hypoxia (Nozaki et al., 1993). Afferent stimulation of the nervous tissue leads to structural reorganization similar to that occurring during development, i.e., proliferation of glial and neural cells, expansion of neuritic arbors, formation of new synapses and angiogenesis (for review see Bailey and Kandell, 1993). These changes could reflect a mobilization of intrinsic growth factors like bFGF.

In contrast to numerous studies describing the pleiotropic effects of bFGF in the nervous system, little is known about the mechanisms that regulate bFGF synthesis, its restricted cellular expression, and how it exerts biological functions. Increases in the levels of bFGF mRNA have been observed in the adult brain tissue following denervation (Leonard et al., 1993) or epileptic seizures (Riva et al., 1992). This finding suggests that changes in neural activity could affect the expression of the bFGF gene. Factors that trigger these changes, their underlying mechanisms and functional significance remain unknown. The elucidation of the regulation of bFGF and bFGF receptor synthesis, represents an important step on the way to a comprehensive understanding of the physiological roles of bFGF.

The comparison of the gene and protein structures of bFGF to other neurotrophic growth factors suggests that the mechanisms controlling both their synthesis and their action are fundamentally different. Unlike brain-derived neurotrophic factor gene (Maisonpierre et al., 1991), bFGF gene does not contain CAAT or TATA box motives (Abraham et al., 1986*a*; Shibata et al., 1991). Little is known about mechanisms through which transcription initiates at TATAA box-deficient promoters and how activity of these promoters is regulated (see discussion in Ackerman et al., 1993). Transcription of the bFGF gene leads to a variety (10 to 1.6 kb) of messenger RNAs that differ in the length of the 3′-translated region (Abraham et al., 1986*a*; Prats et al., 1989; Florkiewicz and Sommer, 1989; Zuniga et al., 1993). Translation of bFGF mRNA initiates from an ATG codon yielding an 18-kD protein and from three CTG codons producing higher molecular weight bFGF isoforms (Florkiewicz and Sommer, 1989; Powell and Klagsbrun, 1991). The structural characterization of bFGF proteins has raised many questions regarding their possible mechanisms of action. bFGF lacks a signal sequence found in other secreted proteins (Abraham et al., 1986*a*,*b*) and is also absent in Golgi, arguing against its incorporation into secretory granules or secretory vesicles (Matsuda et al., 1992; Mignatti et al., 1992). Unlike NGF, or brain-derived neurotrophic factor and many other neurotrophic factors, bFGF is primarily found cell associated. Only the shortest AUG-initiated 18-kD bFGF isoform may associate with the cell membrane (Florkiewicz et al., 1991). Very little or no bFGF is found in the medium of most cultured cells (Schweigerer et al., 1987; Vlodawski et al., 1987; Moscatelli, 1988), and only a small fraction of total bFGF is bound to cell surface heparan sulfate proteoglycans (Bashkin et al., 1992). Nevertheless, the presence of plasma membrane receptors for bFGF, and studies with neutralizing bFGF antibodies have indicated that bFGF may act extracellularly (Sato and Rifkin, 1988). It is possible that leakage from lysed or damaged cells is the primary source of extracellular bFGF (Schechter, 1992).

A convenient model to study mechanisms that control the expression and function of bFGF in neural cells is chromaffin cells of the adrenal medulla. Adrenal medullary pheochromocytes, like the noradrenergic neurons in sympathetic ganglia, are derived from the neural crest (Landis and Patterson, 1981). Their development and functions are regulated by trans-synaptic as well as hormonal signals. The mitotic activity of adrenal medullary cells declines during the development of the adrenal medulla. Nevertheless, the volume and number of cells of the adrenal medulla increase throughout life (Coupland, 1991). Even the adult adrenal medulla may undergo hyperplasia, increased vascularization and, in some instances, neoplastic transformation (Tischler and DeLellis, 1988; Tischler et al., 1988). Stimuli that trigger these changes increase the activity of the nerves innervating the adrenal medulla or directly stimulate acetylcholine receptors on adrenal medullary cells (Malvaldi and Viola-Magni, 1972; for review see Tischler and DeLellis, 1988; Tischler et al., 1988).

In the rat adrenal medulla, the presence of bFGF immunoreactivity is detected for the first time at day 8 of postnatal life (Grothe and Unsicker, 1990). The initial appearance of bFGF and subsequent increase in its expression coincide with the establishment of functional innervation of chromaffin cells suggesting that they are induced by trans-synaptic signals. This apparent regulation of bFGF expression by splanchnic nerve terminals suggests that bFGF could serve as an intermediary in the neural regulation of adrenal medullary cells. Indeed, bFGF stimulates the proliferation of cultured chromaffin cells from neonatal and adult rat adrenal medullae (Stempele et al., 1990; Frodin and Gammeltoft, 1994; Tischler et al., 1993). Unlike NGF, bFGF stimulates proliferation of adrenal medullary cells in the presence of glucocorticoids (Stemple et al., 1988) and therefore could mediate hyperplasia and vascularization of the adult adrenal medulla. bFGF also induces phosphorylation of tyrosine hydroxylase (TH) protein (Rydel and Greene, 1987) and stimulates expression of the TH and pEK genes (Puchacz et al., 1993). Through these effects bFGF could increase the synthesis and release of catecholamines and enkephalins.

To determine if bFGF may mediate afferent stimulation-induced adaptations in the adrenal medulla, we examined whether trans-synaptic or hormonal stimulation of the adrenal medullary cells affects the production and bioavailability of bFGF. As an experimental model, we have used primary cultures of adrenal chromaffin cells which allow the investigation of cellular responses to specific signals in a defined environment. Cultured chromaffin cells express bFGF (Blottner et al., 1989; Puchacz et al., 1993) and retain many functional characteristics of chromaffin cells in vivo (Livett et al., 1983; for review see Stachowiak and Goc, 1992). The synthesis and release of catecholamines, enkephalins and other hormones in cultured adrenal medullary cells are regulated by neurotransmitters and hormones similarly as in the adrenal gland. Signal transduction pathways (second messengers, protein kinases, and nuclear transcriptional factors) that mediate those effects have been characterized (for review see Stachowiak and Goc, 1992; Stachowiak et al., 1994; Goc and Stachowiak, 1994). Using this model we demonstrate that the stimulation of defined neurotransmitter and hormonal receptors and their known second messenger systems increases the expression of the bFGF gene and the synthesis of bFGF protein and affects its subcellular distribution. Our results suggest that bFGF acts as a nuclear mediator of genomic responses to afferent cell stimulation.

Materials and Methods

Materials

Recombinant bovine bFGF (18 kD) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN) and the culture media were from Sigma Chem. Co. (St. Louis, MO). The remaining reagents were obtained from Boehringer Mannheim Biochemicals, Sigma Chem. Co., and Du Pont NEN (Boston, MA).

Cell Cultures

Chromaffin cells from bovine adrenal medullae (BAMC) were prepared by collagenase digestion (Stachowiak et al., 1990*b*) and purified by density gradient centrifugation in Renografin as described in (Puchacz et al., 1993). For RNA and protein analysis, cells were plated in 30-mm wells at a density of 4×10^6 cells/well. Initially, cultures were maintained in Dulbecco's modified Eagles's/F-12 medium containing 10% fetal calf serum, and then changed to serum-free medium supplemented with fatty acid-free bovine serum albumin (0.1 mg/ml) 2 d after plating. At that time many cells became flattened some with neurite-like processes. Drug treatments were initiated after cells were in serum-free medium for 24 h. Forskolin and phorbol esters (PMA or 4 alpha phorbol didecanoate) were added with 0.007% DMSO as vehicle. DMSO had no effect on the expression of bFGF in BAMC.

Isolation of RNA and Northern Analysis of TH and bFGF mRNA

Total RNA was isolated from individual culture dishes by guanidinium isothiocyanate/cesium chloride gradient procedure (Stachowiak et al., 1990*b*). The concentration of total RNA was estimated spectrophotometrically (A260nm). RNA was fractionated by electrophoresis on 1% agarose/5% formaldehyde gels. Ethidium bromide-stained gels were photographed, and the 18S rRNA bands were scanned to estimate the amounts of RNA in individual lanes as previously described (Stachowiak et al., 1990*d*; Goc et al., 1992). RNA was transferred to nitrocellulose or Nytran membranes (Schleicher & Schuell, Keene, NH) and hybridized to ^{32}P-labeled bovine bFGF (Abraham et al., 1986*b*) or tyrosine hydroxylase cDNA (D'Mello et al., 1989). To ascertain that individual lanes contained a similar amount of total RNA and that RNA was completely transferred to the membrane, we compared the amounts of ethidium bromide-stained ribosomal RNA on gel before transfer and on gel and membrane after transfer.

Western Analysis of bFGF

Cultured BAMC (2.5×10^7) were gently detached with a rubber policeman, washed twice with PBS by spinning in microcentrifuge at 3,000 rpm, and were then lysed. Alternatively, cells were washed with PBS and lysed directly on culture dish. Both approaches revealed the same changes in the content of bFGF proteins in BAMC subjected to various treatments. Cells were lysed in 2 ml of ice cold lysis buffer containing 1% NP-40, 0.5% deoxycholate, 20 mM Tris (pH 7.5), 5 mM EDTA, 2 mM EGTA, 150 mM NaCl, and protease inhibitors: 0.01 mM PMSF, 10 ng/ml aprotinin, 10 ng/ml leupeptin and 10 ng/ml pepstatin. Lysates were clarified by centrifugation at 20,000 *g* for 15 min at 4°C, frozen in liquid nitrogen, and stored at −80°C. Centrifugation of lysates yielded a small amount of insoluble material. Protein concentrations in supernatants were determined using Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, VA). Supernatants from different culture dishes, after minor volume adjustments to provide equal amounts of total proteins (0.5–1.5 mg), were incubated with prewashed heparin sepharose for 2 h at 4°C. After washing three times with 20 mM Tris HCl buffer containing 1.0 M NaCl, heparin sepharose-bound proteins were eluted directly into SDS-sample buffer, boiled, and then applied to a 12% SDS-polyacrylamide slab gel with a 3% stacking gel (Florkiewicz and Sommer, 1989). After electrophoresis proteins were transferred to nitrocellulose as described by Florkiewicz and Sommer (1989) and Puchacz et al. (1993). Membranes were blocked in buffer containing 5% powdered milk and incubated with guinea pig anti-18-kD bFGF antisera diluted 1:500 (Florkiewicz and Sommer, 1989), or with bFGF monoclonal antibody (Transduction Laboratories, Lexington, KY) followed by rabbit anti-mouse IgG. Immune complexes were stained by incubation with ^{125}I-labeled protein A. Apparent molecular sizes of bFGF isoforms were determined by comparison to concurrently run protein weight standards. As additional controls, in some experiments extracts of: (*a*) COS cells transfected with plasmids expressing 18-kD bFGF; or (*b*) human glioma cells expressing 18, 22 and 24 kDa bFGF proteins were included. In some experiments media combined from two dishes (5×10^7 cells) were processed and analyzed in a way similar to the cell extracts to determine whether bFGF was present in the media conditioned by BAMC. The levels of individual bFGF isoforms were estimated by densitometric scanning of autoradiograms using Beckman DU70 spectrophotometer. Autoradiograms were exposed for different length of time to ensure that the signals were in the linear range.

Analysis of bFGF in Nuclear and Cytoplasmic Fractions

Isotonic lysis of cells and isolation of nuclei and cytoplasmic fraction were performed as described by Boyle et al. (1985) with minor modifications. Cells were incubated in isotonic nuclear buffer containing 0.1% digitonin, 5 mM sodium phosphate, pH 7.4, 50 mM NaCl, 150 mM sucrose, 5 mM KCl, 2 mM dithiothreitol, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mM PMSF, 10 μM leupeptin, 1 μg/ml aprotinin, 25 μg/ml soybean trypsin inhibitor, and 5 μg/ml pepstatin A, and collected by gently rinsing the plate with buffer. Nuclei were collected from lysed cells by centrifuging at 500 *g* for 10 min at 4°C. The postnuclear supernatant was further centrifuged at 40,000 *g* for 30 min at 4°C to remove cellular debris (P40), and the new supernatant (S40) was reserved for the analysis of cytoplasmic (cytosol + solubilized plasma membrane) bFGF. The crude nuclear pellet was resuspended in nuclear buffer, centrifuged through a cushion of TN buffer (2.5 mM Tris HCl, pH 7.4, 10 mM NaCl) containing 30% sucrose at 1,000 *g* for 10 min at 4°C, and the supernatant was discarded. Nuclei were resuspended in 50 μl of nuclear buffer. Protein content was determined in the aliquots of nuclear and cytoplasmic fractions. All samples were frozen in liquid nitrogen and stored at −80°C until use. After thawing, the nuclear fraction was extracted with 19 volumes of cell lysis buffer containing 1% NP-40, 0.5% deoxycholate. Lysates were clarified by centrifugation at 20,000 *g* for 15 min at 4°C. Nuclear or cytoplasmic extracts from different cell preparations, after small adjustments to yield the same amounts of proteins, were purified on heparin sepharose, and subjected to Western analysis for bFGF as described below.

Immunohistochemical Staining for bFGF

BAMC were plated in eight-chamber slides (Lab-Tek, Naperville, IL). After treatment, the cells were washed with ice-cold PBS and fixed with 2.6% paraformaldehyde in PBS. After washing in PBS with 10 mM glycine, cells were permeabilized for 5 min with 1% Triton X 100 in PBS and blocked in PBS containing 1% bovine serum albumin, 2% goat serum, and 10 mM glycine for 30 min. As primary antibody we used either monoclonal bFGF antibody (UBI, Lake Placid, NY) or polyclonal bFGF antibodies raised in guinea pig against human recombinant bFGF (Florkiewicz and Sommer, 1989), or in rabbit against a fusion protein joining the 155 residues of human bFGF to the amino terminus of β-galactosidase (gift from Dr. J. Abraham, California Biotechnology Inc., Mountain View, CA). Antibodies were diluted in blocking buffer and added to cells for 24–48 hr. After washing with PBS, cells were incubated either with secondary biotynylated antibody for 12-24 h, followed by incubation with avidin-biotin peroxidase (1:400), or with horseradish-conjugated secondary antibody. Cells were washed in PBS and treated with diaminobenzidine-hydrogen peroxide solution, washed again, and coverslipped. Endogenous peroxidase activity was exhausted with 0.1% H_2O_2 prior to the incubation with the secondary antibody. The specificity of bFGF immunostaining in BAMC was indicated by several observations: (*a*) staining was not observed when primary bFGF antibody was omitted or substituted with preimmune serum (Puchacz et al., 1993); (*b*) similar nuclear and cytoplasmic staining was observed using different polyclonal or monoclonal antibodies, and peroxidase or immunofluorescent staining (see Figs. 4 and 8); and (*c*) neutralization of bFGF antibody with an excess of 18-kD bFGF also reduced bFGF staining (not shown). Specificity of the primary antibodies used for histochemical analysis was also ascertained by Western analysis. All bFGF antibodies recognized the same 18-, 22-, and 24-kD bFGF antigens.

Immunofluorescent staining of bFGF was performed by incubating permeabilized BAMC with monoclonal antibodies against bFGF. As the secondary antibody we used fluorescein-conjugated goat anti-rabbit IgG.

Plasmid Constructions

The human bFGF promoter fragment was a gift from R. Florkiewicz (Whittier Institute, La Jolla, CA) (Shibata et al., 1991). The luciferase expression vectors were constructed in our laboratory using the promoter-

less pGL_{2basic} (Promega Biotec, Madison, WI). (−1,800/+314)bFGFLuc was constructed by isolating a 2.1-kb XhoI/SalI fragment from pF2.1-CAT (Shibata et al., 1991) and cloning it into the XhoI site of pGL_{2basic}. (−1,000/+174)bFGFLuc was constructed by inserting a 1,174-bp XhoI/HindIII fragment isolated from pF1.2CAT and inserting it into the HindIII site of pGL_{2basic}. (−650/+314)bFGFLuc was constructed from (−1,800/+314)bFGFLuc by digesting with NsiI and MluI followed by blunt ending with T4 polymerase and recircularization and ligation. (−512/+314)bFGF-Luc was constructed from (−1,800/+314)bFGFLuc using Bal31 nuclease. (−274/+174)bFGFLuc was constructed by digesting (−1,000/+174)bFGF-Luc with NheI followed by recircularization and ligation. The ends of all deletions were determined by DNA sequencing. RSV*b*GAL was described in Edlund et al. (1985).

Transfections and Treatments of BAMC

Cells were transfected using either calcium phosphate precipitation, lipofectin, or electroporation. All transfection methods produced similar results indicating that different cell treatments did not influence regulation of the transfected bFGFLuc genes. For the calcium phosphate method, cells (1×10^6) were treated with 3 μg/35 mm plate of plasmid/calcium phosphate precipitate for 4-8 h followed by two washes with PBS. DME/F12 containing 0.25% BSA was then added. Lipofection was done using 12.5 μl of lipofectin (GIBCO BRL, Gaithersburg, MD) per 1×10^6 cells with 3 μg of plasmid/35 mm plate (Goc and Stachowiak, 1994). The lipofectin was removed 6–8 h later and the cells were refed with DME/F12 with 5% FCS. 24 h later the cells were washed and refed with DME/F12 with 0.25% BSA. The most reproducible transfections of BAMC were obtained by electroporation. The electroporation was performed using 150 μg of plasmid/1 $\times 10^7$ cells in a 0.4-cm-wide cuvette and a Gene Pulser (Bio-Rad Laboratories, Cambridge, MA). An electric pulse of 240 V, using a capacitance of 960 μF was applied in the buffer described by Chu et al. (1988). The electroporated cells were then added to 35 mm plates at a density of 2×10^6 cells/well. Cells were maintained for at least 24 h in serum-free DME/F12 with 0.25% BSA prior to treatment with drugs. Concentrations of forskolin and PMA were 10 and 0.2 μM, respectively, and the time of treatment is given in the text. Luciferase assays were done using a Promega luciferase assay system on a scintillation counter set to single photon counting (Goc and Stachowiak, 1994). Transfection efficiency of different plasmids in the same experiment was normalized by measuring directly the intracellular content of transfected plasmid DNA. To measure the amount of DNA directly, we used a modified procedure (Goc and Stachowiak, 1994) of Keller and Alwine (1984). After luciferase assays were performed the remaining cell lysate was diluted to 500 μl using sterile water. The extract (20–50 μl) was denatured using 0.2 N NaOH containing 5 mM EDTA (pH 8.0), neutralized with Tris-HCl (pH 8.0), and blotted onto a nylon membrane using a dot blot manifold. Filters were hybridized to a probe made from pGem 7Z(−) (Promega Biotec) and exposed for autoradiography. Films were then quantified using a microtiter plate reader at 595 nm. The amount of intracellular DNA was determined by comparing to a serial dilutions of plasmid DNA applied directly to the membrane. In several experiments that examined the effects of forskolin or PMA on the expression of transfected bFGFLuc genes, the results across individual plasmids were normalized also by co-transfecting the bFGFLuc plasmid with RSVbGAL and measuring β-galactosidase and luciferase activities in the same extract.

Treatment of BAMC with bFGF Antisense and Sense bFGF Oligonucleotides

Modified (phosphoro-thio-DNA backbone) sense (s-bFGF) and anti-sense (as-bFGF) oligonucleotides were custom-synthesized by Bio-Synthesis Inc. (Lewisville, TX). Their sequence was identical (s-bFGF; 5'GGG ACC AUG GCA GCC 3') or complementary (as-bFGF; 5'GGC TGC CAT GGT CCC 3) to the 5' region of human bFGF mRNA (Abraham, 1986b), including the first methionine codon (underlined). Oligomers were purified by gel filtration, ethanol precipitated, lyophilized to dryness, and dissolved in culture medium. Cells were incubated with 2.5 μM oligonucleotides for 48 h.

Statistics

Analysis of variance (ANOVA) was used to test for overall statistical significance. Comparison between groups with $N > 4$ was made using Neuman-Keuls post hoc test. Direction effects were inferred based on the relation of mean values.

Results

Stimulation of Acetylcholine and Angiotensin II Receptors Increases Content of bFGF Proteins

To characterize bFGF proteins expressed by cultured BAMC, total cell lysates were purified with heparin-sepharose and subjected to western analysis with bFGF antibodies and ^{125}I-protein A. Polyclonal or monoclonal bFGF antibodies gave the same results. In confirmation of earlier observations (Grothe and Unsicker, 1990; Presta and Rifkin, 1991; Puchacz et al., 1993) we detected proteins that migrated as three separate bands (see Figures 1–4). Their molecular weights (18, 22, and 24 kD) were similar to those of human bFGF proteins (Florkiewicz and Sommer, 1989). Human and rat bFGFs are generated from alternate utilization of CUG or AUG translation initiation codons (Florkiewicz and Sommer, 1989; Powell and Klagsbrun, 1991). Isoforms of bFGF in bovine adrenal medullary cells are likely to be generated through the same mechanisms. In the total cell lysates the ratios of individual isoforms varied slightly between cell preparations, but the 24- and 22-kD isoforms were less abundant than the 18-kD bFGF in all of the experiments (Table I).

Most splanchnic nerve terminals innervating adrenal chromaffin cells contain and release acetylcholine. To determine whether stimulation of cholinergic receptors could increase bFGF content we incubated BAMC with carbachol. Treatment with carbachol increased the content of all three bFGF isoforms. These increases were detected after 6 h and achieved maximal levels (2.6–3.4-fold) after 24 h (Fig. 1 *A*). Carbachol-induced increases in the levels of individual 18-, 22-, or 24-kD isoforms were similar and were statistically significant (Fig. 1, legend). To ascertain whether bFGF induction was mediated by cholinergic receptors, we pretreated cells with a mixture of nicotinic and muscarinic antagonists. When added before the carbachol, D-tubocurarine, and atropine prevented induction of bFGF proteins, while antagonists alone had a small increasing effect on 18-kD bFGF (Fig. 1 *B*). To determine which subtype of cholinergic receptors may induce changes in bFGF content we treated cells with specific nicotinic (nicotine) or mus-

Table I. Relative Abundance of Individual bFGF Isoforms in BAMC

Cell fraction	18 kD	22 kD	24 kD
		(Percent of all isoforms)	
Total cell extract	55 ± 2	27 ± 2‡	18 ± 1‡
Cytosol	55 ± 4	30 ± 2‡	15 ± 2‡§
Nuclei	44 ± 4	25 ± 2‡	31 ± 4*‖

Cells were fractionated into cytosolic (S40) and nuclear fractions as described in the Materials and Methods. Heparin-sepharose purified extracts were subjected to Western analysis using bFGF antibody and ^{125}I-labeled protein A. Autoradiograms were scanned using Beckman 70 spectrophotometer. The abundance of individual bFGF isoforms was expressed as percent of the total bFGF content (18- + 22- + 24-kD isoforms) in each sample. Numbers show mean ± SEM from nine or ten culture dishes obtained from four (cell fractions) or two separate experiments (*total cell extracts*) in which cells were incubated in control medium. ANOVA demonstrated an overall statistically significant difference in the relative abundance of individual bFGF isoforms ($P < 0.005$) and significant difference between cell fractions ($P < 0.01$). Newman-Keuls test: * $P < 0.05$, ‡ $P < 0.01$, different from 18-kD bFGF; § $P < 0.05$ different from 22-kD bFGF; ‖ $P < 0.05$ different from 24 kD in cytosol.

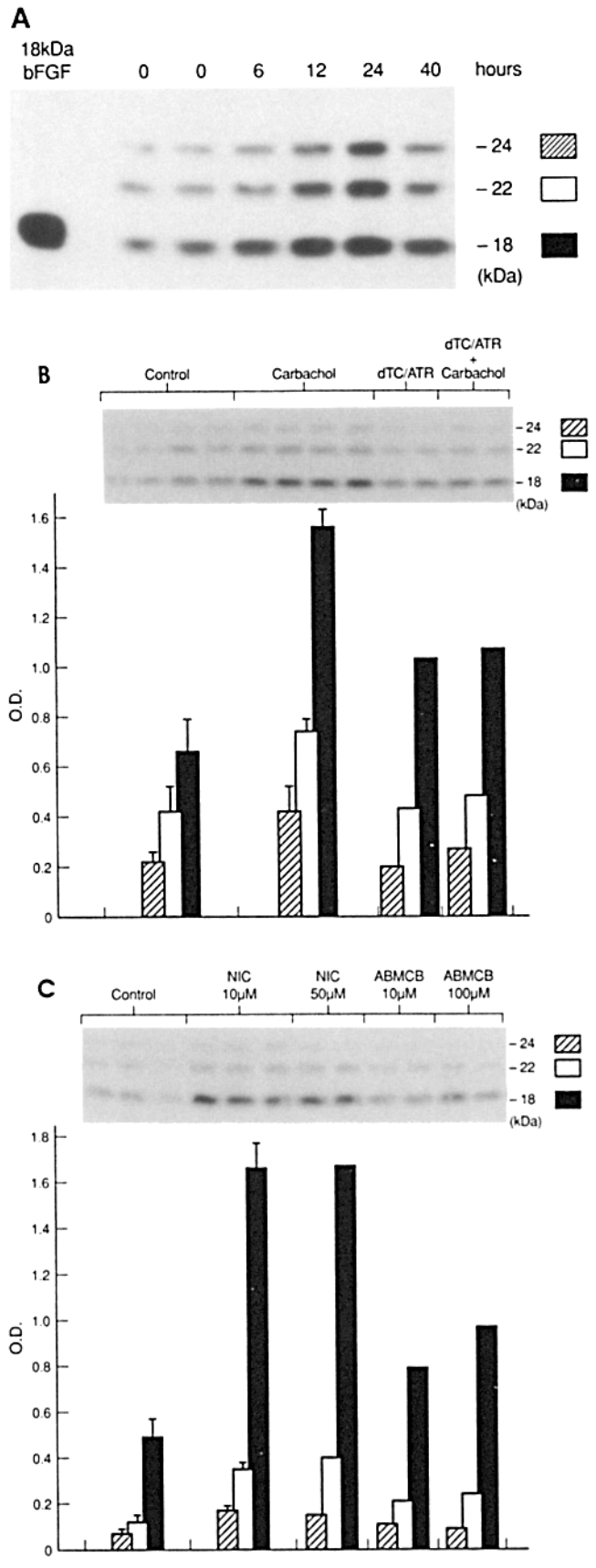


Figure 1. Cholinergic stimulation increases content of bFGF proteins in adrenal medullary cells. BAMC were incubated with cholinergic agonists and antagonists or in control drug-free medium. All cells were harvested at the same time. Total cell extracts were prepared from individual culture dishes (3×10^7 of plated cells/dish). Extracts containing the same amount of protein were purified with heparin sepharose and subjected to Western blot analysis as described in the methods. Autoradiograms (*A–C*) were produced by overnight exposure. Bars represent results of densitometric scanning of autoradiograms. Media combined from two dishes were purified with heparin-sepharose and were processed similar to the cell extracts. The presence of bFGF proteins was not detected in the medium from control cells or cells treated with nicotine or ABMCB even after 1 wk of exposure (not shown). (*A*) Cells were incubated with 200 μM carbachol for the indicated periods of time. (*B*) Cells were incubated in control medium or with 200 μM carbachol for 24 h. Acetylcholine receptors antagonists; d-tubocurarine (*dTC*; 100 μM) and atropine (*ATR*; 1.5 μM) were added to some dishes 30 min before carbachol. Control cultures were treated with d-TC and ATR in the same way. Results of densitometric scanning are summarized on the bar graph. Bars show means ± SEM from (*n*) or averages of two similarly treated culture samples. The analysis of the combined results from experiments (*A*) and (*B*) showed that the increases in bFGF content produced by 24 h of treatment with carbachol (*18 kD*, 2.4 ± 0.1-fold; *22 kD*, 2.1 ± 0.33-fold; *24 kD*, 2.2 ± 0.3-fold) were statistically significant (*18 kD*, $P < 0.001$; *22 kD*, $P < 0.05$; and *24 kD*, $P < 0.01$). (*C*) Cells were treated with nicotinic (nicotine, *NIC*) or muscarinic (acetyl-β methylcholine bromide, *ABMCB*) agonists for 24 h. Bars show means ± SEM from three or averages of two similarly treated culture samples.

carinic (acetyl-β-methylcholine bromide) agonists. Nicotine produced similar increases in bFGF protein content as carbachol indicating that cholinergic stimulation of bFGF expression is mediated by nicotinic acetylcholine receptors (Fig. 1 *C*). The magnitude of stimulation (2.3-fold for 24-kD bFGF, 3.1-fold for 22-kD bFGF and 3.4-fold for 18-kD bFGF) was essentially the same with 10 or 50 μM nicotine. Acetyl-β-methylcholine bromide also increased the levels of bFGF proteins. However, its effects were smaller than the increase produced by nicotine.

Cells in the adrenal medulla contain a high density of angiotensin receptors that stimulate expression of genes encoding transcriptional factors, catecholamine synthesizing enzymes, and proenkephalin (Stachowiak et al., 1990*a,c,d*). Incubation of BAMC for 24 h with a stable analog of angiotensin II, 100 nM sar^1-angiotensin II (s^1-AII), increased bFGF protein levels (Fig. 2). The average increase in 18-kD bFGF content (2.3-fold) was greater than the increases in 22- or 24-kD bFGF (1.4-fold). The increases in the levels of bFGF proteins were elicited by low (0.2–2 nM) concentrations of s^1-AII indicating that they were mediated by high-affinity angiotensin II receptors (not shown).

Expression of bFGF Is Increased by Stimulation of Two Major Signaling Pathways—cAMP and Protein Kinase C

Stimulation of acetylcholine receptors in adrenal medullary cells increases concentrations of cAMP and the activities of cAMP-stimulated protein kinase A (PKA) and calcium/phospholipid-stimulated protein kinase C (PKC) (Guidotti and Costa, 1974; Tuominen et al., 1992). Stimulation of angiotensin receptors also increases PKC activity and cAMP levels in the adrenal medullary cells (Stachowiak et al.,

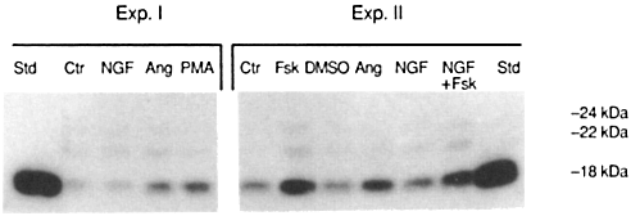


Figure 2. Effect of angiotensin II, nerve growth factor, forskolin, or PMA on bFGF protein levels in bovine adrenal medullary cells. Cells were treated for 24 h with 0.2 μM PMA, 5 μM forskolin (*FSK*), or vehicle (0.007% DMSO). Other culture dishes were incubated with NGF (50 ng/ml), sar[1]-angiotensin II (*Ang*, 100 nM), or without drugs (*Ctr*). *Std*, standard, 10 ng of 18-kD human recombinant bFGF.

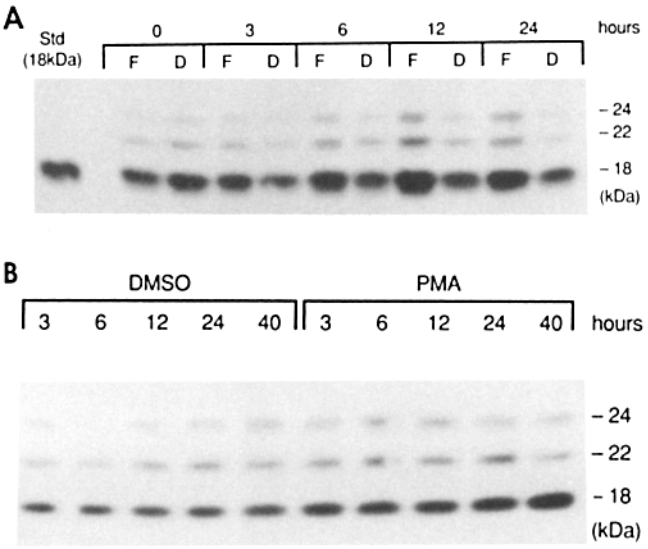


Figure 3. Time course of forskolin- (*A*) and PMA- (*B*) induced changes in bFGF protein levels. Adrenal medullary cells were incubated with 5 μM forskolin (*F*), 0.2 μM PMA or 0.007% DMSO (*D*) for the indicated periods of time.

1990*c*; Boarder et al., 1988). To determine whether the activation of cAMP signaling pathway increases the levels of bFGF, we treated BAMC with adenylate cyclase-stimulating forskolin. In cells treated 24 h with forskolin, the levels of all three bFGF proteins increased (Fig. 2). These changes were observed in each of four experiments that used different BAMC preparations. The average forskolin-induced increases in bFGF proteins levels were 3.9 ± 1.2 (24 kD), 3.4 ± 0.7 (22 kD), and 2.3 ± 0.7-fold (18 kD). An experiment that examined the time course of forskolin-induced changes in bFGF protein levels is shown in Fig. 3 *A*. The results of three independent experiments are summarized in Table II. Incubation with forskolin for 3 to 6 h increased bFGF protein levels minimally. Incubation for 12 h was required to produce larger increases. Maximal increases occurred after 24 h and were sustained during 40 h of treatment. The increases in the levels of high molecular weight bFGF isoforms (24 and 22 kD) exceeded the increases in the levels of 18-kD bFGF.

Cholinergic and AII stimulation of PKC activity could be mimicked by treating BAMC with phorbol esters (Stachowiak et al., 1990*c*). Incubation of BAMC with PKC-stimulating phorbol ester PMA produced gradual, time-dependent increases in the content of bFGF proteins (Fig. 3 *B*). Unlike forskolin, PMA increased predominantly the content of 18-kD bFGF isoform (Figs. 2 and 3 *B*). Thus, stimulation of cAMP and PKC signaling pathways increases cellular content of bFGF in BAMC, and these two pathways exert different effects on the expression of individual bFGF isoforms.

cAMP and PKC Increase Content of bFGF Proteins in Different Subcellular Compartments

To assess the functional significance of the induction of bFGF proteins we examined their localization in adrenal medullary cells. The presence of bFGF proteins was not detected in the medium conditioned by control BAMC or in cells treated with cholinergic agonists (legend to Fig. 1), forskolin or PMA (not shown). In a previous study, we detected bFGF-immunoreactivity (bFGF-IR) in both the cytoplasm and the nuclei of BAMC (Puchacz et al., 1993). To determine the subcellular localization of changes in bFGF content during stimulation of adenylate cyclase or PKC, BAMC were stained with antibodies against bFGF (Fig. 4). Cytoplasmic bFGF staining had a granular character, consistent with the reported presence of bFGF in secretory vesicles (Westerman et al., 1990). Incubation with PMA (12 h) enhanced intensity of bFGF staining in cytoplasm of many cells but had no effect on nuclear staining. In contrast, treatment with forskolin increased bFGF-IR in the nuclei of the majority of cells without increasing cytoplasmic bFGF-IR (Fig. 4 *A*). In many

Table II. Effect of Forskolin on bFGF Content in BAMC

bFGF isoform	3 h		6 h			12 h		24 h		40 h*
	D	F	D	F	D	F	D	F	D	F
	(bFGF content − percent of DMSO control)									
24 kD	73	151 ± 40	103 ± 14	155 ± 37	117 ± 9	279 ± 95	91 ± 6	386 ± 117	92	359
22 kD	77	115 ± 26	113 ± 9	128 ± 33	110 ± 11	265 ± 65	290 ± 20	264 ± 34	32	322
18 kD	88	131 ± 20	109 ± 9	145 ± 3	110 ± 11	153 ± 17	188 ± 3	145 ± 22	82	170
	(2)	(3)	(3)	(3)	(3)	(3)	(3)	(3)	(3)	(1)

Cells were treated with 5 μM forskolin (*F*) or with 0.007% vehicle DMSO (*D*) for 3, 6, 12, 24, or 40 h. In two experiments (Figs. 2 and 3) additional control groups of nontreated cells were included. The levels of bFGF in those cells were similar to those in DMSO-treated cells. The results are expressed as percent of mean bFGF levels in DMSO-treated samples. The levels of bFGF isoforms were estimated by Western analysis (see Fig. 3). Numbers represent results of densitometric scanning of autoradiograms. Numbers show mean ± SEM of (N) samples obtained in independent experiments using different BAMC preparations. * 40-h treatment with forskolin or DMSO was carried out in a separate experiment. One-way ANOVA revealed that overall forskolin significantly increased 24- and 22-kD bFGF ($P = 0.05$) and 18-kD bFGF ($P < 0.005$) content.

cells treated with forskolin the intensity of cytoplasmic staining was reduced. Similar results were obtained in three independent experiments. Fig. 4 *B* shows the time course of forskolin-induced changes in bFGF-IR. Increases in nuclear bFGF-IR were detected in some cells after 1–3 h and reached maximum levels at 12 h. In the nucleus, areas with stronger bFGF-IR, which may represent bFGF in the nucleoli, were found (Fig. 4 *B* and see Fig. 8).

We used Western analysis of fractionated cells to determine subcellular localization of individual bFGF isoforms and to verify changes in bFGF distribution in forskolin- and in PMA-treated cells. bFGF proteins were detected both in the cytoplasmic (S40) and in the nuclear fractions (Fig. 5). The P40 pellet containing cellular debris obtained during isolation of the cytoplasmic fraction contained only trace amounts of bFGF and was not included in further experiments. The nuclear and cytoplasmic fractions contained all three isoforms of bFGF. The contents of 18- and 22-kD bFGFs were six- and fourfold higher, respectively, in the cytoplasm than in the nuclei. The content of 24-kD bFGF was similar in the cytoplasm and in the nuclei. In the cytoplasm the most abundant was the 18-kD isoform, the next was 22 kD, and the least abundant was 24-kD bFGF (Fig. 5 and Table I). In the nuclei the most and the least abundant were the 18- and 22-kD isoforms, respectively. The relative abundance of the 24-kD bFGF isoform was twofold higher in the nuclear than in the cytoplasmic fraction.

Fig. 6 shows an autoradiogram of an experiment in which BAMC were treated with either 5 μM forskolin or 0.2 μM PMA, or they were incubated in control medium containing 0.007% DMSO. Quantitative results obtained from densitometric scanning of autoradiograms produced in three independent experiments are summarized in Tables III and IV. Incubation with forskolin increased levels of all three bFGF isoforms in the nuclear fraction isolated from BAMC (Fig. 4 and Table III). The time course of those changes was similar to that observed in histochemical experiments (Fig. 4 *B*). An elevation in the nuclear content of 18-, 22-, and 24-kD bFGF was observed after 3 h of treatment with forskolin. Incubation for 6 and 12 h produced additional increases. Initially, at 3 h, the elevation of bFGF proteins in the nuclei was accompanied by a small transient decrease in their cytoplasmic content. Later, at 6 and 12 h the contents of bFGF isoforms in the cytoplasmic fraction returned to control levels.

Incubation of BAMC with PMA increased the cytoplasmic content of all three isoforms of bFGF (Fig. 6 and Table IV). These changes were detectable after 3 h. The nuclear content of 24- and 22-kD bFGF proteins was not affected by PMA, while small 1.5- and 1.3-fold increases in 18-kD bFGF in the nuclear fraction were observed after 6 and 12 h of treatment.

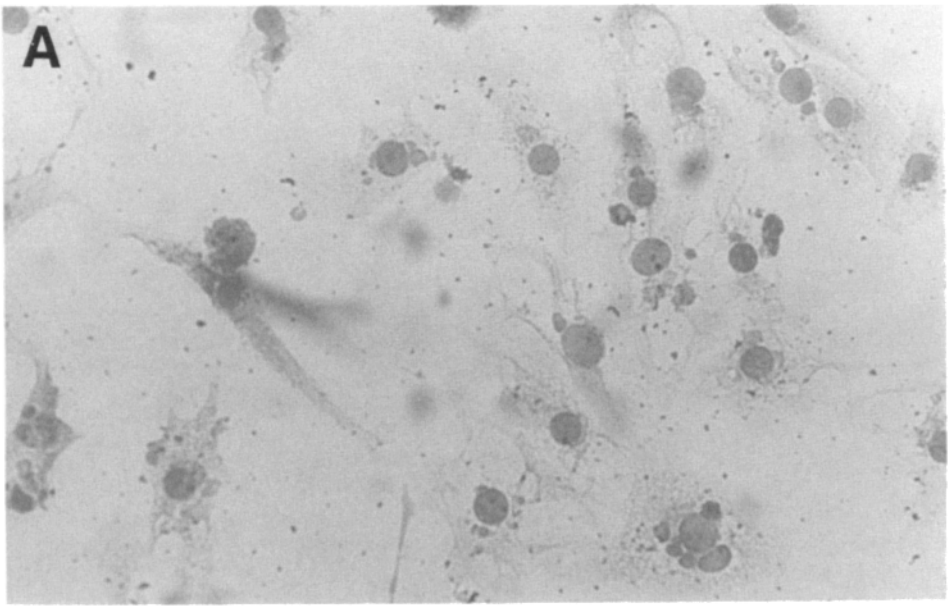
The results of cell fractionation experiments are consistent with the histochemical analyses. They show that increases in bFGFs content produced by PKC stimulation occur in the cytoplasm, whereas stimulation of adenylate cyclase results in accumulation of bFGF proteins in the nuclei.

Increase in bFGF Protein Content Reflects Increased Expression of the bFGF Gene

Short-term (3 h) treatment with forskolin caused no increase in the total cellular bFGF content (Table I). At that time, the increase in nuclear bFGF content was accompanied by a reduction in the cytosolic bFGF levels, suggesting translocation of bFGF from the cytoplasm to the nucleus (Table III). After longer treatment, bFGF content in cytosol returned to control levels, and the nuclear content showed further increases (Table III) in parallel with the total cellular content of bFGF (Table I). To determine whether delayed elevation of nuclear bFGF may reflect reduced turnover or increased synthesis of bFGF, we examined the effects of cycloheximide on forskolin-induced increase in bFGF content. The concentration of cycloheximide (20 μM) used effectively inhibits synthesis of c-*fos* (Stachowiak, M. K., unpublished observations) and general protein synthesis in BAMC (Kelner and Pollard, 1985). Incubation with cycloheximide for 12 h did not reduce bFGF content in the nuclei or in the cytoplasmic fraction (Fig. 7). This demonstrates that bFGF has a low turnover rate; therefore, a reduced metabolism of bFGF could not account for the forskolin-induced increase in bFGF content. Cycloheximide prevented the forskolin-induced increase in nuclear bFGF content consistent with the increase in bFGF synthesis. However, the inhibition of forskolin induction of nuclear bFGF by cycloheximide could be indirect, resulting from inhibited synthesis of other regulatory proteins. Therefore, to specifically inhibit the synthesis of bFGF protein we incubated BAMC with 15-mer antisense (as-bFGF) or sense bFGF (control; s-bFGF) oligonucleotides. This as-bFGF was shown to inhibit expression of bFGF in other cell types (Morrison, 1991). The effects of using as-bFGF were similar to those of cycloheximide. A 2-d incubation with as-bFGF had little or no reducing effect on basal bFGF content, but it prevented an increase in nuclear bFGF content by forskolin (Fig. 8). Together these results suggest that the induction of bFGF protein by forskolin reflects increased synthesis of bFGF rather than decreased turnover.

To determine whether the induction of bFGF proteins reflects increased expression of the bFGF gene, we analyzed the abundance of bFGF mRNA. Bovine bFGF cDNA hybridized predominantly to RNAs that migrated as a 7-kb and a smaller 4.6-kb bands (Fig. 9). Their size was similar to the molecular size of bFGF mRNAs found in other cells and thought to be generated through the use of different polyadenylation sites (Abraham et al., 1986*a*,*b*; Kurokawa et al., 1987). Smaller RNAs that could represent 1.8–2.6-kb antisense bFGF RNA transcribed from the opposite strand of bFGF gene (Zuniga et al., 1993) were not detected. Incubation of BAMC with carbachol increased the levels of 7-kb bFGF mRNA, and, to smaller extent, that of 4.6-kb mRNA (Fig. 9 *A*). These changes were observed after 1.5 h and reached a maximum after 6 h. The level of the 7-kb bFGF mRNA was also increased by forskolin (Fig. 9, *B* and *C*) and by PMA (Fig. 9 *C*). Changes induced by PMA (Fig. 9 *B*) or by forskolin (not shown) were detected after 1.5 h, like the changes produced by carbachol. They were sustained during a continuous 24-h treatment. The effects of PMA and forskolin on bFGF mRNA levels were synergistic suggesting activation through different but interacting mechanisms (Fig. 9 *C*).

In addition to trans-synaptic and hormonal factors, adrenal medullary cells are also regulated by various growth factors. Weich et al. (1991) reported that bFGF stimulates ex-



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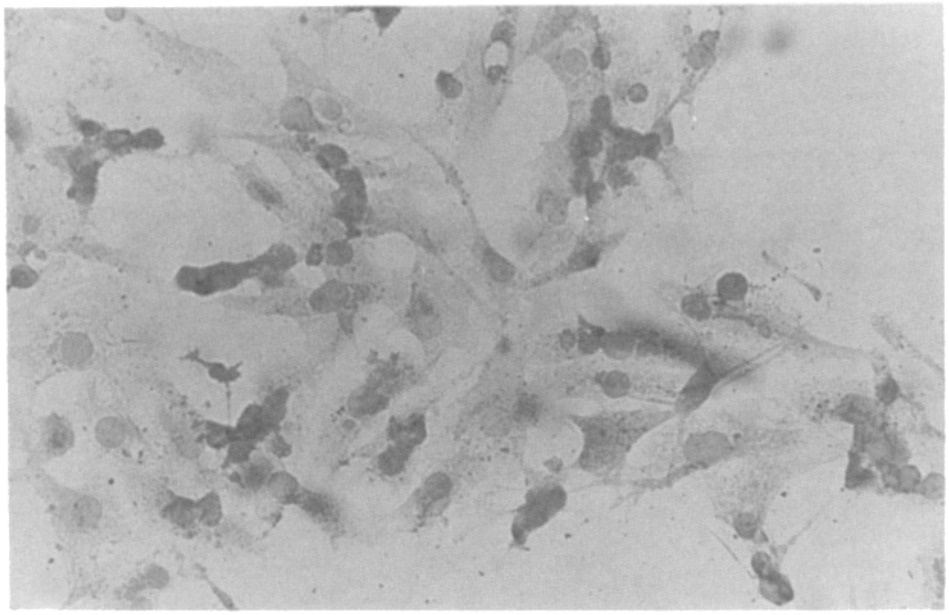
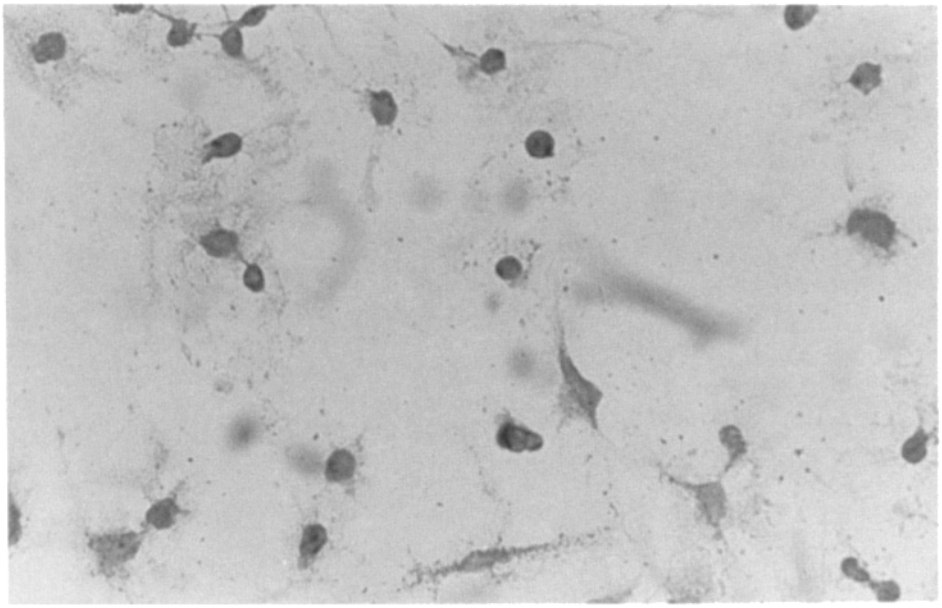
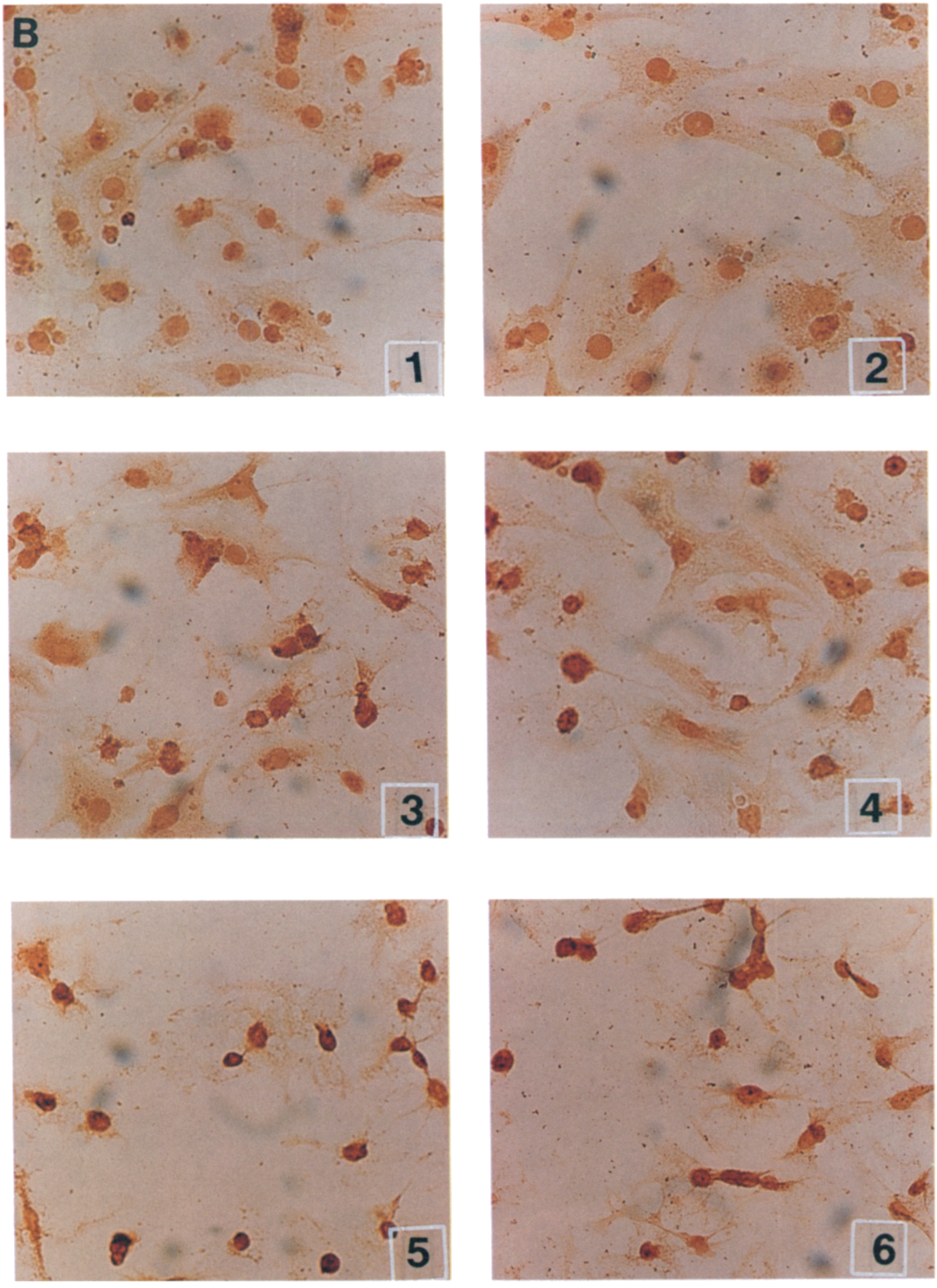


PMA



FORSKOLIN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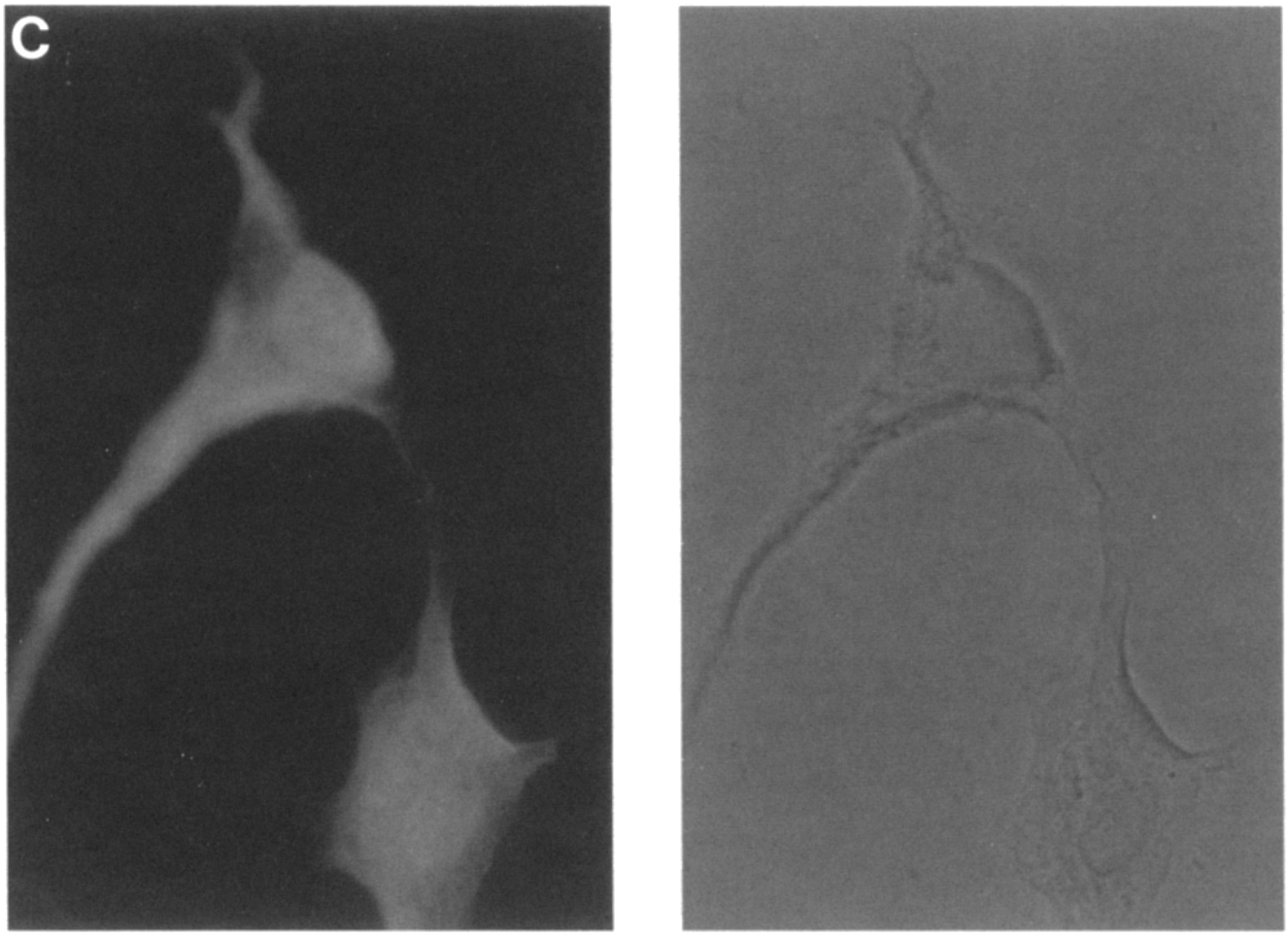

Figure 4. Immuno-histochemical localization of bFGF in BAMC. Cells were stained with polyclonal bFGF (*A* and *B*) or monoclonal (*C*) bFGF antibodies as described in the methods. (*A*) Effect of forskolin and PMA on subcellular distribution of bFGF immunoreactivity in adrenal medullary cells. (*a*) Control; (*b*) BAMC treated for 12 h with 0.2 μM PMA; and (*c*) BAMC treated for 12 h with 5 μM forskolin. (*B*) Time course of forskolin-induced changes in bFGF immunostaining in bovine AM cells. *1*, Control; *2*, cells treated with 0.007% DMSO for 24 h; *3*, forskolin 1 h; *4*, forskolin 3 h; *5*, forskolin, 12 h; *6*, forskolin 24 h. No difference in bFGF immunoreactivity was observed between DMSO-treated and nontreated cells. (*C*) Localization of bFGF immunofluorescence in bovine adrenal medullary cells. BAMC were permeabilized with 1% Triton X-100, and incubated sequentially with monoclonal bFGF antibody and fluorescein-conjugated rabbit anti-mouse IgG.

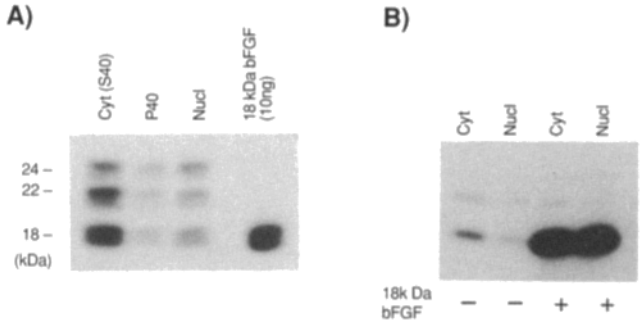

Figure 5. bFGF content in nuclear (*Nuc*) and cytoplasmic (*Cyt*) fractions of BAMC. Fractions were isolated as described in the text. P40 represents pellet obtained after centrifugation of postnuclear crude cytoplasmic fraction at 40,000 *g* that yielded the S40 cytoplasmic fraction. For direct comparison of bFGF content the entire fractions were subjected to heparin-sepharose purification and were loaded on gel. (*A*) BAMC were maintained in control serum-free medium. (*B*) Cells were incubated in control medium with or without 10^{-9} M 18-kD bFGF.

pression of its own gene in bovine endothelial cells. We have previously reported that the incubation of BAMC with exogenous 18 kD results in a time-dependent increase in bFGF immunoreactivity in the nuclei (Puchacz et al., 1993). To determine whether this increase could reflect stimulation of the synthesis of the endogenous bFGF, we examined the effects of bFGF on its own mRNA levels. Treatment with 0.5 nM 18-kD bFGF that increased TH and proenkephalin mRNA levels in BAMC (Puchacz et al., 1993) did not induce bFGF mRNA (Fig. 9 *B*). It also failed to enhance the induction of bFGF mRNA by forskolin. We analyzed the levels of bFGF isoforms in nuclear and in cytoplasmic fractions. Incubation with 18-kD bFGF had no effect on the levels of 22- or 24-kD bFGF (Fig. 5 *B*). A dramatic increase in 18-kD bFGF content in nuclear and cytoplasmic fractions probably represents accumulation of exogenous 18-kD bFGF.

NGF, which stimulates tyrosine kinase activity, also did not increase the content of bFGF proteins in BAMC (Fig. 2). Treatment with forskolin and NGF produced similar increases as did forskolin alone (Fig. 2).

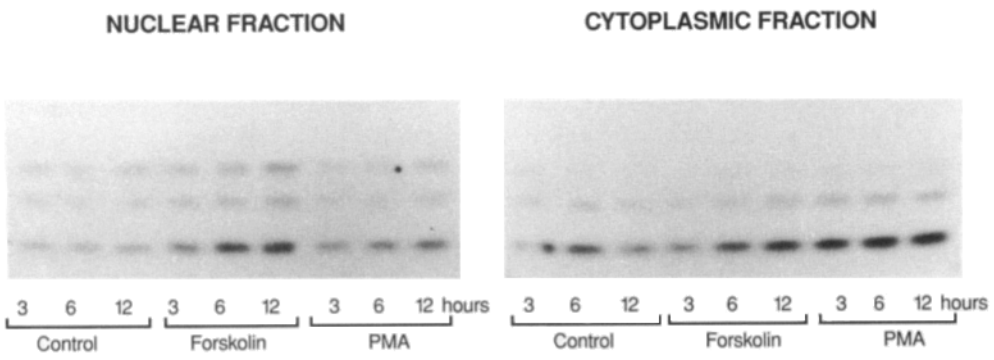

Figure 6. Effect of forskolin and PMA on subcellular distribution of bFGF isoforms in BAMC. Cells were incubated with 5 μM forskolin, 0.2 μM PMA, or 0.007% DMSO for indicated periods of time. Cellular fractions were isolated and bFGF was analyzed as on Fig. 5.

Transcriptional Activation Is Mediated by the 5'-flanking Region of the bFGF Gene

Characterization and Regulation of the bFGF Promoter in BAMC. Using human genomic clones, Shibata et al. (1991) showed that the bFGF gene contains a single transcription start site and mapped its core promoter to sequences located between −480 and +187 (relative to transcription start site). To investigate the molecular mechanisms underlying the induction of bFGF mRNA, we constructed a luciferase reporter plasmid with the promoter sequences isolated from the upstream region of the human bFGF gene. Our initial construct, (−1,000/+174)bFGFLuc contained sequences from −1,000 to +174 bp (+1 transcription start site). In BAMC transiently transfected with this construct, both forskolin and PMA produced time-dependent increases in luciferase expression with maximal induction after 30 h of treatment (Fig. 10). The extent of forskolin and PMA induction varied between 2–5- and 5–20-fold, respectively, depending on the preparation of BAMC used. The phorbol ester (4-α phorbol didecanoate), which does not stimulate PKC, had no effect on the expression of (−1,000/+174)-bFGFLuc (not shown). Treatment of transfected cells with both forskolin and PMA produced a synergistic increase in luciferase activity (Fig. 11). The photon counts obtained in cells transfected with the promoterless pGL_{2basic} plasmid were similar as the reagent blanks and showed no increases in cells treated with PMA or forskolin (Fig. 11, legend).

These results indicate that the −1,000/+174-bp fragment of the bFGF gene contains the *cis*-elements sufficient for transcriptional regulation by PMA and cAMP similar to that observed in the endogenous bFGF gene.

Table III. Effect of Forskolin on bFGF Content in Cytosolic and Nuclear Fractions of BAMC

	3 h		6 h		12 h	
bFGF isoforms	D	F	D	F	D	F
			(bFGF content − percent of DMSO control)			
24-kD nuclei	89 ± 1	186 ± 12	96 ± 18	255 ± 23	123[a]	309 ± 10
Cytoplasm	106 ± 8	94 ± 14	114 ± 8	128 ± 27	80 ± 7	100 ± 10
22-kD nuclei	84 ± 8	160 ± 17	92 ± 3	207 ± 13	123 ± 6	210 ± 26
Cytoplasm	110 ± 8	77 ± 3	107 ± 9	94 ± 1	83 ± 3	90 ± 26
18-kD nuclei	98 ± 2	183 ± 6	89 ± 6	238 ± 8	114 ± 4	283 ± 22
Cytoplasm	106 ± 11	79 ± 5	105 ± 13	109 ± 3	89 ± 3	114 ± 23

Cells were treated with 5 μM forskolin (*F*) or with 0.007% DMSO (*D*) as a vehicle for 3, 6, or 12 h. The results are expressed as percent of mean bFGF levels in DMSO-treated samples. Numbers show mean ± SEM of three independent experiments using different BAMC preparations, except [a] −2 experiments. Forskolin had an overall statistically significant effect on nuclear content of all three bFGF isoforms ($P < 0.001$; one-way ANOVA).

Table IV. Effect of PMA on bFGF Content in Cytosolic and Nuclear Fractions of BAMC

	3 h		6 h		12 h		24 h	
bFGF isoforms	D	PMA	D	PMA	D	PMA	D	PMA
				(bFGF content − percent of DMSO control)				
24-kD nuclei	89	130	74	115	123	91	115	70
Cytoplasm	106	202	119	292	98	267	76	356
22-kD nuclei	125	109	68	105	104	96	104	67
Cytoplasm	104	149	110	183	73	178	115	211
18-kD nuclei	110	112	90	149	81	133	121	107
Cytoplasm	82	175	128	205	79	198	117	265

Cells were treated with 0.2 μM PMA or with 0.007% DMSO (vehicle) for the indicated period of time. The results represent the average from two culture dishes and were combined from three separate experiments using different BAMC preparations. The results are expressed as percent of mean bFGF levels in DMSO-treated control samples.

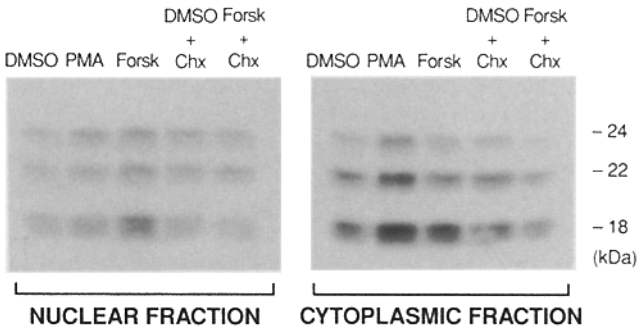

Figure 7. Effect of protein synthesis inhibitor on nuclear translocation of bFGF. Cells were treated for 12 h with 5 μM forskolin (*Fsk*), 0.2 μM PMA, or 0.007% DMSO. Cycloheximide (*Chx*) was added to dishes 30 min before forskolin or DMSO and was present in the medium for the remainder of the experiment.

Identification of Regions of the bFGF Promoter Required for cAMP and PKC Regulation. As shown by our mRNA analysis and transfection experiments, bFGF expression is regulated by cAMP and PMA at the transcriptional level. To identify the regions of the bFGF promoter that are involved in the stimulation of this gene, we conducted a deletion analysis of the bFGF promoter (Fig. 11). Deletion of −1,000- to −274-bp promoter fragment in (−1,000/+174)bFGFLuc produced a significant ($P < 0.0001$) decrease in stimulation by PMA from 5.9- to 1.8-fold and reduced the basal promoter activity (Fig. 11). It also abolished the effect of forskolin and reduced ($P < 0.0001$) stimulation by combined treatment with forskolin and PMA. This outcome indicated that −1,000/−274-bp promoter region supports basal expression of bFGF gene in BAMC and mediates stimulation by forskolin and PMA. Further deletion analysis was performed using (−1,800/+314)bFGFLuc as the starting construct. It contains a larger upstream fragment of the bFGF gene (−1,800 to +314 bp). It produced the same results as those obtained for the −1,000/+174-bp fragment (Fig. 11) showing that the additional −1,800 to −1,000 bp are not needed for cAMP and PMA regulation. Furthermore, the sequences downstream from the transcription start site (+174 to +314 bp), coding for the untranslated region of bFGF mRNA, do not influence the regulation of bFGF promoter activity in BAMC. Plasmid (−650/+314)bFGFLuc showed the same stimulation by forskolin, PMA, and by forskolin + PMA as (−1,800/+314)bFGFLuc. (−650/+314)bFGFLuc has deleted an AP-1-like sequence (TTACTCA). We conclude that this sequence does not participate in cAMP or PMA stimulation of the bFGF gene promoter in transfected BAMC. The next deletion identified a promoter regulatory region located between −651 and −512 bp. Deletion of this region significantly inhibited forskolin stimulation ($P < 0.0005$) and reduced PMA stimulation ($P < 0.0001$). Stimulation by combined treatment with forskolin and PMA was reduced ($P < 0.001$) to the level of PMA alone. This region may also be involved in controlling the basal promoter activity since its deletion decreased the nonstimulated expression of bFGFLuc (Fig. 11).

In the deletion construct (−274/+174)bFGFLuc, stimulation by PMA was reduced twofold ($P < 0.01$) compared to (−512/+314)bFGFLuc indicating that sequences between −512 and −274 also mediated PMA stimulation. (−274/+174)bFGFLuc still exhibited a residual stimulation by PMA and by PMA + forskolin indicating that it contains an additional regulatory element(s).

In conclusion, our deletion analysis indicated the presence of multiple regulatory regions in the bFGF gene promoter. Region I (−650 to −512 bp) mediated the induction by cAMP and PMA and supported basal promoter activity. Two other regions were also involved in the stimulation by PKC. One of them (region II, −512 to −275 bp) does not contain any known PMA-responsive site. The other (region III, −274 to +174 bp) contains sequence homologous to the AP1 binding site located at −243 to −236 bp within a dyad symmetry element.

Regulation of the bFGF gene by cAMP appeared to be mediated by a novel *cis/trans* mechanism, since we found no sequences similar to any known cAMP responsive elements (CRE) in the promoter region responsible for cAMP stimulation. Since induction of many genes by cAMP is mediated by the constitutively expressed CREB factor and is not sensitive to translational inhibitors (Walton and Rehfuss, 1992) we examined whether induction of bFGF mRNA is affected by cycloheximide. Addition of 20 μM cycloheximide to culture medium prior to forskolin prevented induction of bFGF mRNA, suggesting that cAMP stimulates bFGF gene expression by acting through inducible or unstable intermediate protein(s) (Fig. 12). The induction of TH mRNA known to be mediated by CRE and CREB (Huang et al., 1991) was still observed in the presence of cycloheximide.

Discussion

Afferent Stimulation Increases Expression of bFGF in AM Cells

bFGF is a pleiotropic growth factor that has a broader target cell specificity than NGF or other known neurotrophic proteins. Specific effects of bFGF on cell growth, differentiation or survival are the consequence of the time- and location-restricted expression of bFGF and bFGF receptors in the nervous system. Observations that the overexpression of bFGF leads to deregulated growth and neoplastic transformation of cells (Couderc et al., 1991; Quatro et al., 1991) further emphasize the importance of the fine regulation of bFGF expression in the nervous system. In contrast to a long list of the known effects of bFGF on neuronal, glial, or vascular cells, little is known about the mechanisms that control expression of bFGF and how it produces its biological effects. bFGF is often viewed as a trophic/mitogenic protein deposited in extracellular matrix, that acts in a tonic fashion on membrane receptors of the surrounding cells (Baird and Walicke, 1989; Vlodavsky et al., 1991). The present study offers a different view of bFGF as an intracellular cytoplasmic-nuclear protein, expression of which is regulated by trans-synaptic and hormonal stimuli, and which may serve as a direct intracrine mediator of genomic responses to afferent cell stimulation.

In this study we show that stimulation of acetylcholine receptors increases the content of bFGF proteins in adrenal

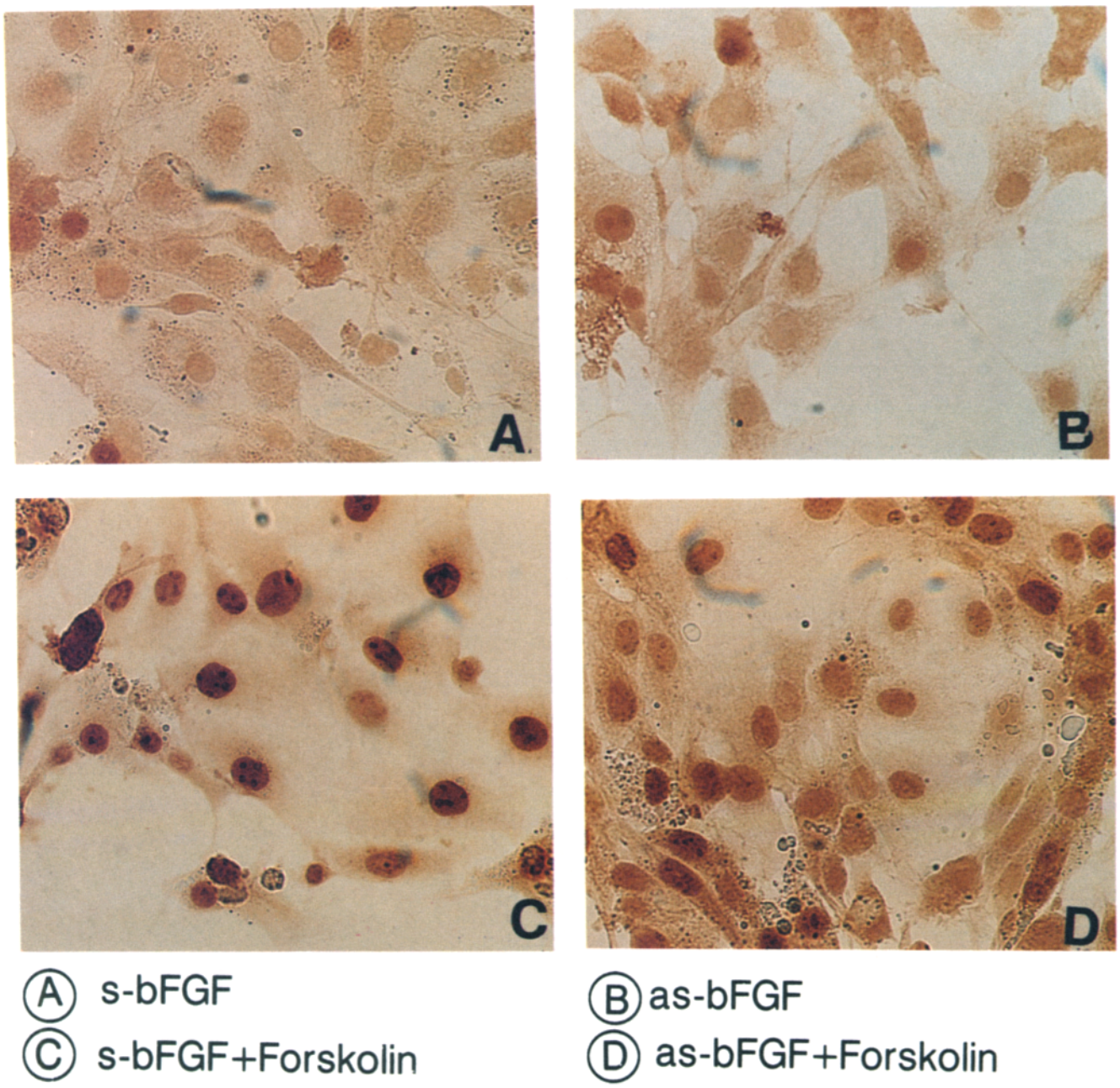

Figure 8. Effect of antisense bFGF oligonucleotide on forskolin-induced increase in bFGF protein content. BAMC were incubated for 48 h with 2.5 μM sense (*s-bFGF*) or antisense (*as-bFGF*). During last 24 h, cells were treated with 5 μM forskolin or 0.007% DMSO (control). Cells were stained with monoclonal bFGF antibodies as on Fig. 4.

medullary cells. Nicotine in concentrations that specifically activate nicotinic receptors was more potent than the muscarinic agonist acetyl-β methylcholine bromide. Hence trans-synaptic induction of bFGF is likely to be mediated by a nicotinic subtype of acetylcholine receptors. To the best of our knowledge, this is the first demonstration that the activation of neurotransmitter receptors increases expression of a growth factor protein. Acetylcholine is the main neurotransmitter and secretagogue released from the splanchnic nerve terminals onto adrenal medullary cells and controls the functions and biology of adrenal medullary cells. Acetylcholine and its receptors are also broadly distributed in the central and peripheral nervous systems. Their functions include opening ion channels, changing membrane potential in neuronal and glial cells, or the stimulating release and synthesis of neurotransmitters (Lukas and Bencherif, 1992). By in-

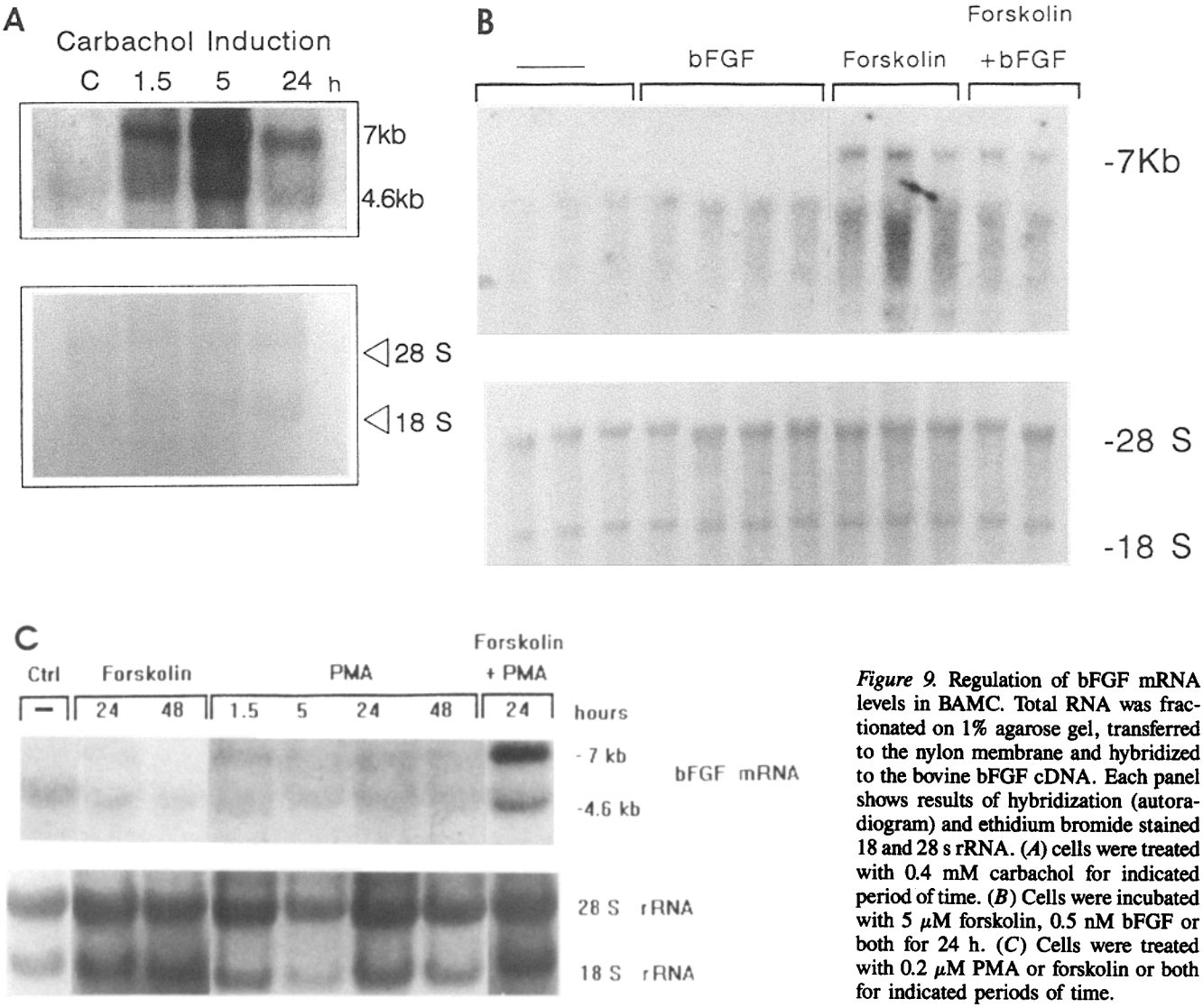

Figure 9. Regulation of bFGF mRNA levels in BAMC. Total RNA was fractionated on 1% agarose gel, transferred to the nylon membrane and hybridized to the bovine bFGF cDNA. Each panel shows results of hybridization (autoradiogram) and ethidium bromide stained 18 and 28 s rRNA. (*A*) cells were treated with 0.4 mM carbachol for indicated period of time. (*B*) Cells were incubated with 5 μM forskolin, 0.5 nM bFGF or both for 24 h. (*C*) Cells were treated with 0.2 μM PMA or forskolin or both for indicated periods of time.

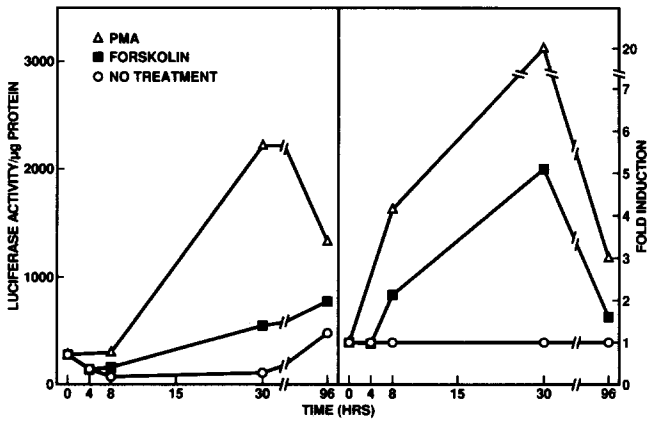

Figure 10. Induction of bFGFLuc by forskolin and PMA. Cells were transfected with 3 μg of (−1,000/+174)bFGFLuc using calcium phosphate as described in the Materials and Methods. Cells were first starved for serum for 24 h followed by drug treatment at the indicated times. Extracts were prepared and protein measured. 200–400 μg of protein was used/luciferase assay.

creasing expression of bFGF, acetylcholine could also regulate cell proliferation and neurite and synapse formation in developing and mature nervous tissue.

Angiotensin II, an effector peptide of the renin-angiotensin system, is another potent activator of adrenal medullary cells. Stimulation of AII receptors induces expression of c-*fos*, c-*jun*, and other related early response genes, followed by transcriptional activation of the TH and PEK genes and increased synthesis and release of adrenal medullary hormones (for review see Stachowiak and Goc, 1992). The present study indicates that the scope of functions of AII receptors in adrenal medullary cells includes the induction of bFGF protein. AII receptors are found on many peripheral and central neurons and glial cells (Sumners et al., 1989). Itoh et al. (1993) reported recently that angiotensin increases synthesis of a bFGF-like factor in smooth muscle cells and indicated that this effect is essential for the mitotic response to AII stimulation. Thus, the increase synthesis of bFGF may be a general response to angiotensin stimulation, common to a variety of cells.

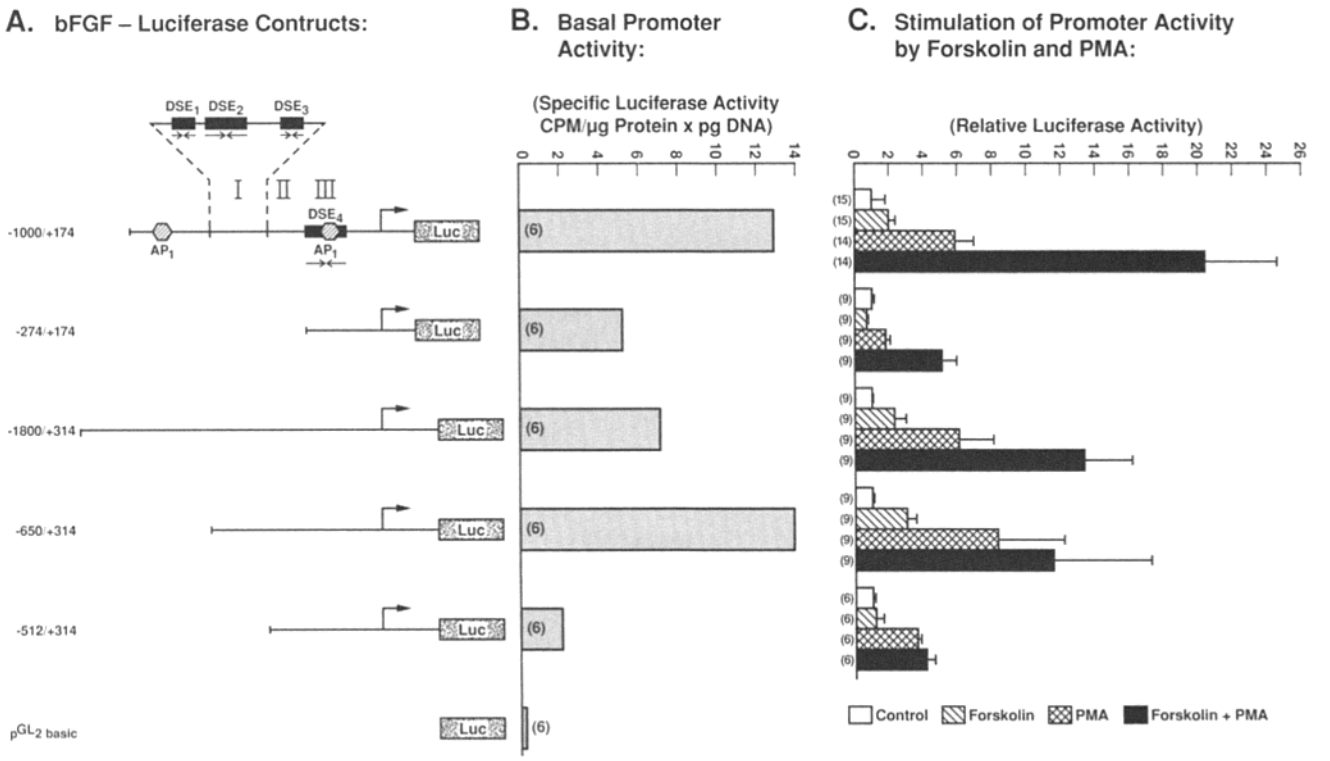

Figure 11. Deletion analysis of the bFGF promoter and mapping of the cAMP and PMA-responsive regions. BAMC were transfected using DNA lipofection or electroporation. (*A*) Schematic representation of the different bFGFLuc expression vectors used in this study. The arrow indicates the transcriptional start site of the bFGF gene. The dashed lines indicate the regions of the bFGF promoter implicated in cAMP regulation and basal level expression of the bFGF promoter (*I*) and in PMA stimulation (*II* and *III*). Also shown are the locations of the AP-1–like sites. The dyad symmetry elements (DSEs) shown are potential regulatory sites discussed in detail in the text. Plasmid construction is described in the Materials and Methods. (*B*) Basal promoter activities of individual bFGFLuc plasmids. Luciferase activity was normalized to the amount of plasmid DNA present in transfected cells. Results are the mean of six culture dishes from two different cell preparations. (*C*) The bar graphs shows levels of induction of luciferase activity in cells treated for 24 hours with forskolin, PMA, or forskolin plus PMA. Luciferase activity in individual samples was normalized per amount of plasmid DNA present in transfected cells or per amount of proteins in cell extract used for luciferase assay. Both methods produced similar results and data were combined. The same results were obtained when luciferase activity was normalized to β-galactosidase activity expressed from co-transfected RSVbGAL plasmid (not shown). For each plasmid, luciferase activity was expressed relative to the activity in nonstimulated cells. Bars represent means ± SEM of (*n*) culture dishes in two or three independent experiments. ANOVA demonstrated an overall statistically significant effect of all treatments (forskolin $P < 0.001$; PMA $P < 0.0001$; and Forskolin + PMA, $P < 0.00001$) on the expression of bFGFLuc genes. Results of the comparisons between individual groups using Newman-Keuls post hoc test are discussed in the text. Luciferase activities in promoterless pGL_{2Basic} plasmid were as follows: *Control*, 0.213 ± 0.14; *Forskolin*, 0.25 ± 0.15; *PMA*, 0.20 ± 0.07 ($n = 8$); *Forskolin + PMA*, 0.36 ± 0.11 cpm/µg protein × pg DNA ($n = 6$).

Direct stimulation of adenylate cyclase with forskolin or PKC with PMA increased bFGF protein content, indicating that cholinergic and AII stimulations may be mediated by these two signaling pathways. cAMP and PKC are two second messengers that could be accessed by a variety of neurotransmitter or hormonal receptors. Therefore transsynaptic and hormonal regulation of bFGF expression could represent a general phenomenon of the entire nervous system. Receptors that activate both pathways or the simultaneous activation of cAMP and PKC by different receptors may have an enhanced effect on bFGF expression as indicated by the synergism between adenylate cyclase and PKC stimulations.

bFGF was previously shown to stimulate its own gene expression in bovine endothelial cells (Weich et al., 1991) or in human astrocytes (Stachowiak, M. K., *Int. Soc. Dev. Neurosci. Symp.* San Diego, CA, 1994). Although bFGF stimulated expression of *c-fos*, *c-jun*, TH, and proenkephalin genes in BAMC (Puchacz et al., 1993), it had no effect on the expression of the endogenous bFGF gene (Fig. 9 *B*). Bovine adrenal medullary cells also contain functional receptors of NGF (Acheson et al., 1984). NGF, however, had no effect on bFGF content (Fig. 3). Therefore, neither bFGF nor NGF is likely to mediate regulation of bFGF gene expression in adrenal medullary cells, and induction of bFGF is not a universal response to receptor stimulation.

Increased Expression of the bFGF Gene Underlies Induction of bFGF Protein

The experiments using cycloheximide and antisense bFGF oligonucleotides indicated that bFGF has a low turnover rate and that the induction of bFGF protein reflects increased synthesis of bFGF proteins. The increased expression of the bFGF was indicated by the results of our northern analyses. Bovine bFGF cDNA hybridized to a 7- and 4.6-kb bFGF mRNAs that may be generated through the use of different polyadenylation sites.

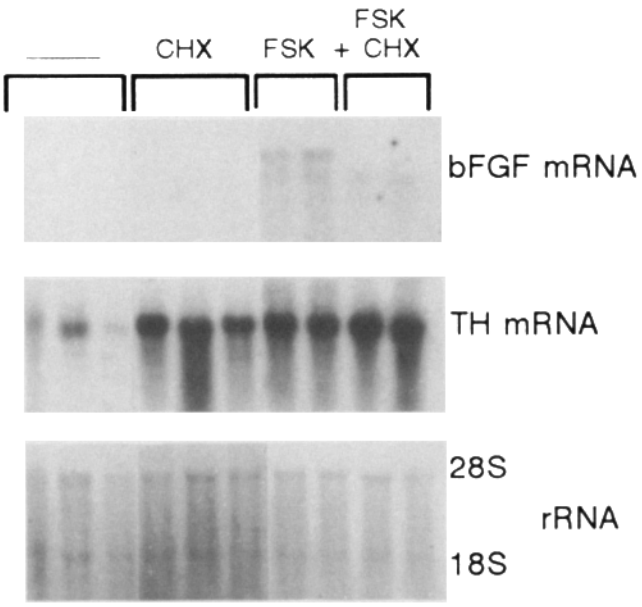

Figure 12. Cycloheximide prevents the induction of bFGF mRNA by forskolin. Cells were incubated for 12 h in control medium or with 5 μM forskolin. Cycloheximide was added to some cultures 30 min before the start of incubation. The same membrane was hybridized to bovine TH cDNA, washed, and rehybridized to bFGF cDNA.

In nonstimulated BAMC the levels of 7-kb bFGF mRNA were below or close to the detection limit of our assay. This suggests that either this mRNA is very unstable and is degraded immediately after translation, or that the 4.6-kb bFGF mRNA is translated into bFGF proteins in nonstimulated BAMC. Stimulation of BAMC increased levels of both mRNAs indicating that each of them could contribute to the induction of bFGF protein.

Transcriptional Stimulation of the bFGF Gene Is Mediated through Unique Regulatory Sequences

Induction of bFGF mRNA could reflect an increase in mRNA stability as well as increased transcription of the bFGF gene. Transfection experiments using bFGF promoter–reporter gene constructs demonstrated that changes in steady-state levels of bFGF mRNA reflect, at least partially, increased transcription of bFGF gene and are mediated through the sequences upstream from its core promoter region. Those experiments indicate that sequences between −1,000 and +174 bp of the bFGF gene support basal expression of bFGF in BAMC and are sufficient for transcriptional regulations by cAMP and PKC similar to those observed in the endogenous bFGF gene. An additional 800-bp upstream or 140-bp downstream from this region did not influence regulation of bFGF promoter activity. Similar to the induction of endogenous bFGF mRNA, stimulation of bFGF promoter activity by cAMP and PKC was synergistic indicating participation of separate interacting pathways. Deletion analysis revealed that cAMP and PKC stimulations were mediated partially through different promoter regions. cAMP stimulation was mapped to a 138-bp promoter fragment (region I) 512-bp upstream from the transcription start site. This same region was also found to support basal promoter activity in BAMC. The majority of cAMP-inducible genes have either CRE (TGACGTCA) or AP-2 binding elements (CCCCAGGC). Only a very small number of genes do not belong to these categories (for review see Walton and Rehfuss, 1992). The −650/−512-bp region of the bFGF gene shows no homology to CRE, AP-2, or atypical elements conferring cAMP stimulation in the human steroid 21-hydroxylase ($P\text{-}450_{C21}$) or bovine steroid 17-α-hydroxylase. Computer analysis of −650/−512 bp of the bFGF gene promoter fragment revealed the presence of sequences homologous to sites recognized by topoisomerase II and strings of A/T rich sequences. Both are characteristic of the matrix attachment regions found in other genes (Phi-Van and Stratling, 1990). Another interesting feature of this region is the presence of three unique dyad symmetry elements (Fig. 7; *DSE*). These types of elements are known to extrude cruciform-like structures using energy derived from negative DNA supercoiling (Mizuuchi et al., 1982) and to bind dimeric transcriptional factors (Johnson and McKnight, 1989).

Induction of reporter gene by cAMP through CRE or AP-2 sites is rapid (within 30 min) and insensitive to cycloheximide, a translational inhibitor, indicating recruitment of preexisting transcriptional factors (Walton and Rehfuss, 1992). Induction of bFGF mRNA by forskolin was sensitive to cycloheximide suggesting participation of inducible or rapidly metabolized intermediary proteins. The induction of TH mRNA, which may be mediated by CRE (Huang et al., 1991), was unaffected by cycloheximide. This suggests that different transcription regulatory factors confer cAMP responsiveness to bFGF and TH genes in the adrenal medullary cells. We propose that the stimulation of bFGF promoter by cAMP is mediated through *cis* elements different from those currently known and may require novel transcription factors.

Sequences that mediate PKC stimulation were mapped within 511 to −264 bp (region II) of the bFGF gene promoter. The cAMP-responsive region I (−650 to −512 bp) may also participate in PKC stimulation. These two regions do not contain known PMA-responsive elements (AP-1, AP-2, NFkB, SRE) found in other genes, suggesting a mechanism of activation different from those currently known. Residual stimulation by PMA of (−274/+174)bFGFLuc (stimulation was not observed in promoterless pGL_{2basic} plasmid) suggested that region III of bFGF promoter (−274 to +174 bp) may contain additional PKC-responsive element(s). One putative regulatory element may be the TGAGTCA sequence. It is homologous to the AP-1 binding element found in other genes (Angel et al., 1987), and it overlaps with a 20-bp dyad symmetry element. Expression of c-*fos*, c-*jun*, and related proteins, components of the AP-1 factor is increased during hormonal or trans-synaptic stimulation of the adrenal medullary cells (Stachowiak et al., 1990*a*,*d*; Goc et al., 1992). It is possible that they may play a supporting role in the activation of bFGF by PKC in adrenal medullary cells.

Regulation of bFGF Protein Content in Adrenal Medullary Cells Also Involves Posttranscriptional Mechanisms

Although stimulation of both cholinergic and AII receptors increased bFGF protein content, the pattern of changes produced by neurotransmitter and hormonal stimulations was

different. The acetylcholine analog, carbachol, produced similar increases in the levels of all three isoforms whereas AII predominantly increased the 18-kD bFGF isoform. Even more distinct differences were observed after direct stimulation of individual second messenger pathways. Stimulation of adenylate cyclase predominantly increased 24- and 22-kD bFGF levels, whereas activation of PKC increased the content of 18-kD bFGF more. A differential regulation of bFGF isoforms content has not been described. It could reflect differential translation of individual bFGF proteins or could also be a result of preferential turnover of individual isoforms. In two experiments we found that cells treated with cycloheximide and forskolin had reduced levels of 18-kD bFGF compared with cells incubated with cycloheximide alone, while the levels of the 22- and 24-kD bFGFs were less affected. Thus, in addition to increasing overall synthesis of all bFGF proteins, stimulation of adenylate cyclase could accelerate the metabolism of 18-kD bFGF leading to a larger increase in the content of higher molecular weight bFGF isoforms.

The biological significance of the presence of different bFGF isoforms is still unclear, even though the present study shows that their levels could be differentially regulated during cell stimulation and that their subcellular distribution is different. Earlier studies by Quatro et al. (1991) and Couderc et al. (1991) showed that the over-expression of recombinant 18-kD bFGF has a different effect on cell growth and tumorigenic potential than the overexpression of the higher molecular weight bFGFs. The effects of differential regulation of bFGF isoforms on the biology and functions of adrenal medullary cells remains to be determined.

bFGF May Act as an Intracrine Nuclear Factor in Adrenal Medullary Cells

In this report we established the nuclear and cytoplasmic expression of bFGF proteins in BAMC using subcellular fractionation and western analysis of heparin-sepharose purified cytoplasmic and nuclear extracts and immunohistochemical techniques for the detection of different epitopes of bFGF. All three bFGF isoforms were present in the cytoplasmic and nuclear fractions, consistent with earlier reports that used different cells (Brigstock et al., 1991; Florkiewicz et al., 1991; Gualandris et al., 1993). In the nuclei, levels of all three isoforms were similar. In contrast in the cytoplasm, the 18-kD bFGF was two to fourfold more abundant than the 22- and 24-kD bFGF. The mechanisms controlling nuclear translocation of bFGF are still contested. The differential compartmentalization of individual isoforms may be associated with nuclear localization sequences present in the amino-terminal extension of 22- and 24-kD bFGFs (Couderc et al., 1991). Such a sequence is absent in 18-kD bFGF, which translocates to the nucleus less efficiently than the higher molecular weight isoforms. Nevertheless, since the 18-kD bFGF is also found in the nucleus, additional nuclear translocation sequence(s) may be present downstream from the first AUG codon, or translocation may be mediated in association with a carrier protein.

In the present study the nuclear staining for bFGF was most striking, especially in cells treated with forskolin. Within nuclei, a particularly high concentration of bFGF was found in the nucleoli. The nuclear presence of bFGF immunoreactivity has also been reported in hippocampal neurons (Woodward et al., 1992), in neurons of the trigeminal nerve (Matsuda et al., 1992), in human astrocytes (Stachowiak, M. K., *Int. Dev. Neurobiol.* 1994. San Diego, CA, 1994), and in endothelial cells (Yu et al., 1993). The cytoplasm of BAMC showed a generalized staining for bFGF. Darker staining granules were also found suggesting that a portion of cytoplasmic bFGF may be associated with vesicles. The analysis of higher magnification photographs suggested apparent association of bFGF with the vesicular membrane and its absence inside vesicles (Stachowiak, M. K., unpublished observations). Araujo and Cotman (1992) reported the presence of a bFGF-like substance in cell culture medium of both neuronal or glial cells and its increase after treatment with growth factors or cytokines. Whether this substance, however, represented a partially degraded or intact bFGF protein is unknown. In an attempt to determine whether bFGF is released from BAMC, we analyzed the culture medium for the presence of bFGF using Western blot analysis. No 18-, 22-, or 24-kD bFGF were detected in the medium conditioned for 24 h by control cells or by cells treated with nicotine, PMA, or forskolin. Hence, if bFGF is released from BAMC the amount of extracellular bFGF must be very small relative to its intracellular content. We did not detect bFGF-IR between cultured BAMC that might suggest deposition of bFGF in extracellular matrix. Increases in bFGF content in BAMC were observed regardless whether cells were lysed directly on the dish or were first detached and washed extensively in PBS to remove extracellular matrix. This indicates that the observed changes reflect increases in the intracellular content of bFGF.

As shown for the first time in our studies, the subcellular distribution of bFGF proteins is affected by treatment of cells with agents other than bFGF itself. Angiotensin II or carbachol increased bFGF-IR both in the cytoplasm and in the nuclei of BAMC (not shown). Independent stimulation of individual receptor signaling pathways revealed that subcellular compartmentalization of bFGF is a regulated process. Activation of PKC increased the cytoplasmic content of bFGF proteins, whereas stimulation of adenylate cyclase resulted in nuclear accumulation of bFGFs. Association of bFGF with secretory granules of BAMC (Westerman et al., 1990) indicates that bFGF may be released to extracellular medium, interact with plasma membrane, undergo receptor-mediated endocytosis, and accumulate in the nuclei. Our earlier observation that bFGF added to culture medium accumulated in the nuclei of BAMC suggested such a mechanism (Puchacz et al., 1993, see also Fig. 5 *B*). Although we cannot rule out this mechanism, our data support nuclear translocation independent of release. The absence of intact bFGF in culture medium suggests a direct transfer of bFGF from the cytoplasm to the nucleus. The initial increase in the nuclear content of bFGF in forskolin-treated cells was accompanied by a decrease in cytoplasmic levels of bFGF and preceded the increase in the total cell content of bFGF. During this first phase previously synthesized bFGF could be translocated from the cytoplasm to the nucleus. The long-term accumulation of bFGF in the nucleus during continuous stimulation of adenylate cyclase depended on the synthesis of new bFGF molecules. However, an increase in bFGF synthesis, although necessary, is insufficient for the long-term accumulation of nuclear bFGF. Stimulation of PKC, which also increased expression of the bFGF gene and total bFGF

content, did not lead to nuclear accumulation of bFGF proteins. The mechanisms that regulate cytoplasmic/nuclear distribution of bFGF proteins could involve posttranslational modification of bFGF such as phosphorylation. The rate of nuclear translocation of some transcriptional factors is stimulated by forskolin and correlates with their increased phosphorylation (for review see Whiteside and Goodbourn, 1993). bFGF contains separate consensus sequences that account for its phosphorylation in vitro by cAMP-stimulated PKA and by PKC (Feige and Baird, 1989). It is possible that nuclear translocation of bFGF in BAMC is caused by its phosphorylation with PKA and cytoplasmic accumulation by phosphorylation with PKC. However, whether PKA and PKC phosphorylate bFGF in vivo has not been determined; hence, it is also possible that the same results could be achieved by modulating the activities of associated proteins.

With the observations that bFGFs have no signal sequence, remain cell-associated, and may not be secreted by a classical mechanism, a regulated targeting of those proteins to the nucleus, shown in this study, may be of particular importance. Electron microscopic studies of bFGF immunopositive neurons showed deposits of bFGF-IR in euchromatin but not in heterochromatin, suggesting that endogenous bFGF is conveyed into a transcriptionally active zone of chromatin (Matsuda et al., 1992). In BAMC bFGF is also associated with chromatin and with other nuclear components (Joy, A., J. Shapiro, and M. K. Stachowiak, manuscript in preparation). The existence of the mechanism regulating nuclear/cytoplasmic compartmentalization of endogenous bFGF demonstrated in this study suggests that bFGF may play a role in the regulation of gene expression by extracellular factors. Nakanishi et al. (1992) demonstrated that bFGF affects in vitro gene transcription when added to the nuclear extract. Bouche et al. (1987) showed that bFGF stimulates activity of the RNA polymerase I by acting directly in the nuclei. Hence, accumulation of bFGF in the nuclei could have a direct effect on gene expression and synthesis of ribosomal RNA in BAMC. The direct nuclear action of bFGF provides a novel mechanism through which peptide growth factors/hormones may regulate gene expression. The existence of such a mechanism, in addition to classical signaling systems activated by the membrane receptors, could contribute to specificity of transcriptional effects of different factors that activate the same second messenger systems.

bFGF as a Mediator of Cellular Memory

In the nervous system, information is stored at several different cellular levels (Goelet et al., 1986). Short-term memory is generated through covalent modifications of existing macromolecules that participate in transmission of signals within and between neural cells. The longer-lasting memory of afferent stimulus depends on changes in the synthesis of those macromolecules. Permanent memory may involve structural remodeling of neural tissue (i.e., alterations in neuritic arbors, numbers of synaptic contacts, proliferation of neuronal and glial cells, and angiogenesis) (Bailey and Kandel, 1993). These structural changes require coordinated regulation of a great number of genes coding for the functional and structural components of the nervous tissue. We have proposed that this program of coordinate gene expression is activated by the same pleiotropic growth factors that control ontogenic development. Our finding that the trans-synaptic and hormonal stimulation of adrenal medullary cells increases expression of bFGF is consistent with this proposition.

Hypothetical Mechanisms of Functional and Structural Adaptations to Afferent Stimulation of Adrenal Medulla

In the adrenal medulla, trans-synaptic or hormonal stimulation produces an instantaneous release of catecholamines and enkephalins from the chromaffin cells. Short-term stim-

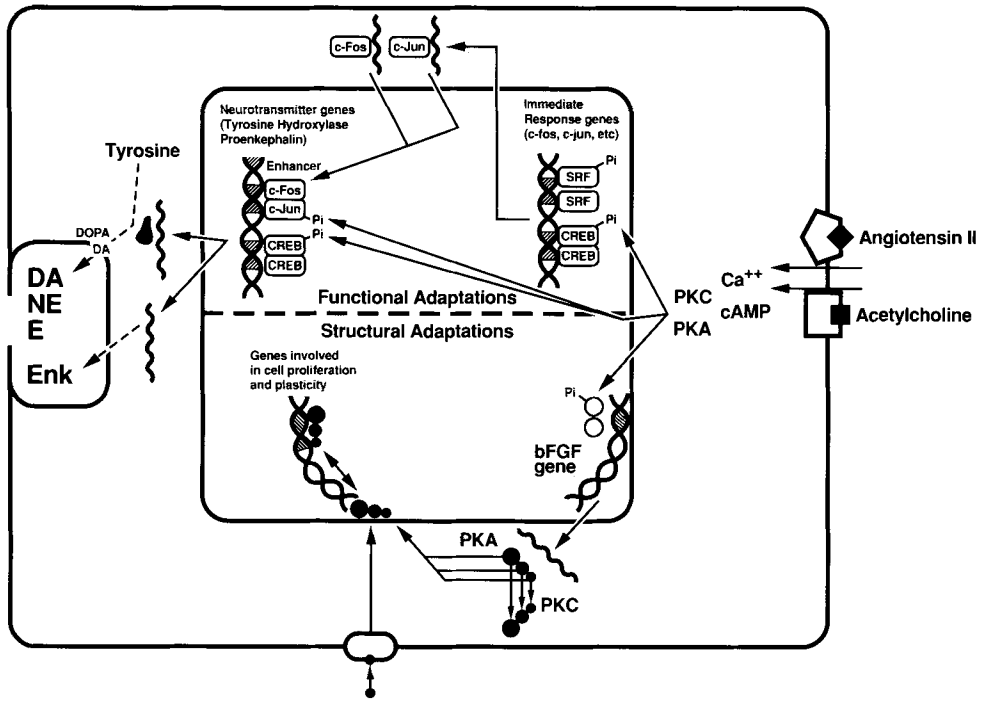

Figure 13. Hypothesis: molecular mechanisms of functional and structural adaptations to stimulation in adrenal medullary cells. *DA*, dopamine; *NE*, norepinephrine; *E*, epinephrine; *Enk*, enkephalins; *Pi*, phosphorylated proteins; *SRF* and *CREB*, factors binding to serum responsive element and CRE, respectively; *PKA*, protein kinase A; *PKC*, protein kinase C.

ulation also induces phosphorylation and activation of TH leading to a transient enhancement in catecholamine production and release. A longer or more intense stimulation of chromaffin cells increases the expression of TH and proenkephalin genes resulting in longer-lasting increases in the synthesis and release of adrenal medullary hormones. These changes occur without apparent proliferation of chromaffin cells and are referred to as functional adaptations (Stachowiak and Goc, 1992; Fig. 13). Long-term afferent stimulation produces structural changes in adrenal medullary tissue that involve proliferation of chromaffin and other adrenal medullary cells (Tischler and DeLellis, 1988). Increasing the number of cells and total content of adrenal medullary hormones may permanently enhance the secretory function of the adrenal medulla.

One mechanism for the induction of structural adaptations could be the recruitment of the mitogenic, angiogenic, and neurite promoting factor, bFGF. Depending on whether PKC or cAMP signaling pathways are activated, bFGF proteins accumulate in the cytoplasm or in the nucleus. Nuclear bFGF proteins may interact directly with chromatin and could thereby regulate expression of batteries of genes engaged in cell proliferation and other types of structural and functional changes. In addition to intracrine pathways, extracellular bFGF may be taken up by the chromaffin cells and also accumulate in the nuclei.

Although TH or proenkephalin genes are activated by the same receptors and second messengers, the nuclear mechanisms that stimulate the bFGF gene transcription are different. Transcriptional stimulation of the TH gene is conveyed by c-*fos* and c-*jun* interacting with the AP-1 binding element (Stachowiak et al., 1992; Goc et al., 1992; Goc and Stachowiak, 1994) and by constitutively expressed proteins that interact with the CRE (Huang et al., 1991). Similar mechanisms appear to mediate stimulation of the proenkephalin gene (Sonnenberg et al., 1989). For the bFGF gene, stimulation is conveyed by unique regulatory regions and may involve novel transcriptional factors. The existence of separate nuclear mechanisms conveying extracellular stimulation to bFGF and to neurotransmitter biosynthetic genes could allow independent modulation of their transcriptional activation by other factors. This in turn would allow adrenal medullary tissue to respond in a graded fashion to afferent stimulation: (*a*) through increased synthesis of adrenal medullary hormones, and (*b*) proliferation of chromaffin cells and angiogenesis.

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