

Differentiating and mature neurons express the acidic fibroblast growth factor gene during chick neural development

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Summary

We have previously isolated and characterized acidic fibroblast growth factor (aFGF) from the embryonic chick brain. To analyze the expression of the gene encoding this growth factor a cDNA clone was isolated. The predicted amino acid sequence was found to be highly conserved (90%) between human and chick. Using single-stranded DNA probes, aFGF gene expression was detectable at day 3.5 in the embryonic chick brain. The mRNA level of the 1.7 kb transcript increased during embryonic development and reached the highest level in the adult brain. *In situ* hybridization results confirmed these developmental changes and revealed a localized expression in neurons. In the adult, Purkinje cells, deep cerebellar and brainstem neurons showed a high level of aFGF mRNA. In the embryonic

brain, localized expression in neurons was detectable from day 6 onward. aFGF mRNA was also present in neurons of the peripheral nervous system. In dorsal root ganglia, aFGF was found to be expressed after embryonic day 6. Cells of blood vessels and the ependyma did not express detectable amounts of aFGF mRNA. These results suggest that aFGF may have a function as a differentiation or maintenance factor for postmitotic neurons or as a growth or differentiation factor for other cells in the nervous system mainly in later stages of development.

Key words: fibroblast growth factor, gene expression, *in situ* hybridization, neuron, angiogenesis.

Introduction

Polypeptide growth factors of the fibroblast growth factor (FGF) family have recently been implicated in the regulation of important embryonic processes such as mesoderm induction, angiogenesis and neuronal survival (Folkman and Klagsbrun, 1987; Woodland, 1989; Barde, 1989; Risau, 1990). Acidic and basic FGF are the best-studied growth factors of the FGF family. Both polypeptides are potent mitogens for a wide variety of mesodermal and ectodermal cells (for reviews see: Burgess and Maciag, 1989; Rifkin and Moscatelli, 1989; Folkman and Klagsbrun, 1987; Thomas and Gimenez-Gallego, 1986; Risau, 1990). Like other multifunctional mitogens, they have differing biological effects on different cell types. For example, bFGF was found to stimulate the growth of many cell types but inhibited the growth of Ewing sarcoma cells (Schweigerer *et al.* 1987). Both factors have been isolated mainly from adult brain tissue but are also present in the embryonic brain (Risau *et al.* 1988) as well as in other adult and embryonic tissues (Folkman and Klagsbrun, 1987; Risau, 1990). Since these factors at very low concentrations stimulated endothelial cell proliferation, migration and invasion *in vitro* and also angiogenesis in model systems *in vivo*, they seemed to be the prototype of angiogenic growth factors (Mosca-

telli *et al.* 1986; Rifkin and Moscatelli, 1989; Montesano *et al.* 1986; Risau, 1990). Furthermore, a number of reports have indicated that FGFs (basic FGF was used in most cases) can have neurotrophic activity *in vitro* (Walicke *et al.* 1986; Morrison *et al.* 1986) and *in vivo* (Sievers *et al.* 1987; Anderson *et al.* 1988).

Our interest in the FGFs emanated from our attempts to characterize embryonic angiogenesis factors. We have isolated soluble growth and angiogenic factors from the embryonic brain (Risau, 1986) which were subsequently identified as acidic and basic FGF (Risau *et al.* 1988). However, these factors are also present in the adult brain in which angiogenesis does not occur and cells have a low turnover under physiological conditions. Therefore, their activity as growth factors in the adult brain may be regulated by as yet unknown mechanisms. Our knowledge about the possible regulation of FGF activity would be much enhanced if the expression of these growth factors could be correlated with cellular proliferation or differentiation *in vivo*. It is thus essential to investigate the developmental pattern of gene expression and to identify the cell types that express the genes. Previously, those studies were hampered by the fact that the FGF mRNA seemed to be unstable and expressed at a very low level (Abraham *et al.* 1986a; Jaye *et al.* 1986). For example, bFGF cDNA clones were found to

be present in libraries at very low levels when compared to the quantity of the growth factor in the tissues. Furthermore, several of those clones represented unspliced transcripts suggesting that the cytoplasmic mRNA is unstable and that the protein is stored in tissues (Abraham *et al.* 1986b). Thus, the cellular origin and regulation of FGF gene expression remained unknown.

Here we report the cloning of the chick aFGF gene and its expression during embryonic brain development using a sensitive hybridization technique based on single-stranded DNA probes. By using this method and an optimized *in situ* hybridization technique, we have identified aFGF gene expression in neurons. The observation that the mRNA level dramatically increased during neuronal development suggest that aFGF may have a function in later stages of the development of the nervous system after neuronal proliferation has ceased.

Materials and methods

Animals and tissues

Fertilized eggs were incubated for the desired period of time, and embryos were staged according to Hamburger and Hamilton, (1951). Organs were removed, frozen immediately in liquid nitrogen and stored at -80°C .

DNA extraction and analysis

Genomic DNA was isolated according to Hermann and Frischauf, (1987). 10 μg samples were digested with various restriction enzymes, electrophoresed in 0.6% agarose gels, transferred in 0.4 N NaOH to Genescreen Plus membrane (DuPont) and hybridized overnight in a solution containing 5 \times SSPE, 5 \times Denhardt's, 0.5% SDS at 65 $^{\circ}\text{C}$ with 1–3 $\times 10^6$ cts $\text{min}^{-1} \text{ml}^{-1}$ of ^{32}P -labelled human aFGF cDNA (Jaye *et al.* 1986). Blots were washed in 0.3 \times SSPE, 0.5% SDS at 65 $^{\circ}\text{C}$ and exposed at -70°C to Fuji films for up to 6 days.

RNA extraction and analysis

Total cytoplasmic RNA was isolated according to the method of Chomczynski and Sacchi (1987). Enrichment for poly(A) $^{+}$ -containing fractions was achieved by oligo(dT) column chromatography (Aviv and Leder, 1972). 5 μg aliquots of poly(A) $^{+}$ RNA were electrophoresed in agarose gels containing 0.66 M formaldehyde and transferred to Hybond N membrane (Amersham) according to the manufacturer's instructions. Hybridizations were performed overnight in 5 \times SSPE, 5 \times Denhardt's, 0.5% SDS at 65 $^{\circ}\text{C}$ with 1–3 $\times 10^6$ cts $\text{min}^{-1} \text{ml}^{-1}$ of ^{32}P -labelled single-stranded DNA followed by high-stringency washes at 65 $^{\circ}\text{C}$ in 0.1 \times SSPE, 0.5% SDS. Filters were autoradiographed at -70°C on Fuji or Kodak films.

DNA sequencing

The complete nucleotide sequence of the genomic *EcoRI*–*XhoI* fragment and the chick aFGF cDNA clone was derived from systematic deletion subclones produced by the method of Lin *et al.* (1985). Sequencing was done on alkali-denatured plasmid templates using the Sequenase System (USB). The cDNA sequence was determined on both strands, the genomic clone was sequenced on one strand.

Genomic and cDNA library construction

A partial genomic library was constructed after electroeluting (Biotrap, Schleicher and Schüll) the size fraction around 6.5 kb of an electrophoresed genomic *EcoRI* digest. The fragments were cloned into the lambda-gt 10 vector and 120,000 phages were screened using the human cDNA probe labelled by random hexanucleotide priming (Boehringer). One clone (GAF 1) out of 6 positives was chosen for further analysis. The inserted DNA fragment of GAF 1 as well as various subfragments were introduced into the Bluescript vector (Stratagene). A cDNA library from E15 chick brain poly(A) $^{+}$ RNA was prepared using a Pharmacia cDNA synthesis kit. 500,000 primary plaques were analysed for aFGF containing sequences using the genomic *SacII*–*BamHI* fragment as a hybridization probe. The insert of the positive clone CAF 1 was subcloned into the Bluescript Vector.

Preparation of single-stranded DNA probes

RNA transcribed from linearized plasmid templates containing the chick cDNA clone served as a template for reverse transcription reactions to generate single-stranded DNA. 1 μg of either sense or antisense RNA was incubated with 100 units MMLV reverse transcriptase (BRL) for 1 h at 37 $^{\circ}\text{C}$ in 20 μl of a buffer containing 1.8 i.u. μl^{-1} RNAase inhibitor (Pharmacia), 2.5 mg ml^{-1} random hexanucleotides (Boehringer), 100 $\mu\text{g ml}^{-1}$ actinomycin D (Boehringer), 0.5 mM dCTP, 0.5 mM dTTP, 0.5 mM dGTP, 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM MgCl_2 , 10 mM DTT and 100 $\mu\text{Ci } ^{35}\text{S}$ -dATP (for *in situ* hybridization) or 100 $\mu\text{Ci } ^{32}\text{P}$ -dATP (for northern hybridization). Reactions were stopped and the RNA template was hydrolyzed by incubation in 0.2 N NaOH at 65 $^{\circ}\text{C}$ for 10 min. After neutralization with 1 vol 0.4 N HCL, 1/50 vol 1 M Tris–HCl (pH 7.5), unincorporated nucleotides were removed by Biogel P10 spun column chromatography. The probe was precipitated with ethanol and redissolved in the desired volume of hybridization buffer.

Preparation of tissue sections

Unfixed chick brains or whole embryos were embedded in Tissue Tek (Miles), frozen and sectioned on a Leitz Cryostat. 10 μm sections were mounted on organosilane-treated slides (Uhl, 1986), dried at 55 $^{\circ}\text{C}$ and postfixed in 4% paraformaldehyde in PBS for 15 min at room temperature. After two washes in PBS for 5 min each, sections were dehydrated in increasing ethanol concentrations and stored desiccated at -80°C .

In situ hybridization

Sections were brought to room temperature, treated with 40 $\mu\text{g ml}^{-1}$ predigested Pronase (Sigma) in 50 mM Tris–HCl, 5 mM EDTA, pH 8 for 10 min, incubated for 30 s in 0.2% glycine in PBS, washed in PBS for 1 min and fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. Acetylation was performed by immersion in fresh 100 mM ethanolamine and 25 mM acetic anhydride. After rinsing in PBS for 5 min, each section was covered with 25 μl hybridization buffer containing 2 \times SSPE, 2 mg ml^{-1} tRNA, 2 mg ml^{-1} BSA, 50% formamide, 400 $\mu\text{g ml}^{-1}$ polyadenylic acid, 10 mM DTT, 100 nM SdATP, 500 $\mu\text{g ml}^{-1}$ heparin and prehybridized for 1 h at 37 $^{\circ}\text{C}$. For hybridization, sections were incubated with 1–2 $\times 10^6$ cts min^{-1} of ^{35}S -labelled single-stranded anti-sense DNA in the above buffer for 6 h at 37 $^{\circ}\text{C}$. On each slide a control section was hybridized with labelled sense DNA under identical conditions. Sections were then washed overnight in 2 \times SSPE, 50% formamide, 20 mM β -mercaptoethanol and dehydrated. Autoradiography was performed

using Kodak NTB2 emulsion. Exposure times varied from 8 days to 30 days. Finally, sections were stained with 1% fast green, 0.5% cresyl violet or with 0.02% toluidine blue and mounted. Conditions employed for *in situ* hybridization were optimized using a random hexanucleotide primed chicken β -actin probe (Kost *et al.* 1983). Variables that were found significantly to affect the signal-to-noise ratio were protease treatment, temperature of the hybridization and washing steps. In contrast, pretreatment of sections with HCl and/or hot SSC did not increase the hybridization signal and were therefore omitted. Hybridization times of 6 h were found to be sufficient to detect even the low abundant FGF message and, in addition, led to reduced background levels.

Results

Cloning of the chick aFGF gene

Human aFGF cDNA hybridized to a single 6.5 kb *EcoRI* fragment of chick genomic DNA detected under high-stringency conditions (Fig. 1A). A partial genomic library containing DNA fragments of corresponding size was constructed in a lambda gt10 vector and screened using the human cDNA probe. Several clones were identified. One of these, GAF1 (Fig. 1B), was further analyzed. Restriction and Southern analysis showed that only the 1 kb *EcoRI-XhoI* fragment indicated in Fig. 1B contained sequences homologous to human aFGF. The nucleotide sequence of this fragment (Fig. 2) revealed an open reading frame with high homology to the N-terminal sequence of human acidic FGF. The predicted amino acid sequence matched our previously published N-terminal amino acid sequence (Risau *et al.* 1988). The exon-intron structure of the presumptive first exon of the human aFGF gene was also conserved in the chick genome (Fig. 2). Typical eucaryotic promoter elements (CAAT and TATA; boxed in Fig. 2) were present in the 5' region of the ATG codon. Thus, we believe that this clone represents part of the chick aFGF gene.

The *SacII-BamHI* fragment of clone GAF1 (Fig. 2) was used to isolate a corresponding cDNA clone from a cDNA library prepared from embryonic day 15 chick

brain mRNA (preliminary northern hybridizations had shown that aFGF mRNA was expressed at this developmental stage, see Fig. 4). Only one phage plaque out of 500 000 gave a positive hybridization signal. The nucleotide sequence of this clone (CAF1) is shown in Fig. 2. At its 5' end, the first 42 nucleotides diverged from the genomic sequence. We cannot exclude at present that this is due to a cloning artefact. Translation is probably initiated by the ATG (underlined in Fig. 2) because an in-frame stop codon (TGA; also conserved in the human sequence) is present preceding this ATG. The 1419 nucleotides of the CAF1 clone constitute the entire coding region and a long 3' noncoding region. Since longer transcripts are detected in northern blots (Fig. 4), CAF1 does not represent a full-length cDNA clone. There are 16, 19 and 16 predicted amino acid exchanges (out of 155) in the chick sequence compared to the human, bovine and rat sequence, respectively (Fig. 3). Therefore, this gene is highly conserved during evolution.

Developmental expression of the aFGF gene

Chick aFGF cDNA labelled by nick translation or hexanucleotide primers resulted in very weak hybridization signals in northern blots of chick brain poly(A)⁺ RNA. This is consistent with previous observations indicating a low level of expression of this gene (Jaye *et al.* 1986). The more sensitive riboprobes were found to hybridize unspecifically to 28S ribosomal RNA. Therefore, we used single-stranded DNA probes generated by reverse transcription using hexanucleotide primers and *in vitro* synthesized RNA as a template. The method allows the detection of less than 1 pg of aFGF mRNA as determined by dot-blot analysis of poly(A)⁺RNA (not shown).

Fig. 4 shows that a 1.7 kb mRNA hybridizing to the chick aFGF cDNA is present in the embryonic chick brain. It was already detectable after 3.5 days of embryonic development and dramatically increased relative to actin mRNA (not shown). High levels of mRNA were found in the adult brain. Upon longer exposure times (Fig. 4) at least three additional mRNA

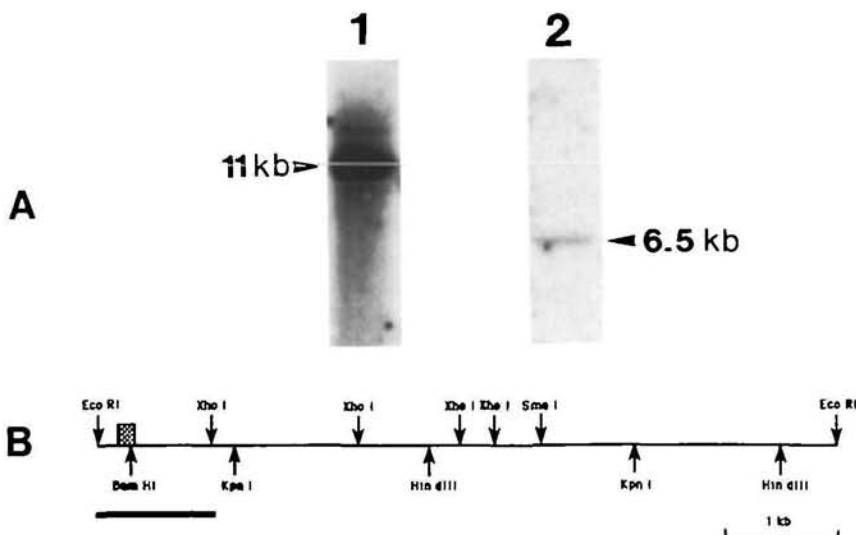


Fig. 1. Cloning of chick genomic aFGF. (A) Southern blot hybridization of human aFGF cDNA to *EcoRI*-digested human (lane 1) and chick (lane 2) genomic DNA. Fragments of 11 kb in human DNA (as described (Jaye *et al.* 1986)) and 6.5 kb in chick DNA are indicated. (B) Restriction map of the genomic chick aFGF clone GAF 1. The *EcoRI-XhoI* fragment (indicated by the bar) containing the coding region (stippled bar) was used for nucleotide sequencing.

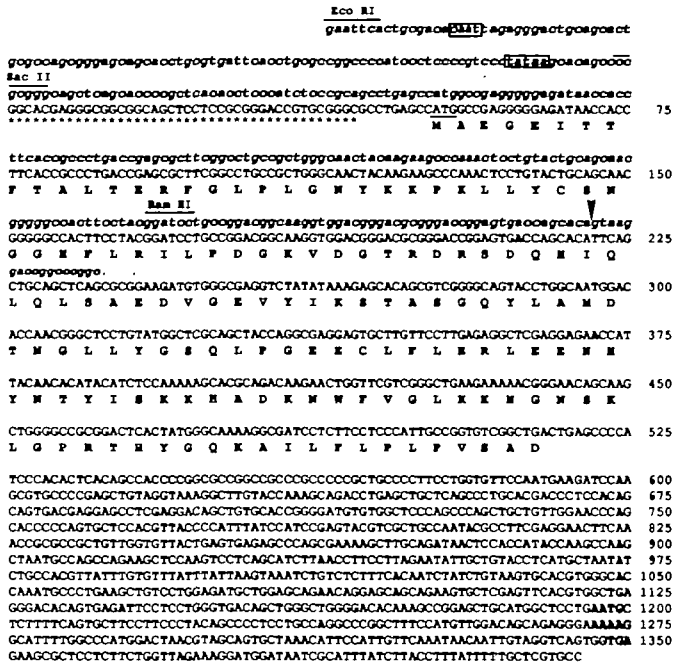


Fig. 2. Nucleotide sequence and predicted amino acid sequence of chick genomic and cDNA clones. The sequence of the genomic clone is given in lower case letters, the cDNA sequence in upper case letters. The *SacII*–*BamHI* fragment of the genomic clone was used to isolate the cDNA clone. CAAT and TATAA sequences are boxed, the sequence diversity between the genomic and cDNA clones is indicated by asterisks and the splice acceptor site is indicated by the arrowhead. The putative translation initiation codon ATG is underlined and the predicted amino acids are given in the single letter code.

species of about 2.7, 3.2 and 3.4 kb could be detected. It is presently unknown whether the additional transcripts represent precursors.

In situ hybridization

Initial attempts using established protocols for *in situ* hybridization of *Drosophila* or mouse tissue sections were not successful. By using antisense cDNA probes of high specific activity and optimizing hybridization conditions, specific hybridization patterns as compared to sense cDNA probes were observed (Figs 6–10). In sagittal sections of the adult chick brain, as schematically indicated in Fig. 5, the highest level of aFGF mRNA was found in the Purkinje cells of the cerebellum (Fig. 6A,B), deep cerebellar neurons (dentate nucleus; Fig. 6C) and neurons of the brainstem (Fig. 6D). No specific hybridization was detectable in neurons of the molecular layer and granular layer. A

lower level of aFGF expression was found throughout the telencephalon (Figs 6B; 8A,B), particularly in a dorsostrual region which overlaps with the hyperstriatum accessorium (van Tienhoven and Juhasz, 1962). Figs 7A–D show higher magnifications of particular regions indicated in Fig. 6 to reveal the hybridization signals at the cellular level. A high density of grains is found over the large neuronal perikarya whereas cells of adjacent blood vessels were essentially devoid of grains. The majority of glial cells appear to be unlabeled. To examine the anatomical regions of aFGF expression in more detail hybridizations were performed on frontal serial sections of the adult brain (Fig. 8). The results are consistent with the observation that aFGF mRNA is present in the majority of telencephalic neurons of the adult brain.

During brain development, aFGF mRNA is present in deep cerebellar neurons of the dentate nucleus at embryonic day 15 whereas much weaker signals are detected in the molecular layer (Fig. 9A). At this developmental stage, the Purkinje cells are present within this layer and probably give rise to this signal. A comparison of identified brain regions like the hyperstriatum accessorium region (referred to above) revealed developmental changes in aFGF gene expression during neuronal development. At embryonic day 12, the pattern of aFGF expression in this region is already similar to that observed in the adult (Fig. 6B) whereas at day 9 expression was restricted to neurons localized at the periphery (Fig. 9C).

As illustrated in Figs 6–9 endothelial cells and smooth muscle cells of blood vessels (cortical capillaries, veins and pial vessels; Figs 6, 7) as well as ependymal cells (of the lateral ventricles; Fig. 8A) and choroid plexus epithelial and endothelial cells (Fig. 8B) did not express aFGF mRNA at a detectable level.

In situ hybridizations of earlier developmental stages (days 6 and 9) were performed on sections of entire embryos. We therefore could also examine aFGF expression outside the central nervous system. Our observations suggest that at these stages aFGF mRNA is predominantly, if not exclusively, expressed in the central and peripheral nervous system. We also observed significant hybridization associated with other organs (e.g. stomach, gut). Preliminary analyses indicate that this is due to aFGF gene expression in nerve cell complexes that innervate these organs (e.g. ganglion coeliacum, ganglion mesentericum). As an example for the expression in the peripheral nervous system cervical dorsal root ganglia (DRG) are shown in Fig. 10. At embryonic day 6, no specific expression was observed (Fig. 10A). Expression commenced at day 9 and increased until day 12 (Fig. 10C). The grains were

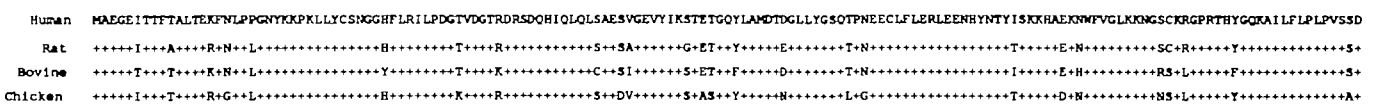


Fig. 3. Conservation of aFGF amino acid sequence. Comparison of amino acid sequences of human, bovine (Alterio *et al.* 1988) and rat (Goodrich *et al.* 1989) aFGF proteins with the chick protein. Identical amino acids in all species are represented by +.

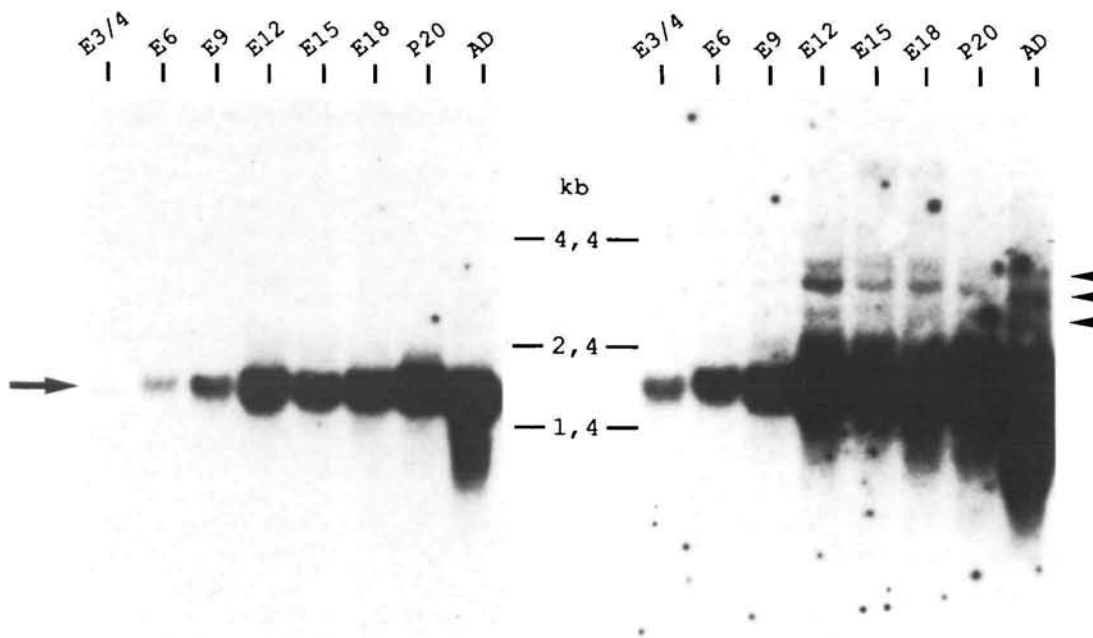


Fig. 4. Expression of the aFGF gene during embryonic brain development. Northern blot hybridization of chick brain poly(A)⁺RNA from the different developmental stages indicated on the top of each lane (E, embryonic day; P, postnatal day; AD, adult). The same blot was exposed for 18 h (left) and 7 days (right). Transcripts in addition to the major 1.7 kb RNA showing up after longer exposure times are indicated by arrowheads and probably represent nuclear precursors.

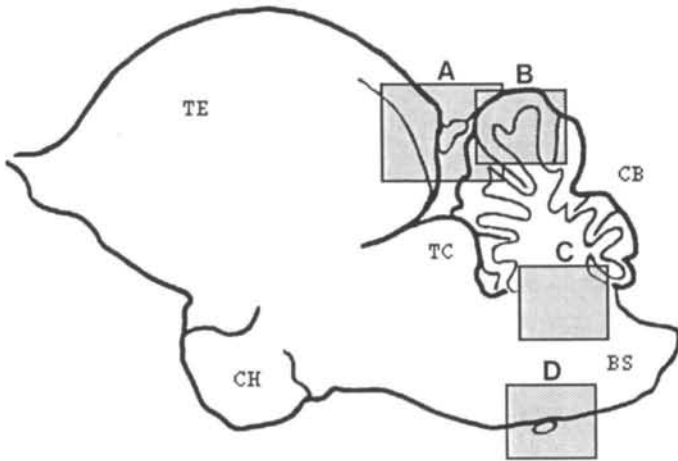


Fig. 5. Schematic representation of a sagittal section of an adult chick brain. Sections A–D shown in Fig. 6 are indicated by the stippled squares. TE, telencephalon; CH, chiasma opticum; TC, Tectum; CB, cerebellum; BS, brainstem.

concentrated over the large cell bodies of neurons (Fig. 11A,B). The increase in expression in peripheral neurons is consistent with the changes observed in the central nervous system.

Discussion

Acidic and basic FGF isolated from tissues or cells are potent mitogens for a wide variety of cells. They have been implicated in a number of important physiological

processes such as angiogenesis, mesoderm induction, neuronal survival and wound healing (Folkman and Klagsbrun, 1987; Woodland, 1989; Barde, 1989; Risau, 1990). Expression of bFGF mRNA has been detected in the *Xenopus* oocyte and embryo when mesoderm induction occurs (Kimelman and Kirschner, 1987). Endothelial cell growth factor activity and angiogenesis-inducing activity have been characterized from tumor and embryonic tissues and identified as bFGF and aFGF (Shing *et al.* 1984; Libermann *et al.* 1987; Folkman and Klagsbrun, 1987; Risau *et al.* 1988). The FGFs also supported the *in vitro* survival of embryonic neurons (Walicke *et al.* 1986; Morrison *et al.* 1986). These results supported the view that the FGFs may have a role as growth and inducing factors. However, direct evidence for such a role is lacking because the biological roles of the FGFs have been determined mainly using *in vitro* assays. Only a few experiments have been performed to try to interfere with proposed functions of the FGFs *in vivo*.

In vivo studies intended to interfere with FGF functions can only be reasonably well designed if the cellular origin and level of expression are known. This has been impossible in the past, probably due to the low abundance of FGF mRNAs. Our results presented here demonstrate that the mRNA level of the 1.7 kb aFGF transcript increased during embryonic development and was high in the adult brain. These results were consistent with our previous results showing an increase in mitogenic activity during embryonic chick brain development (Risau, 1986). Furthermore, our *in situ* hybridization results reveal these developmental changes at the cellular level. Cortical and peripheral

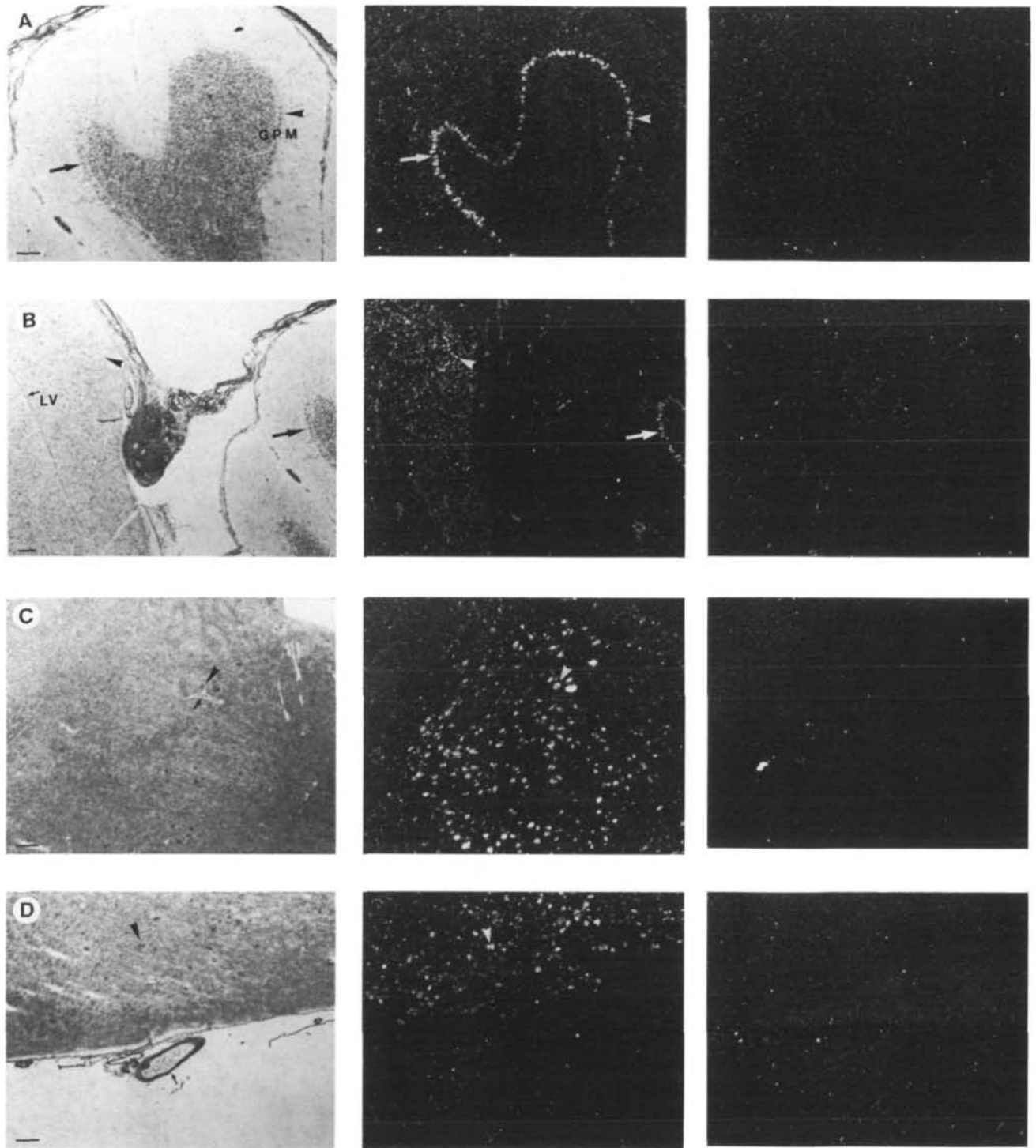


Fig. 6. Localization of aFGF transcripts in adult chick brain. Bright-field (left column) and dark-field (middle and right columns) micrographs of adult chick brain sagittal sections (A–D) as indicated in Fig. 5 were hybridized with ^{35}S -labelled aFGF probes, autoradiographed and stained. Exposure time 11 days. The left-hand dark-field micrographs are of sections hybridized with the single-stranded antisense DNA probe and correspond to the bright-field micrograph; the right-hand dark-field micrographs are of adjacent sections hybridized with the control aFGF sense probe. The regions indicated by arrowheads in A–D are shown in higher magnification in Fig. 7. Purkinje cells of the cerebellum (arrows in A,B), deep cerebellar neurons (C) and neurons of the telencephalon of a dorsorostral region which overlaps with the hyperstriatum accessorium (B, arrowhead) and brainstem (D) show a high level of aFGF expression. Arrows point to blood vessels (c, cerebral vein; d, blood vessel of the pia) and the lateral ventricle (LV). G, granule layer; P, Purkinje layer; M, molecular layer of the cerebellum. Bar, represents 0.2 mm.

neurons were found to have very little aFGF mRNA in early stages of neurogenesis but the level of expression increased dramatically in later stages of neuronal development. Before embryonic day 9, the level of

aFGF gene expression in the central nervous system was very low as determined by northern blots. At day 6, we did observe a low level of specific *in situ* hybridization in the anterior part of the telencephalon

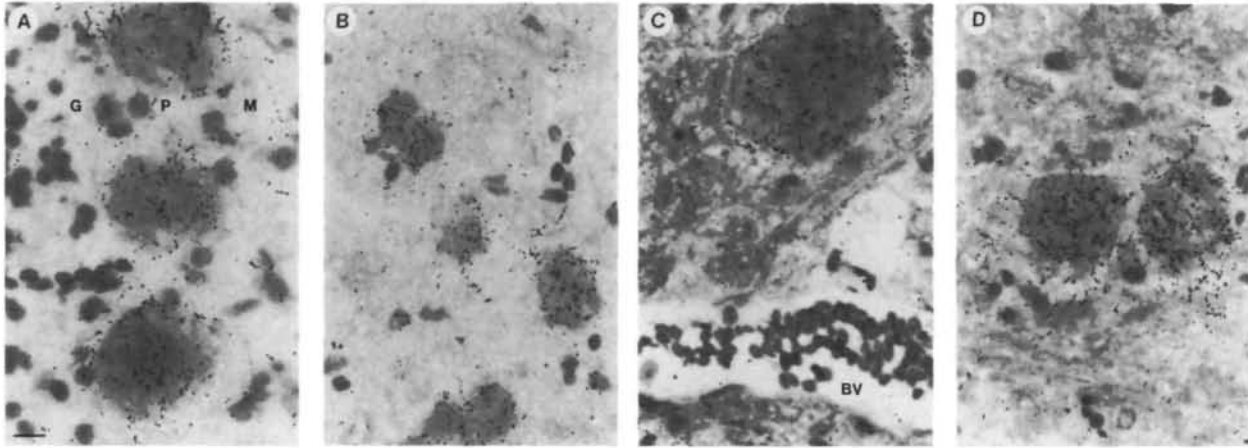


Fig. 7. Localization of aFGF transcripts at the cellular level. Higher magnification of the bright-field micrographs of the regions indicated by arrowheads in Fig. 6. A high density of grains is present over the neuronal perikarya whereas glial cells and cells of the blood vessel (BV in C which is also indicated by the arrow in Fig. 6C) are devoid of grains. Abbreviations are as in Fig. 6. Bar, represents 10 μ m.

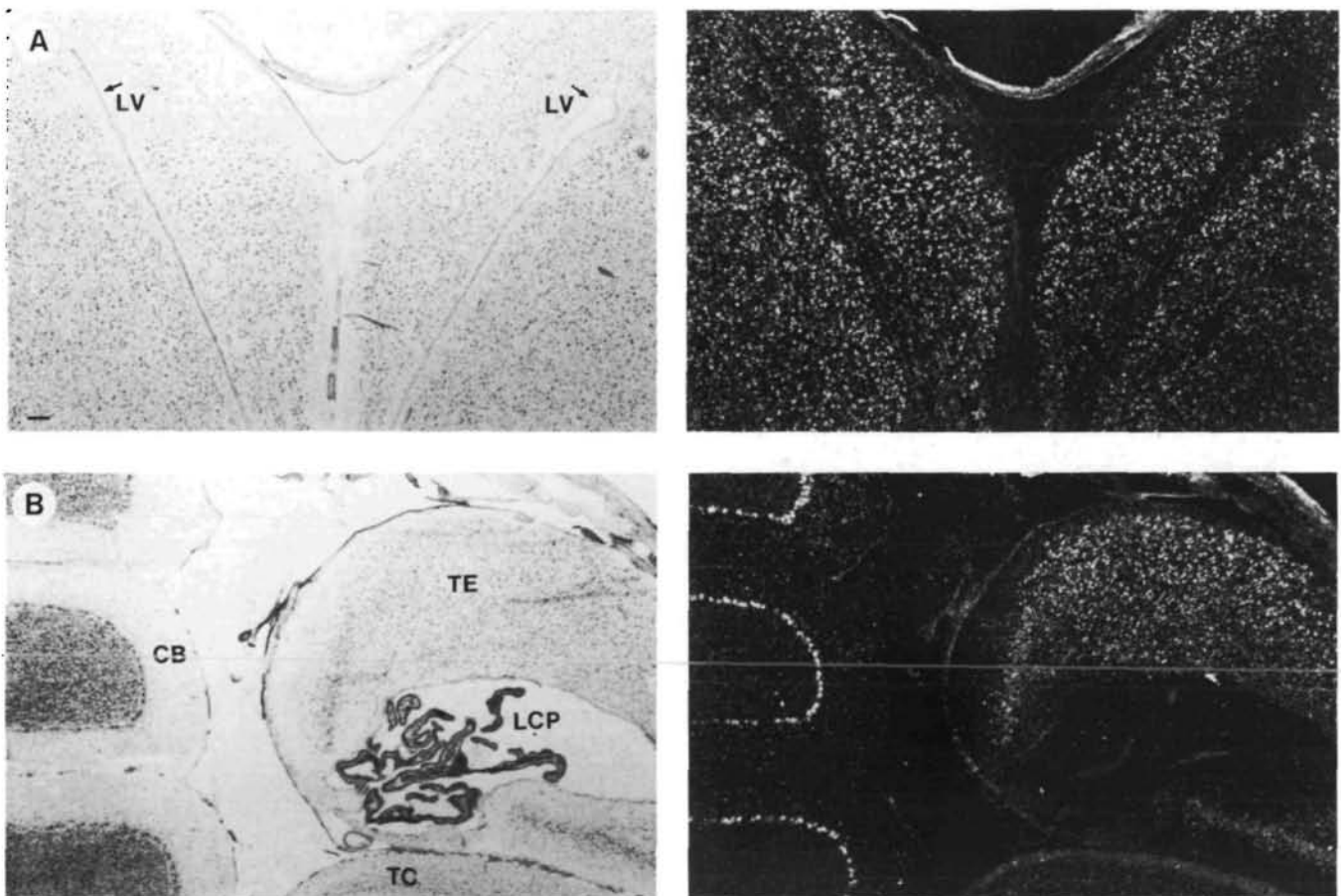


Fig. 8. Localization of aFGF transcripts in frontal sections of the adult chick brain. Purkinje cells of the cerebellum (B-CB) and the majority of neurons in all layers in the telencephalon (B-TE) express aFGF mRNA. The area in (A) represents the middle part of the hyperstriatum accessorium. Ependymal cells lining the lateral ventricles (A-LV) and choroid plexus (B-LCP) appear to be negative. Exposure time 16 days. TC, Tectum. Bar, represents 0.2 mm.

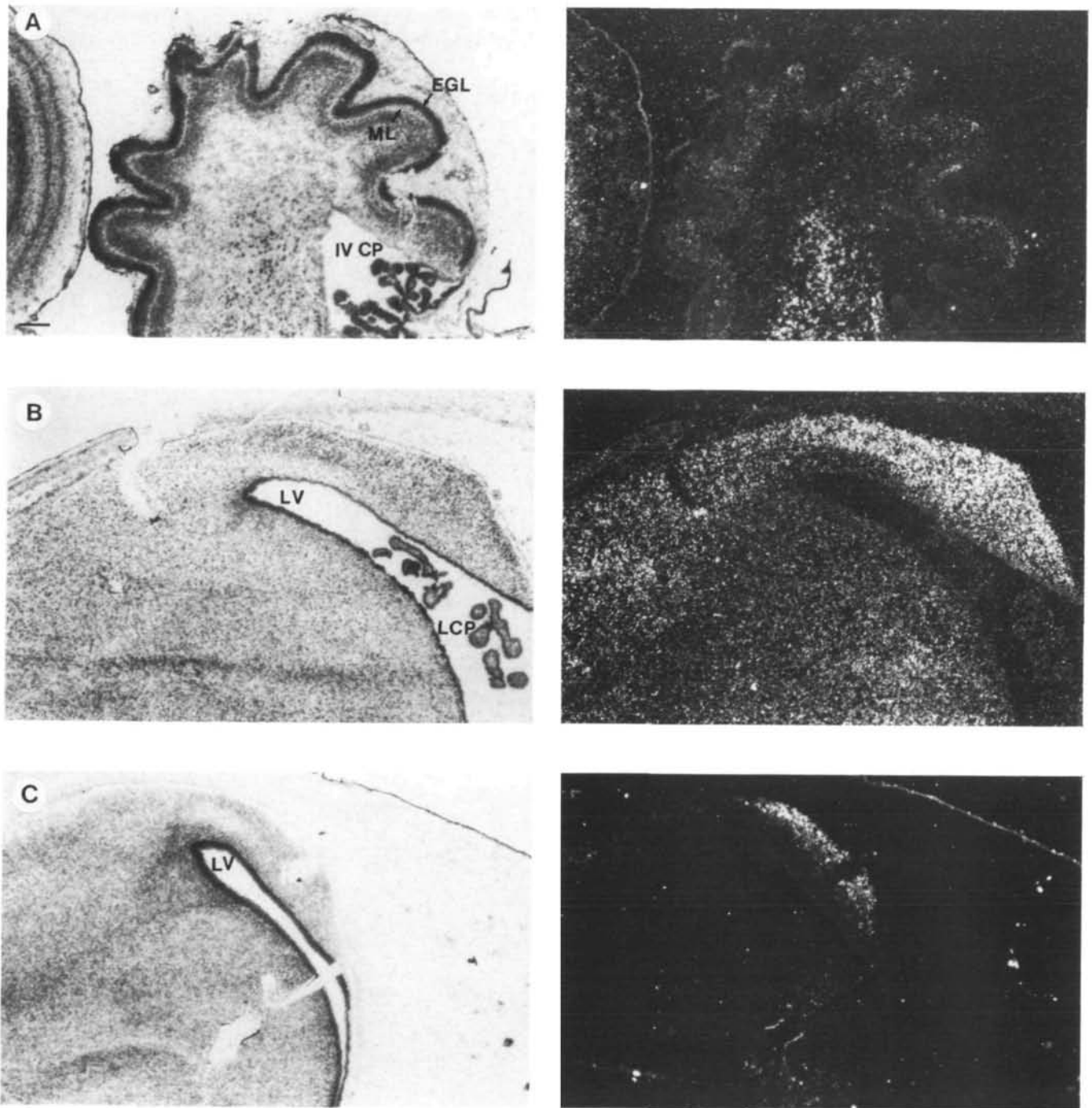


Fig. 9. aFGF expression during embryonic brain development. Sagittal sections of embryonic day 15 (A), day 12 (B) and day 9 (C) brains. aFGF mRNA is present in deep cerebellar nuclei (A) and weakly in the molecular layer (ML) where Purkinje cells can be found at this stage of development. Exposure times: (A) 18 days, (B) 8 days, (C) 18 days. EGL, external granular layer; IV CP, choroid plexus of the fourth ventricle. In a telencephalic region of the hyperstriatum accessorium, specific expression was observed throughout the layers at embryonic day 12 (B) whereas at day 9 (C) it is restricted to the peripheral neurons where expression is also highest in the 12 day embryonic brain. LV, lateral ventricle; LCP, choroid plexus of the lateral ventricle. Bar, represents 0.2 mm.

(not shown). Therefore, we can conclude that the level of aFGF mRNA is low between 3 and 9 days of embryonic brain development and that there is no switch in the cell type that expresses the gene during development. Studies are in progress using polymerase

chain reaction techniques to determine the level of aFGF gene expression in earlier stages of embryonic development. In any case, the pattern of aFGF gene expression does not correlate with endothelial cell proliferation and angiogenesis in the brain because

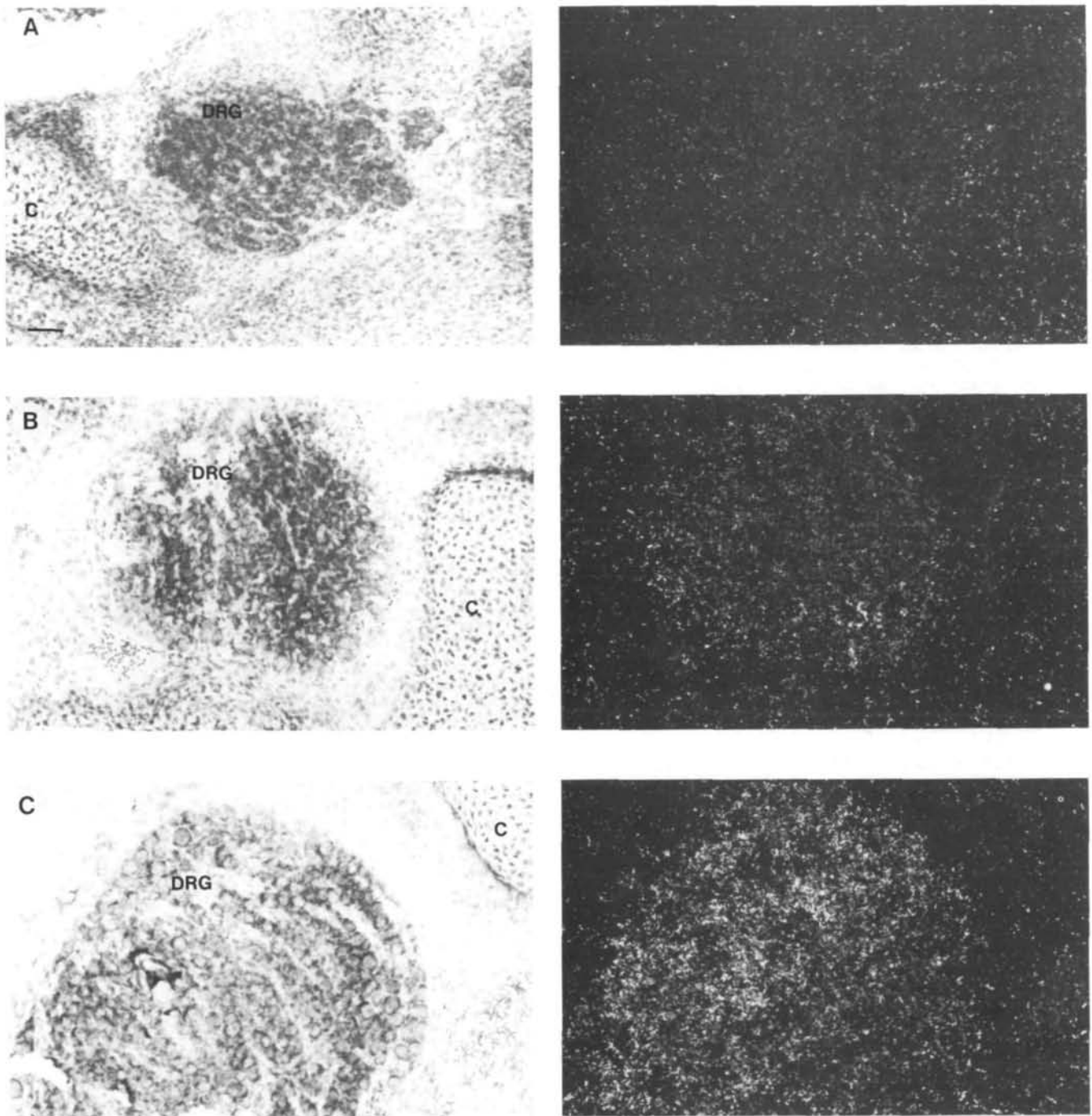


Fig. 10. Localization of aFGF transcripts in dorsal root ganglia during embryonic development. Transverse (A) and sagittal (B,C) sections of cervical dorsal root ganglia (DRG) of 6 day (A), 9 day (B) and 12 day (C) chick embryos. Increasing amounts of the aFGF transcript are present during embryonic development. Exposure times: (A) 11 days, (B) 18 days, (C) 8 days. C, cartilage. Bar, represents 5 μ m.

these processes do occur in the embryonic but not adult brain. However, it is possible that other mechanisms regulate or inhibit aFGF activity during later stages of development (see below). Our results indicate that the abundance of aFGF in the brain is due to local synthesis rather than to transport of the protein *via* blood or neuronal processes.

In the chick CNS, neurons are postmitotic after 4 to 9

days of embryonic development (Fujita, 1964; Wilson, 1978; Jacobson, 1978). The production of Purkinje cells in the cerebellum ceases already after 2 days while dorsal root ganglion neurons are postmitotic after 7 days (Jacobson, 1978; Hanaway, 1967; Hamburger and Levi-Montalcini, 1949). Our data on the temporal and spatial pattern of aFGF expression indicate that aFGF gene expression is initiated in postmitotic neurons and

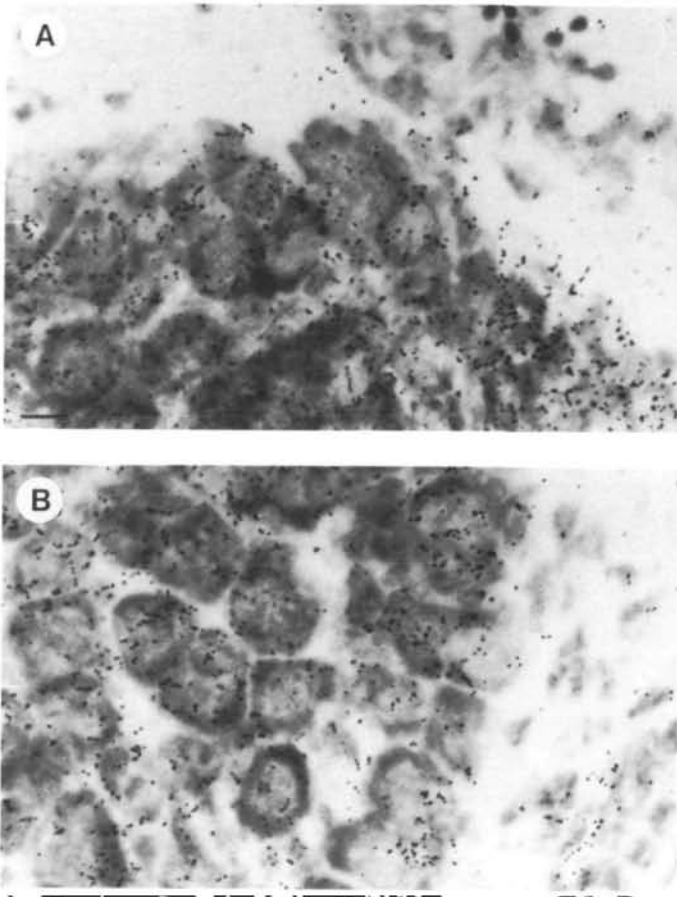


Fig. 11. Localization of aFGF transcripts in dorsal root ganglia at the cellular level. A high density of grains is present over neuronal perikarya in the dorsomedial (A) and ventrolateral (B) portion of a 12 day embryonic DRG. Other cells in the ganglia and outside are negative. Exposure time: 8 days. Bar, represents 10 μm .

raise the possibility that aFGF has a role in neuronal function such as determination of neurotransmitter phenotype, axonal growth or synaptogenesis after neuroblast proliferation has ceased. Another member of the family of FGF-related genes, *int-2*, was localized to mouse Purkinje cells. Unlike the aFGF gene *int-2* gene expression appeared to be restricted to early stages of Purkinje cell differentiation (Wilkinson *et al.* 1989).

It is noteworthy that not all adult neurons expressed the gene. This is most strikingly seen in the cerebellum where neurons of the molecular and granular layers are negative. Further work is necessary to analyze aFGF gene expression in specific sets of neurons. Interestingly, *in vitro* experiments indicate that FGFs can exert their neurotrophic activity on neurons from different brain regions (hippocampus: Walicke *et al.* 1986; cerebral cortex: Morrison *et al.* 1986; Walicke, 1988; striatum, septum and thalamus: Walicke, 1988; cerebellum: Hatten *et al.* 1988; ciliary ganglia and spinal cord: Unsicker *et al.* 1987; retina: Lipton *et al.* 1988)

which is consistent with the widespread expression of aFGF in the nervous system.

Cells of the vascular wall (e.g. endothelial cells and smooth muscle cells), ependymal cells and most cortical glial cells did not express detectable amounts of aFGF mRNA. Interestingly, aFGF expression has been found in cultured smooth muscle cells and glial cells (Winkles *et al.* 1987; Ferrara *et al.* 1988). In the brain *in vivo*, neither cell type was found to express this gene suggesting that aFGF mRNA is upregulated upon *in vitro* culture conditions.

Recently, immunolocalization studies have demonstrated that aFGF protein is present in neurons (Huang *et al.* 1987; and our unpublished results). bFGF has also been localized in neurons by immunohistology (Janet *et al.* 1987) and *in situ* hybridization (Emoto *et al.* 1989). It will be interesting to investigate whether aFGF and bFGF are coexpressed in certain neurons.

A major problem concerning the physiological role of the aFGF and bFGF proteins is their lack of a hydrophobic signal sequence required for the secretion via the classical secretory pathway. The lack of a hydrophobic signal sequence is shared by human interleukin-1, which is approximately 30% homologous to aFGF (Abraham *et al.* 1986a; Gimenez-Gallego *et al.* 1985), and the platelet-derived endothelial cell growth factor (PD-ECGF) which is not significantly homologous (Ishikawa *et al.* 1989). In contrast to the FGFs, interleukin-1 has been found to be released by cultured cells (Hazuda *et al.* 1988). Thus, it is possible that the FGFs may be sequestered inside the cell and may not have direct access to target cells (Abraham *et al.* 1986a; Jaye *et al.* 1986). Alternatively, they may be released by other mechanisms, or may only be released after cell death following tissue injury (wounding, tumors, inflammation, ischemic damage) or programmed cell death. Programmed neuronal cell death indeed occurs during the development of the nervous system and may lead to the release of aFGF. It may then act as an angiogenic factor and/or neurotrophic factor. Alternatively, aFGF may act as a growth factor for other cells, particularly glial cells which are known to be mitogenically stimulated by aFGF (Pettmann *et al.* 1985). However, this activity as well as the angiogenic activity have to be controlled in the adult brain to prevent aberrant cell proliferation. It is conceivable that in the adult the FGFs may act as general 'rescue' factors (including angiogenic and neurotrophic activity) released by damaged cells after lesioning as has been discussed previously (Barde, 1989) or may act as a physiological factor if released in limiting amounts by intact cells.

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