

Basic Fibroblast Growth Factor in the Chick Embryo: Immunolocalization to Striated Muscle Cells and Their Precursors

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Abstract. The identification of acidic and basic fibroblast growth factors (FGFs) in a number of embryonic tissue extracts has implicated these growth factors in the regulation of a variety of embryonic events including angiogenesis, eye development, and muscle differentiation. Lack of information concerning the cellular distribution of the growth factor within these tissues has made it extremely difficult to assign developmental roles to FGF. We have localized bFGF in the developing chick embryo using immunohistochemical techniques and our monospecific polyclonal rabbit anti-human bFGF IgG. The spatial pattern for bFGF

localization was highly specific. The anti-human bFGF antibodies recognized striated muscle cells and their precursors in 2–6-d chick embryos. Myocardium, somite myotome, and limb bud muscle all stain positively for bFGF. In addition, the anti-human bFGF antibodies localized specifically to the cell, rather than to the extracellular matrix or nucleus of myotubes. The localization of bFGF demonstrated here provides further support for the hypothesis (Clegg et al., 1987; Seed et al., 1988) that this growth factor is involved in muscle development.

BASIC fibroblast growth factor (bFGF)¹ has a widespread distribution in adult tissues as determined by biochemical, biological, and immunological analysis of tissue extracts (see reviews in references 10, 16). However, little is known concerning its localization or its physiological role in these tissues. In vitro, bFGF has mitogenic activity for a variety of mesodermal cells including endothelial cells (4, 5, 6) and myoblasts (2), as well as for cells of neuroectodermal origin (6, 23). When supplied exogenously to tissues of living organisms such as the chick embryo chorioallantoic membrane, bFGF is capable of stimulating angiogenesis (3, 19, 28). Whether endogenous bFGF is involved in regulating these processes in the organism, has yet to be determined.

More recently, research has begun to focus on possible roles of both the acidic and basic forms of FGF in embryogenesis. A role for bFGF in the induction of mesodermal tissue, an early developmental event crucial to the normal development of the embryo, has been suggested by the demonstration that bFGF can mimic the effect of vegetal pole mesodermal inducing factors in *Xenopus* blastula (29). In addition, mRNA for bFGF has been identified in amphibian oocytes and embryos (14). The expression of the int-2 proto-oncogene, which is related to FGF, has recently been studied

in early mouse embryos, and its pattern of localization suggests possible roles in cell migration during gastrulation and inductive events during neurulation (33). It has been suggested that FGF may also be important later in development, during organogenesis. The acidic form of FGF (aFGF) has been identified in embryonic kidney extracts (25), while a heparin-binding growth factor which has very similar properties to aFGF has been identified in extracts of embryonic brain (24). In addition, bFGF has been found in the brain, retina, and vitreous of 11-day chick embryos (18). The presence of these factors has been temporally correlated with angiogenesis in these tissues, suggesting that aFGF and bFGF may regulate embryonic vascularization. bFGF has also recently been identified in extracts of 2.5–13-d chick embryos and in chick embryo limb buds (27). A temporal correlation between FGF levels in the limb and muscle cell differentiation in the same tissue was noted in this study and may suggest a role for FGF in the regulation of muscle development in the embryo. bFGF was previously shown to regulate the differentiation of cultured myoblasts (2).

The identification of FGF in tissue extracts of embryonic organs, and the correlation of its presence with particular developmental events is suggestive of a role for this growth factor in organogenesis. The ability to determine the cellular localization of the growth factor within these tissues during their development would provide further information which would aid in the elucidation of the role played by FGF in development. In this paper, we localize bFGF to striated muscle cells and their precursors in the embryonic chick using

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1. *Abbreviations used in this paper:* aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor.

specific polyclonal anti-human bFGF antibodies (11) in conjunction with immunohistochemical methods.

Materials and Methods

Fertile eggs from White Leghorn chickens were obtained from Spafas Inc. (Norwich, CT) and maintained in a humidified incubator at 37°C. Embryos were staged according to Hamburger and Hamilton (7) before dissection.

Preparation of Antibodies

Polyclonal anti-human bFGF antibodies were raised in rabbits and gamma globulin fractions were prepared as previously described (11).

Affinity-purified anti-human bFGF IgG was prepared by applying anti-human bFGF gamma globulins onto a column of human recombinant bFGF conjugated to agarose beads (Affigel-10; Bio-Rad Laboratories, Richmond, CA). Affinity-purified antibodies were then eluted with 0.1 M glycine (pH 2.5) after extensive washing of the column with PBS (PBS = 0.15 M NaCl in 50 mM sodium phosphate buffer, pH 7.4). Fractions were immediately neutralized and dialyzed overnight against PBS. The flow through from the affinity column was collected and subjected to two additional rounds of chromatography on the bFGF affinity column to prepare anti-human bFGF-depleted gamma globulin for use in control studies. When tested in an ELISA for immunoreactivity with bFGF, the anti-human bFGF-depleted antibodies contained no activity.

Immunocytochemistry

Chick embryos of appropriate stages were directly fixed in Bouin's fixative (15:5:1 saturated aqueous picric acid/formaldehyde/glacial acetic acid) overnight. The fixed embryos were dehydrated through a series of ethanol solutions, cleared in xylene and then infiltrated and embedded in Paraplast Plus (Monoject Scientific, St. Louis, MO). Sections were cut using a rotary microtome and were then prepared for immunohistochemistry using a modification of the method of D. Anderson (personal communication). Sections were first placed in xylene and then rehydrated through a series of ethanol solutions (70–100%). Sections were washed in a saturated lithium chloride/70% ethanol solution and then were incubated for 30 min at room temperature in 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. Sections were then incubated for 30 min at 37°C in PBS containing 5% normal goat serum and 0.1% NP-40 (Sigma Chemical Co., St. Louis, MO) to block nonspecific antibody binding.

Sections were then incubated overnight at 4°C in the appropriate primary antibody diluted in PBS containing 5% normal goat serum and NP-40. Anti-human bFGF gamma globulins were diluted 100–1,000-fold while the affinity-purified antibodies were diluted to 30 µg/ml. Since the same pattern of staining was seen with both the gamma globulin fraction of the anti-human bFGF serum and affinity-purified IgG, we used in most cases the more readily available gamma globulin preparation to stain sections. In all cases, either nonimmune rabbit gamma globulins or anti-human bFGF depleted gamma globulins were included as controls.

Bound antibody was detected using the ABC method (9) with reagents supplied by Vector Laboratories, Inc. (Burlingame, CA) in their Vectastain ABC Kit. Sections were incubated for 30 min at 37°C in biotinylated goat anti-rabbit IgG, and then for 30 min at room temperature in the ABC reagent (avidin-biotin complex coupled to peroxidase). To visualize the antigen-antibody complexes, sections were then incubated for 8–10 min at room temperature in 0.4 mg/ml 3,3'-diaminobenzidine (Sigma Chemical Co.) in PBS containing 0.003% hydrogen peroxide. Sections were counterstained in 0.125% methylene blue. Photographs were taken with a Nikon automatic camera using Panatomic X black and white film (Eastman Kodak Co., Rochester, NY).

Preparation of Embryo Extracts and Western Blot Analysis

For Western Blot analysis, extracts of embryos at different developmental stages were prepared in the following manner. Embryos were dissected from the yolk and placed briefly in PBS. When several embryos were collected, they were transferred to 20 mM Tris/3 mM EDTA containing 0.5 M NaCl and 0.1 mM PMSF and homogenized briefly in a homogenizer (Dounce, Vineland, NJ) fitted with a teflon pestle. The homogenized material was sonicated at 10 W for a total of 5 min (1-min pulses) while on ice, and then

centrifuged in an Eppendorf microfuge (Brinkman Instrument Co., Westbury, NY) at 16,000 *g* to pellet insoluble material. The Bradford assay (1) was used to determine protein concentrations of the extracts.

The samples were diluted in sample buffer and subjected to SDS-PAGE according to the method of Laemmli (15). Proteins were transferred to nitrocellulose membranes (30) and the membranes were then probed with rabbit anti-human bFGF gamma globulin or affinity-purified IgG as previously described (11), with the following modifications. To detect antigen antibody complex, alkaline phosphatase-conjugated goat anti-rabbit antibody (Promega Biotec, Madison, WI) was used in conjunction with the Protoblot Western Blot System (Promega Biotec).

Results

Identification of bFGF in Chick Embryo Extracts

We have recently shown that specific polyclonal anti-human bFGF antibodies inhibited bFGF-like activity; that is the stimulation of microvascular endothelial cell plasminogen activator production from cultured chick embryo fibroblasts (20). To determine whether extracts of whole chick embryos also contain a bFGF-like molecule, western blot analyses were done on extracts of chick embryos at several stages of development. A single band with an approximate molecular weight of 18,600 was detected in extracts of 4- (stage 24), 5- (stage 26), and 6-d (stage 29) chick embryos using affinity-purified anti-human bFGF IgG (Fig. 1, lanes A, B, and C). No such reactivity was ever detected on blots probed with anti-human bFGF-depleted gamma globulin (Fig. 1, lanes D, E, and F). These immunoblots and the inhibition data described above (Moscatelli et al., 1986) demonstrate that these antibodies recognize chick bFGF and therefore, might be useful in detecting bFGF in the chick embryo by immunohistochemical methods.

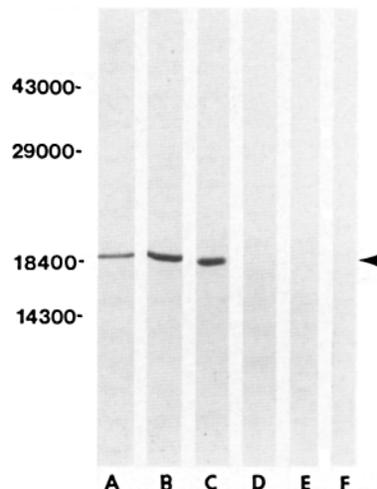


Figure 1. Immunoblot identification of bFGF in extracts prepared from chick embryos. Equal amounts (45 µg) of extracts from 4-, 5-, and 6-d chick embryos were electrophoresed on SDS polyacrylamide gels and transferred to nitrocellulose. Duplicate blots were incubated with either affinity-purified anti-bFGF (lanes A–C) or anti-bFGF-depleted antibodies (lanes D–F) at the same concentration. A protein with a molecular weight of 18,600 was present in extracts from all stages studied. This protein was not recognized by anti-bFGF-depleted antibodies. Lanes A and D, 4-d embryo extract; lanes B and E, 5-d embryo extract; lanes C and F, 6-d embryo extract.

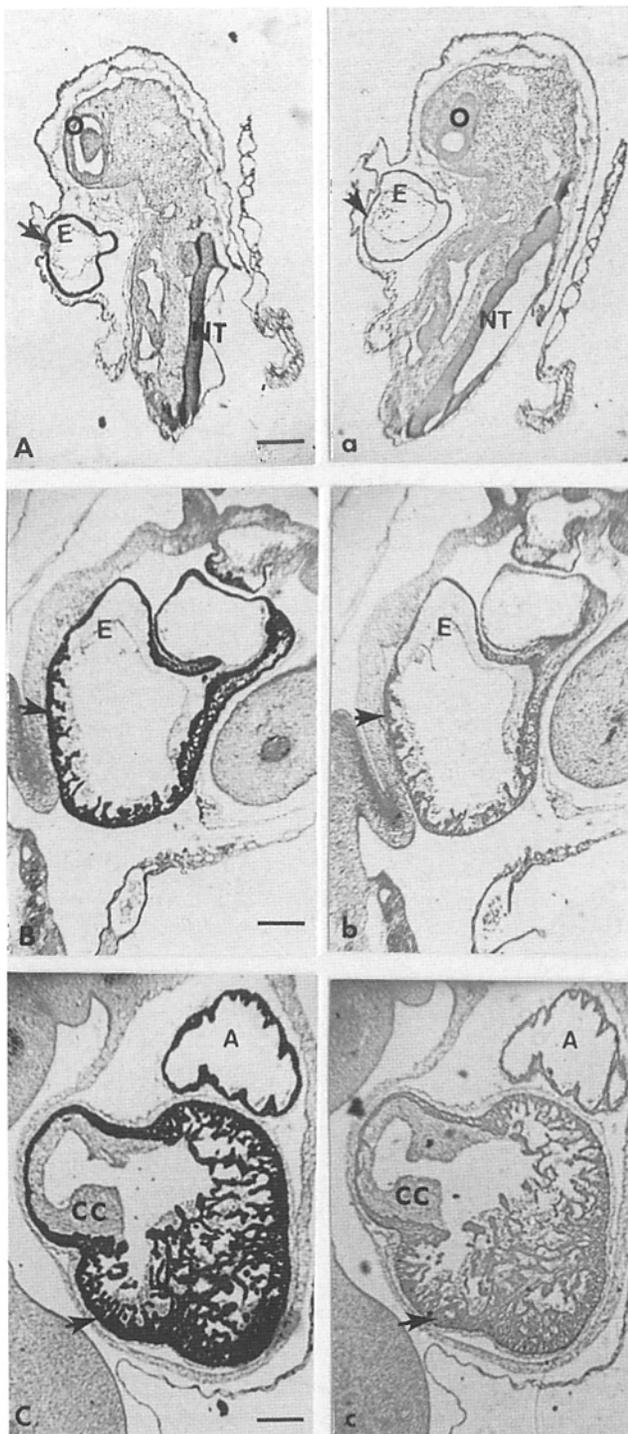


Figure 2. Immunohistochemical identification of bFGF in myocardial tissue of the chick embryo. Sections through 2- (stage 12; *A* and *a*), 3- (stage 19; *B* and *b*) and 4-d (stage 24; *C* and *c*) embryos were incubated with either anti-human bFGF gamma globulins (uppercase letters) or anti-bFGF-depleted gamma globulins (lowercase letters). In the developing heart, only the myocardium (arrow) stained positively for bFGF. Although not apparent in these black and white photographs, the neural tube (*NT*) and developing eye (*o*) were counterstained dark blue with methylene blue. *E*, endocardium; *A*, atrium; *CC*, cardiac cushions. Bars, 0.2 mm.

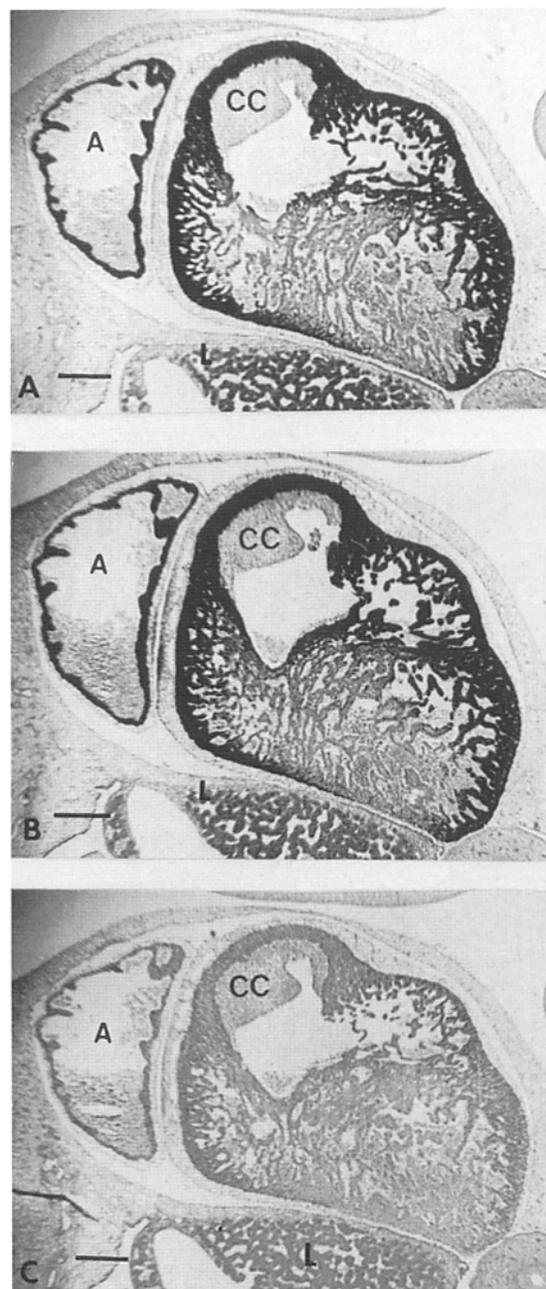


Figure 3. A comparison of staining patterns with anti-bFGF gamma globulins and affinity-purified anti-bFGF antibodies in the 5-d (stage 26) heart. Anti-bFGF gamma globulins (*A*) stained embryonic myocardium at 5 d. Affinity-purified anti-human bFGF antibodies (*B*) gave an identical staining pattern as the gamma globulin fraction. Anti-human bFGF-depleted gamma globulins did not stain the myocardium (*C*). The liver (*L*) is not stained with the antibodies but is counterstained dark blue. *A*, atrium; *CC*, cardiac cushions. Bars, 0.2 mm.

Immunolocalization of bFGF in the Chick Embryo

A striking pattern of immunoreactivity was obtained with the anti-human bFGF antibodies in sections through 2–6-d embryos. At all stages studied, the developing heart stained positively for bFGF (Fig. 2). Staining was specific for the myocardial tissue only. This specificity was readily apparent

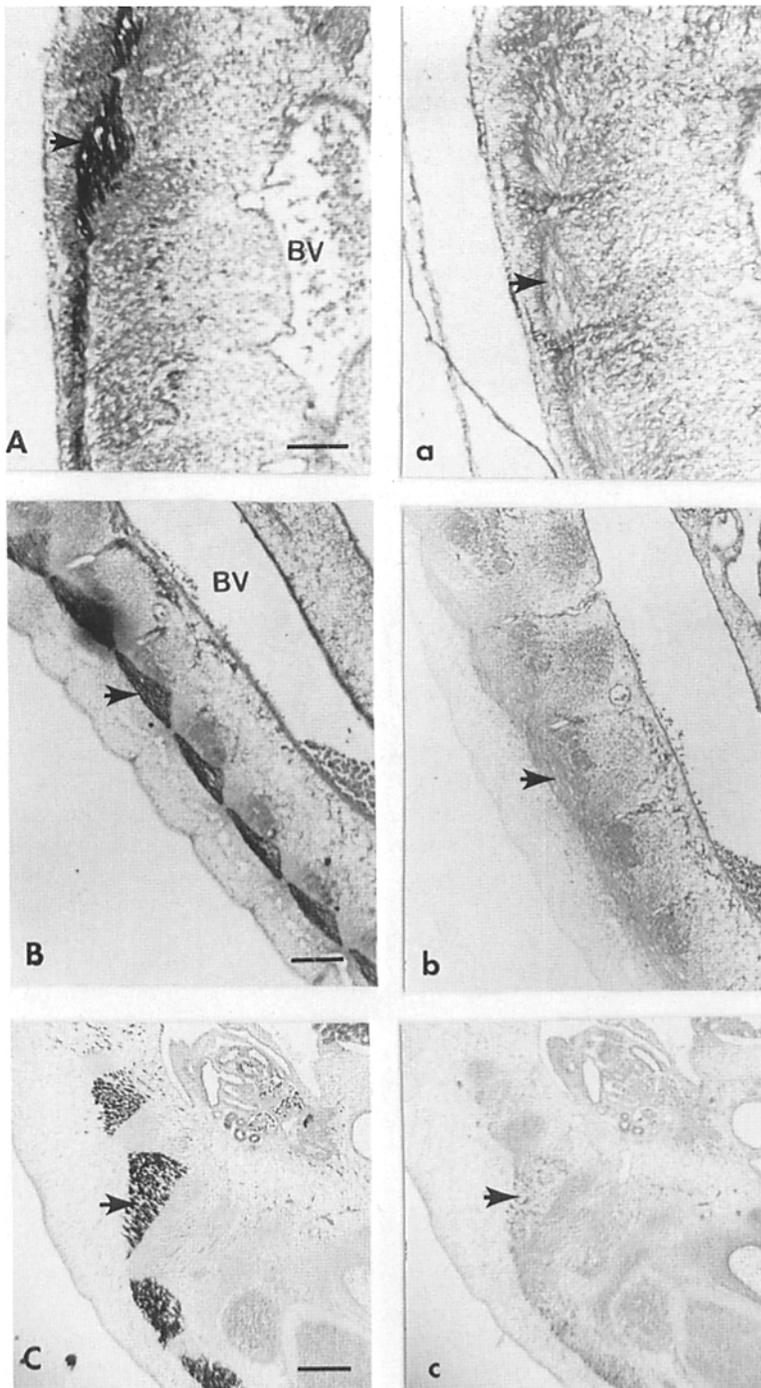


Figure 4. Immunohistochemical identification of bFGF in myotomes of the chick embryo. Sections through 3- (stage 19; *A* and *a*), 4- (stage 24; *B* and *b*), and 5-d (stage 26; *C* and *c*) embryos were incubated with either anti-human bFGF gamma globulins (uppercase letters) or anti-bFGF-depleted gamma globulins (lowercase letters). The myotomes (arrow) were the only regions of the somites which stained with the anti-bFGF gamma globulins. Neither the sclerotome nor dermatome was stained. Bars: (*A*) 0.05 mm; (*B*) 0.3 mm; (*C*) 0.2 mm.

in 48-h embryos (stage 12) when the heart is merely a single tube consisting of an outer epimyocardial layer and an inner endocardial layer separated by a wide space filled with extracellular ground substance. The intensity of the staining was constant throughout the myocardium of 2- (stage 12), 3- (stage 19), and 4-d (stage 24) embryos. However, by 5 d (stage 26) (Fig. 3) the intensity of staining was much decreased in the most internal regions of the heart, and this pattern persisted in 6-, 12-, and 15-d hearts (preliminary observations). Interestingly, in sections through 12-d hearts in which the dorsal aorta is prominent, it is apparent that the

anti-bFGF antibodies did not stain the endothelium or smooth muscle of the blood vessel wall (data not shown).

In addition to the myocardium, somite myotome, consisting of skeletal muscle cell precursors, also stained intensely with the anti-bFGF antibodies in sections through 3-6-day embryos (stages 19 to 29) (Figs. 4, 5, and 6). The remaining two regions of the somites, the sclerotome and dermatome, did not react with the anti-human bFGF antibodies. In sections through 6-d-old embryos in which the developing vertebral column can be identified (Fig. 6 *A*), the myoblasts remaining in the somite area, between adjacent developing

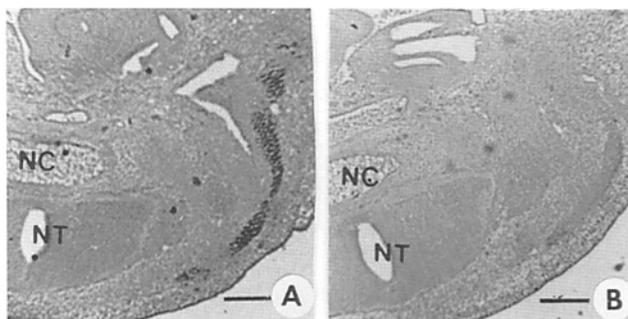


Figure 5. bFGF staining in a transverse-section through the myotome of a 4-d embryo. Anti-bFGF gamma globulins were diluted 1:1,000 (A) and incubated with sections through a 4-d embryo. A positive response was obtained, while no staining was apparent with anti-bFGF-depleted antibodies (B). NT, neural tube; NC, notochord. Bars, 0.2 mm.

vertebrae, continued to be intensely stained with the anti-bFGF antibodies even though some of the cells were beginning to differentiate into multinucleated myotubes (Fig. 6 B). These cells were darkly stained, while neither the nuclei, nor the spaces between cells stained with the antibody. This pattern of staining of myoblasts and myotubes is most consistent with a cytoplasmic localization of bFGF. The same staining pattern was apparent in the myocardial tissue (data not shown).

In sections through 4-d (stage 24) embryos, in which the limb buds were easily recognizable, single cells that appeared to be migrating into the bud, away from the trunk were stained with the anti-bFGF antibodies (Fig. 7 A). In some regions of the limb bud, adjacent to areas in which chondrogenesis was beginning to occur, small groups of these cells aggregated into clusters. These are presumably myoblasts which go on to form the skeletal muscle of the limb. In fact, in some sections through 5- and 6-d limbs, in-

tensely stained multinucleated myotubes like those described above were present (Fig. 7, B, C, and D). In 12-d limbs some staining of the skeletal muscle remained but it was not nearly so intense as in the earlier limbs (preliminary observation).

No positively stained structures were identified in sections through the head of 3- and 4-d embryos. However, sections through the head of 5-d embryos contained small groups of stained cells (Fig. 8). At the base of the developing tongue were individual stained cells beginning to aggregate (Fig. 8 A). Adjacent to the ventral surface of the embryonic eye, bundles of stained cells (Fig. 8 B) were seen which are the developing extra-ocular muscles.

Discussion

We have localized bFGF in the chick embryo using specific antibodies in conjunction with immunohistochemistry. To date, most studies have focused on the identification of FGF in tissue extracts or in cultured cells, neither of which provides information on its cellular distribution during development. This study provides information concerning the cellular localization of the growth factor within embryonic tissues, and to our knowledge, represents the first report on the immunohistochemical localization of bFGF in an intact embryo during the initial stages of organogenesis.

Recently, both aFGF and bFGF have been identified in extracts of embryonic tissues, suggesting that these growth factors may be involved in the regulation of developmental processes. The presence of FGF in embryonic brain (aFGF-like: 24; aFGF and bFGF: 18) and kidney (aFGF: 25) has been correlated with vascularization of these tissues. This is consistent with the well-known angiogenic activity of FGF in *in vivo* model systems (3, 19, 28). However, based on the myriad of biological activities of FGF demonstrated *in vitro*, such as the promotion of neuronal survival and neurite outgrowth (8, 31), the stimulation of protease production in capillary endothelial cells (19), and the stimulation of prolif-

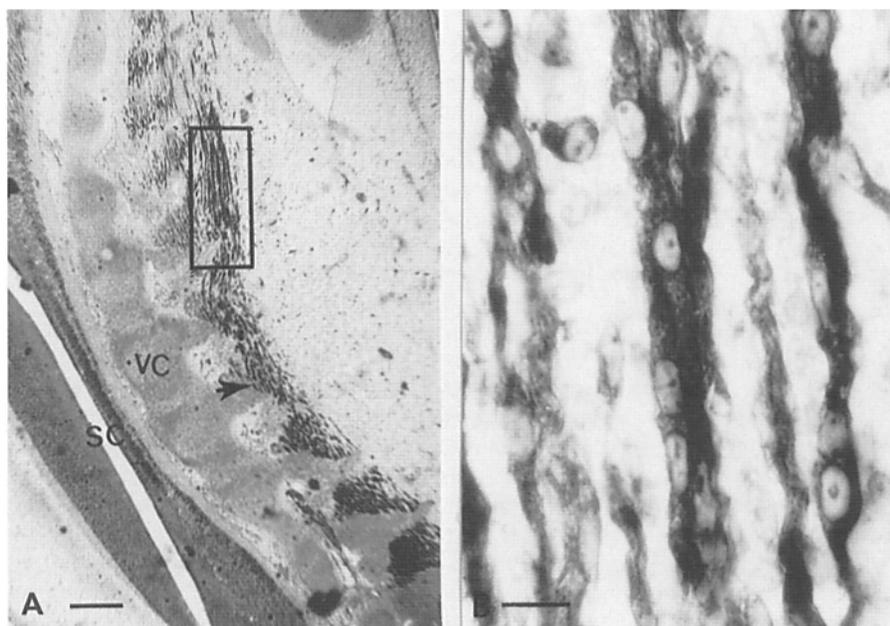


Figure 6. Immunohistochemical localization of bFGF in multinucleated myotubes of 6-d chick embryo myotomes. Sections through a 6-d (stage 29) embryo were stained with anti-bFGF gamma globulins. The cells of the myotome, which are beginning to differentiate into intercostal muscle cells stained positively (A, arrow). A higher magnification (B) of part of the region within the box in A, demonstrates the multinucleate nature of the myotubes. Neither the spinal cord (SC) nor the developing vertebral column (VC) stained positively with the antibody. No staining of the myotubes was apparent with anti-bFGF-depleted antibodies. Bars: (A) 0.2 mm; (B) 10 μ m.

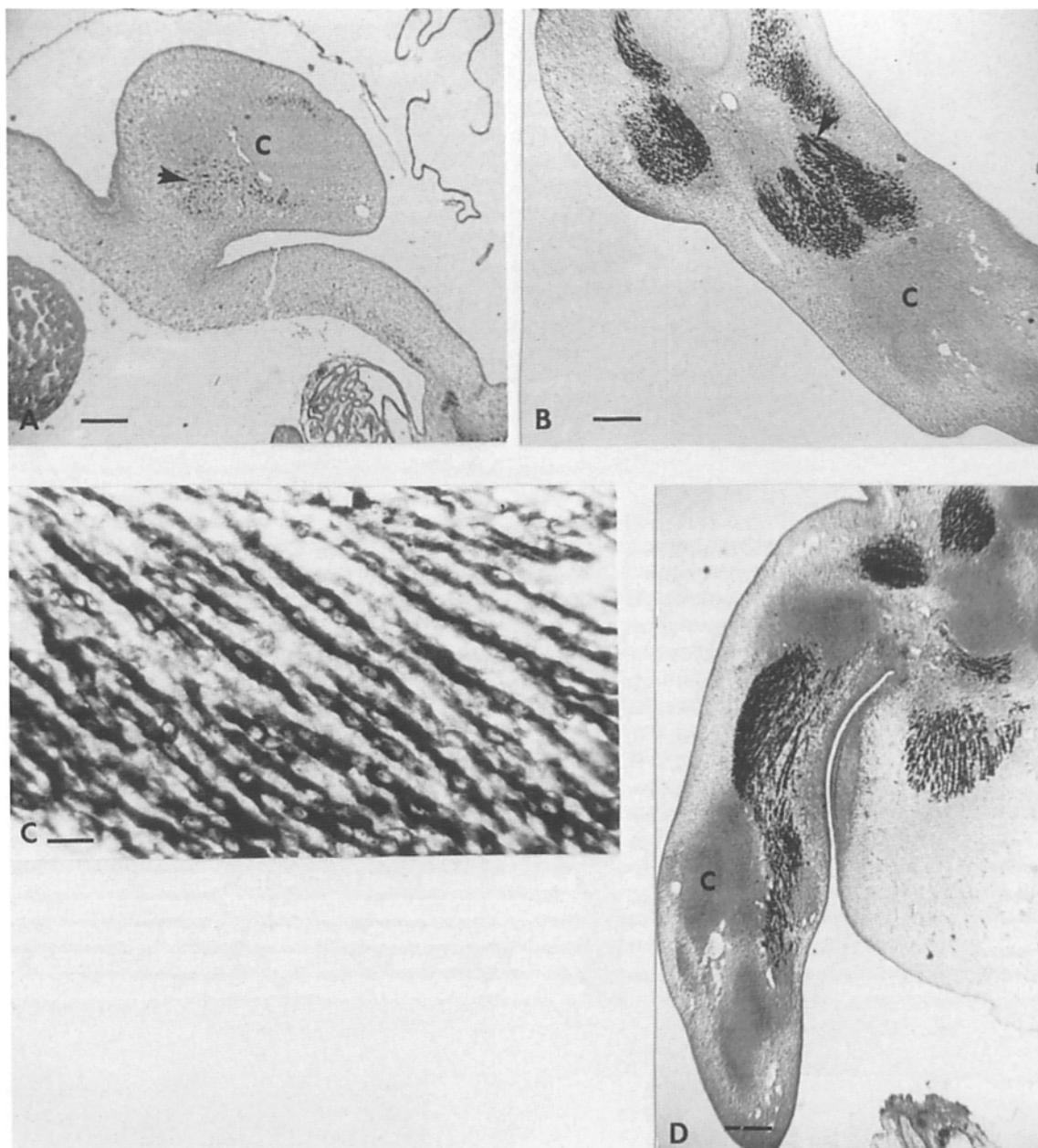


Figure 7. Anti-bFGF gamma globulins stain a population of cells in the chick limb bud. Sections through 4- (stage 24; *A*), 5- (stage 26; *B*), and 6-d (stage 29; *D*) chick embryos were incubated with anti-bFGF gamma globulins. In a cross section through a 4-d limb bud (*A*) single immunoreactive cells (*arrow*) were seen surrounding areas of chondrogenesis. In a longitudinal section through a 5-d limb (*B*) stained cells aggregated into discrete bundles. In some sections, cells appear as long tubular structures (*arrow*), which are presumably differentiating myotubes. In a longitudinal section through a 6-d limb bud (*D*), bundles of myotubes stained positively with anti-bFGF gamma globulin. The multinucleate structure of the myotubes can be seen more clearly at higher magnification (*C*). No staining was observed when anti-bFGF-depleted antibodies were used. *C*, areas of chondrogenesis. Bars: (*A*, *B*, and *D*) 0.2 mm; (*C*) 0.02 mm.

eration of a wide variety of mesodermal cells including endothelial cells (4) and skeletal muscle myoblasts (2), it seems likely that the regulation of angiogenesis may not be the only role for FGF during development. In fact, a recent report demonstrates that FGF can act as a mesodermal-inducing factor during amphibian embryogenesis (14, 29).

The regulation by FGF of multiple events associated with organogenesis, has been suggested by the identification of FGF in a variety of embryonic tissues. Mascarelli et al. (18)

have identified both aFGF and bFGF in 11-d chick embryo retina and vitreous, neither of which is a vascularized tissue. They have suggested that FGF may be involved in some inductive events in the eye. In addition, Seed et al. (27) have identified FGF in chick limb buds and have hypothesized a role for FGF in muscle differentiation.

The demonstration here that bFGF can be localized to developing myocardium, somite myotome, and differentiating muscle in head, trunk, and limbs, not only extends the

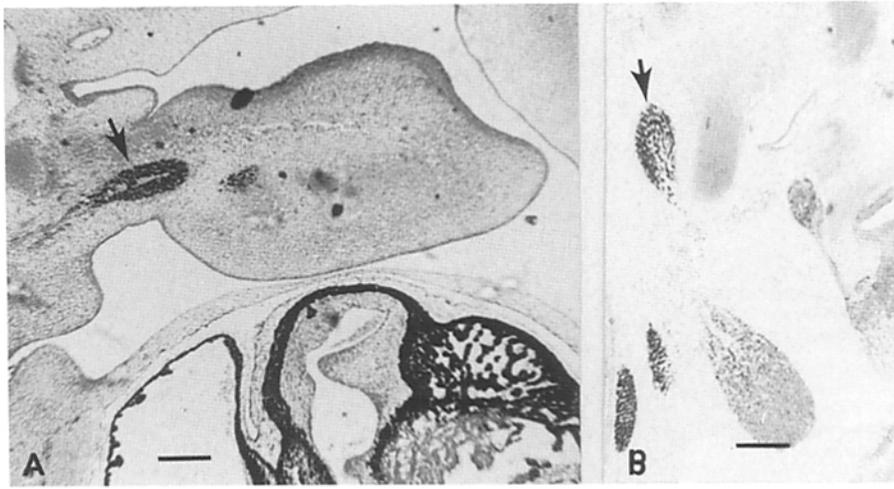


Figure 8. bFGF-containing cells are present in the head region of the 5-d chick embryo. When sections through 5-d (stage 26) chick embryos were incubated with anti-bFGF gamma globulins only the developing muscle at the base of the tongue (A, arrow) and the extra-ocular muscles (B, arrow) stained positively for bFGF. Bars, 0.2 mm.

finding of Seed et al. (27) by identifying a source for the FGF which can be detected in the chick embryo limb bud, but also provides a further example of a spatial pattern for bFGF inconsistent with a sole role for this growth factor in angiogenesis. In the developing heart, the formation of the endocardium, which is made up of endothelial cells, precedes the appearance of the myocardium (17), which stained intensely with our anti-bFGF antibodies (Fig. 2). In several sections, blood vessels are apparent in regions in which no anti-bFGF antibody staining was detectable (Figs. 4-7). However, it must be kept in mind that other tissues may contain bFGF, but in quantities too small to be detected with our antibodies, and that these antibodies recognize only the basic form of FGF (11). In addition, we have looked at early organogenesis in the chick embryo (days 2-6), and cannot comment on the role of bFGF in embryonic events occurring after this time.

The presence of bFGF in embryonic chick striated muscle is consistent with the results of Kardami et al. (12, 13), who have purified a bFGF-like protein from adult skeletal muscle. Receptors for FGF have been detected on a cultured myoblast cell line (21). Both aFGF and bFGF stimulate the proliferation of these cells and repress their terminal differentiation (2). In addition, a recent study demonstrates that a loss of FGF binding to the surface of these cells is correlated with their terminal differentiation (22). These data seem to suggest a possible autocrine role for FGF in myoblast differentiation. Further evidence for the importance of FGF in the regulation of muscle differentiation comes from studies on the effect of FGF on primary clonal cultures of chick embryo myoblasts (26). While FGF delayed the terminal differentiation of myoblasts derived from 7-12-d-old chick embryo wings, some myoblasts derived from 4- and 5-d chick embryo wing buds were actually dependent on FGF for their differentiation. The detection of bFGF in striated muscle myoblasts by immunohistochemistry suggests the possibility that bFGF may regulate muscle development in the embryo. Since the localization of bFGF does not distinguish between the myoblast as a source and/or target for this protein, whether bFGF may act to regulate muscle differentiation in an autocrine manner cannot be commented on. It is possible that bFGF may be at work early in muscle development, stimulating the migration of cells from the myotome. Although there

has been no report of the stimulation of myoblast migration by bFGF it is known that bFGF stimulates chemotaxis of capillary endothelial cells (19). The detection of bFGF in small groups of cells which seem to be migrating from the somites into the limb bud, suggests such a role for bFGF.

Recently, bFGF has been identified in the extracellular matrix of bovine aortic and corneal endothelial cells (32). However, in our studies, bFGF seems to be mainly intracellular. It is possible that small amounts of this protein are present in the matrix of embryonic striated muscle but is undetectable at the level of sensitivity of the immunohistochemical technique. However, it is also possible that during the particular developmental stages studied here (stages 12-29), bFGF is not deposited into the matrix.

The highly specific spatial pattern for bFGF localization generated by staining with both a gamma globulin fraction of anti-bFGF serum and affinity-purified anti-bFGF antibodies has aided in the generation of a testable hypothesis for the role of bFGF in organogenesis.

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