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Expression of Fibroblast Growth Factor by F9 Teratocarcinoma Cells as a Function of Differentiation

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Abstract. F9 teratocarcinoma stem cells treated with retinoic acid (RA) and dibutyryl cAMP (but_2 cAMP) differentiate into embryonic parietal endoderm. Using heparin-affinity chromatography, endothelial cell proliferation assays, immunoprecipitation, and Western analysis with antibodies specific for acidic and basic fibroblast growth factors (FGFs), we detected biologically active FGF in F9 cells only after differentiation. A bovine basic FGF cDNA probe hybridized to 2.2-kb mRNAs in both F9 stem and parietal endoderm cells and to a 3.8-kb mRNA in F9 stem cells. A genomic DNA probe for acidic FGF hybridized to a 5.8–6.0-kb mRNA in both F9 stem and parietal endoderm cells, and to a 6.0–6.3-kb mRNA only in parietal endoderm cells. Although these FGF mRNAs were present in the stem cells, we could find no evidence that F9 stem cells synthesize FGFs, whereas differentiated F9 cells synthesized both acidic and basic FGF-like proteins. We conclude that biologically active factors with properties characteristic of acidic and basic FGF are expressed by F9 parietal endoderm cells after differentiation. Differentiating embryonic parietal endoderm thus may serve as a source of FGF molecules in the developing blastocyst, where these factors appear to play a central role in subsequent embryogenesis.

F9 murine teratocarcinoma stem cells are analogous in many respects to the cells of the inner cell mass of the mouse blastocyst (Martin, 1980). Within 3–4 d after treatment of monolayer cultures with retinoic acid (RA)[1] and dibutyryl cAMP (but_2 cAMP), F9 stem cells irreversibly differentiate into a homogeneous population of cells indistinguishable from extraembryonic parietal endoderm (Strickland et al., 1980). These parietal endoderm cells synthesize plasminogen activator, laminin, type IV collagen, and membrane-bound heparan sulfate (Strickland et al., 1980; Wang et al., 1985; Kapoor and Prehm, 1983), keratins (Vasseur et al., 1985; Singer et al., 1986), and H-2 antigen (Croce et al., 1981). This system has been used by a number of workers as a model for the study of the differentiation of embryonic inner cell mass cells and has provided a means for identifying the biochemical and molecular events involved in this process.

Another application for this system has been the study of tumorigenesis. Teratocarcinoma stem cell lines characteristically undergo continuous proliferation in vitro and are tumorigenic in vivo, whereas the differentiated cells are not tumorigenic (Martin, 1980). These observations led investigators to compare the growth requirements of stem and differentiated cells in a number of teratocarcinoma cell lines (Heath et al., 1981; Gudas et al., 1983; Heath and Isacke, 1984). Several lines acquire specific growth factor receptors as a function of differentiation. For example, differentiated PC-13 and OC-15S1 cells bind increased amounts of epidermal growth factor (EGF) (Rees et al., 1979) and insulin (Heath et al., 1981) after differentiation. These studies suggest that the inability of these differentiated cells to form tumors correlates with an increased responsiveness to external signals of growth control.

In contrast, undifferentiated teratocarcinoma stem cells were reported to secrete growth factors. For example, PSA-1-G and F9 stem cells secrete a platelet-derived growth factor (PDGF)-like molecule (Gudas et al., 1983; Rizzino and Bowen-Pope, 1985). Another undifferentiated teratocarcinoma cell line, PC13, secretes an uncharacterized growth factor, embryonal carcinoma-derived growth factor, which has been shown to stimulate the proliferation of fibroblasts and the PC13 differentiated cells (Heath and Isacke, 1984). Both of these growth factors are secreted by stem cells and are either absent or present at lower levels in the media of the corresponding differentiated cells. Although it is not

1. *Abbreviations used in this paper*: a, acidic; b, basic; but_2 cAMP, dibutyryl cAMP; EGF, epidermal growth factor; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; RA, retinoic acid; TGF-β, transforming growth factor beta.

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known what roles growth factors play in the developing embryo, evidence is rapidly emerging that dramatic changes occur in the profiles of growth factor production as a function of differentiation. We have examined the F9 stem and differentiated teratocarcinoma cells for the presence of another well-characterized class of cellular mitogens, the fibroblast growth factors (FGFs).

In recent years, FGFs have been identified in numerous normal tissues, and in association with a number of solid tumors (for reviews see Folkman and Klagsbrun, 1987; D'Amore and Braunhut, 1988). These factors have been purified to homogeneity using their unusual affinity for the glycosaminoglycan heparin (Shing et al., 1984). It has emerged that there are two classes of heparin-binding growth factors, class 1 and class 2, which are synonymous with acidic (a) and basic (b) FGF, respectively.

FGF has been identified in many tissues, including pituitary (Bohlen et al., 1984), placenta (Gospodarowicz et al., 1985*a*), corpus luteum (Gospodarowicz et al., 1985*b*), adrenal gland (Gospodarowicz et al., 1986), and kidney (Baird et al., 1985). Furthermore, endothelial cell mitogens associated with a number of tumors, including a rat chondrosarcoma, a human melanoma, and a hepatoma, are structurally related if not identical to bFGF (Klagsbrun et al., 1986). aFGF appears to have a more limited distribution and has been found in hypothalamus (Klagsbrun and Shing, 1985), retina (D'Amore and Klagsbrun, 1984), testis (Feig et al., 1983), and bone (Hauschka et al., 1986), and in association with some human tumor cell lines (Lobb et al., 1986).

The amino acid sequence for bovine bFGF is known (Esch et al., 1985) and 7.0- and 3.7-kb mRNA transcripts have been identified in bovine adrenal cortex-derived and bovine brain-derived capillary endothelial cells (Schweigerer et al., 1987*a*). Rodent tissues screened with a cDNA encoding rat bFGF revealed a single 6.0-kb mRNA in rat hypothalamus and brain cortex tissue (Shimasaki et al., 1988), and human fibroblasts express mRNA transcripts encoding bFGF of 7, 3.7, 2.2, and 1.5 kb in size (Steinfeld et al., 1988).

The amino acid sequence of aFGF has been determined (Gimenez-Gallego et al., 1985) and a single, 4.8-kb mRNA transcript encoding aFGF has been reported in human brain stem using Northern analysis (Jaye et al., 1986). There are few reports in the literature on the sizes of the transcripts encoding aFGF in other species.

Factors with properties similar but not identical to the FGFs have been detected in F9 cells (van Veggel et al., 1987; Rizzino et al., 1988) and may relate to the presence in F9 cells of mRNA transcripts encoding two oncogenes in the FGF family, *hst 1* and *int-2* (Yoshida et al., 1988). The biologic properties of the protein products of these genes are now known, but the message sizes are distinctly different from mRNA encoding the FGFs (Yoshida et al., 1988). F9 stem cells express high amounts of a 3.0-kb transcript for *hst 1*, which decreases after differentiation. Conversely, mRNAs encoding *int-2* of 2.9, 2.7, 1.8, and 1.6 kb are detected in greater abundance in F9 parietal endoderm cells (Yoshida et al., 1988). The significance of the differential expression of mRNAs encoding these two oncogenes in F9 cells and their presence during early embryogenesis is not known. In this report, we examine the expression of FGF in F9 cells as a function of differentiation.

Materials and Methods

Materials

RA (all-*trans*) and most other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). [^{35}S]Methionine (1,131 Ci/mmol) was obtained from New England Nuclear (Boston, MA). The cDNA clones for basic and acidic FGF were generously provided by Dr. John Fiddes (California Biotechnology, Inc., Mountain View, CA). Heparin-Sepharose was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Reagents used for Na_2DodSO_4 PAGE were from Bio-Rad Laboratories (Richmond, CA). Tissue culture reagents were from Gibco Laboratories (Grand Island, NY).

Cell Culture

The F9 teratocarcinoma stem cells were grown on gelatinized tissue culture (Falcon Labware, Oxnard, CA) dishes in DME containing 10% heat-inactivated calf serum. For differentiation studies, cells were treated with RA (1×10^{-6} M), but$_2$ cAMP (2.5×10^{-4} M), and theophylline (400 μM) for at least 72 h. Details of this differentiation protocol have been described previously (Wang et al., 1985).

Bovine capillary endothelial cells from adrenal cortex were provided by Dr. J. Folkman (The Children's Hospital, Boston, MA). The endothelial cells were grown in DME containing 10% calf serum and cells were maintained on gelatinized tissue culture dishes from Nunc (Vanguard International, NJ). Endothelial cell proliferation studies were conducted by plating the cells at 1×10^4 per well in 24 multiwell gelatinized dishes. The cells were allowed to attach overnight and washed, and 0.5 ml of fresh media was added with aliquots of fractions to be tested. 72 h later cells were washed and trypsinized and cell numbers were determined electronically with a counter (Coulter Electronics, Inc., Hialeah, FL). The SK hepatoma cells, provided by Dr. Michael Klagsbrun (The Children's Hospital, Boston, MA), were cultured in DME plus 10% calf serum.

Preparation of Cell Extracts

F9 stem and parietal endoderm cells were harvested from monolayer cultures by rinsing in cold PBS three times, followed by trypsinization, or in some experiments removal by scraping with a rubber policeman. Approximately 1×10^8–1×10^9 stem or RA-treated cells were harvested, depending on the experiment. Cells were washed again in PBS, centrifuged at 2,000 rpm for 10 min at 4°C, resuspended rapidly in a small volume (0.6–1.2 ml) of 1 M NaCl and 0.01 M Tris-HCl buffer, pH 7.6, and sonicated on ice (model W370; Heat Systems-Ultrasonics Inc., Farmingdale, NY; microprobe setting 2, twice for 10 s each). Cell extracts were then incubated for 1 h on ice in the presence or absence of protease inhibitors: 1 μg/ml leupeptin, 2.75 μg/ml pepstatin, and 175 μg/ml PMSF as indicated. After centrifugation at 10,000 rpm for 5 min at 4°C in a microfuge, the supernatant, in a volume of ~0.4–1.0 ml, was stored at −20°C before being assayed for mitogenic activity. In some experiments, affinity-purified antiserum to porcine transforming growth factor (TGF-β) (1:1,000) (R&D Systems, Inc., Minneapolis, MN) was added directly to the extract or pooled fractions for 1 h at 4°C. Staphylococcal protein A-Sepharose was then added to the suspension and the mixture incubated overnight at 4°C with mixing, the beads were pelleted at 1,000 *g*, and the supernatants were assayed in parallel with an untreated extract for their effect on capillary endothelial cell growth.

Preparation of Conditioned Media

Conditioned media were prepared from F9 stem and parietal endoderm cell cultures that had been treated with RA plus but$_2$ cAMP for 4 d by washing ~5×10^8 F9 cells three times with sterile PBS. The cells were then placed in DME plus 10% calf serum with RA and but$_2$ cAMP for 48 h. Approximately 14 ml of DME was used for each flask of 5×10^7 cells. The medium was then centrifuged at 5,000 rpm for 12 min at 4°C and frozen at −20°C.

Column Chromatography

Cell extracts, prepared as described above, were diluted to 0.1 M NaCl, in PBS buffer, pH 7.6, and applied to a column of heparin-Sepharose (1 × 20 cm, 10 ml) that had been previously washed with 3.0 M NaCl and PBS buffer, pH 7.6, and reequilibrated with PBS buffer, pH 7.6. After loading the sample, the column was clamped overnight at 4°C to allow for maximal binding. The column was then washed with 3 column vol of PBS buffer, pH

7.6, at a flow rate of 10 ml/h at 4°C. For studies on fractionation of conditioned media, the conditioned media or an equivalent volume of unconditioned media were subjected to heparin-Sepharose chromatography by loading the entire sample at a rate of 10 ml/h onto a heparin-Sepharose column (1.5 × 30 cm, 40 ml) prewashed and equilibrated as described above. The 3T3 cell stimulatory activities were separated on the heparin-Sepharose column with a gradient of 0.1–2.0 M NaCl, or in some cases, up to 3.0 M NaCl in PBS buffer, pH 7.6, at a flow rate of 10 ml/h. Column fractions were screened for their ability to stimulate DNA synthesis in quiescent BALB/c 3T3 cells by established methods (Klagsbrun et al., 1977). Briefly, a small aliquot (5–50 μl) of each fraction was added to contact-inhibited and nutrient-starved monolayers of 3T3 cells in the presence of methyl-[^{3}H]thymidine. 48 h later, the stimulation of DNA synthesis in the 3T3 cultures was determined by measuring the amount of [^{3}H]thymidine incorporated into TCA-precipitable material by scintillation spectrometry (Klagsbrun et al., 1977).

Radiolabeling of Intracellular Proteins

To determine if FGF was being actively synthesized by F9 cells after differentiation, 4-h labeling studies were conducted using F9 stem and fully differentiated F9 parietal endoderm cells. The parietal endoderm cells were obtained by growing F9 teratocarcinoma stem cells in DME containing 10% calf serum with RA (1×10^{-6} M), but$_2$ cAMP (2.5×10^{-4} M) and theophylline (400 μM) for 5 d, with refeeding on day three. For labeling, the cells were rinsed with methionine-free DME and then incubated for 4 h in 16 ml/dish of medium consisting of methionine-free DME, 5% dialyzed calf serum, and [^{35}S]methionine (1,094 Ci/mmol) at 5 μCi/ml. After 4 h at 37°C, the radiolabeling medium was removed, the cells were washed three times with cold PBS, protease inhibitors were added, and cell extracts were harvested and prepared as described above. Approximately 6×10^8 F9 stem cells and 1×10^8 RA- and but$_2$ cAMP-treated cells were radiolabeled.

Immunoprecipitation

Samples were diluted with distilled water to lower the salt concentration to 0.1 M NaCl. Gelatin (Difco, Detroit, MI), Triton X-100, and sodium deoxycholate were then added to yield final concentrations of 2 mg/ml, 0.5%, and 0.5%, respectively. Finally, preimmune antiserum (1:300) or polyclonal antiserum specific for basic or acidic FGF (1:300) was added and samples were incubated for 1 h at 4°C with shaking. The anti-FGF antisera were directed against synthetic peptides representing (*a*) amino acids 1-12 of the amino terminus of bFGF; and (*b*) amino acids 59–90 of the carboxyl terminus of aFGF. Staphylococcal protein A-Sepharose was then added to the suspension and the mixture incubated overnight at 4°C with mixing. The antigen-antibody complexes were pelleted by centrifugation and the beads were washed several times in PBS and finally with deionized distilled H_2O before elution directly into Na_2DodSO_4-PAGE sample buffer and gel electrophoresis (Laemmli, 1970).

Western Blot Analysis

Cell extracts from 1×10^8 F9 stem cells or differentiated parietal endoderm prepared as described above were diluted with distilled water to lower the salt concentration to 0.1 M NaCl. Prewashed heparin-Sepharose beads were added to the extracts and incubated overnight at 4°C with mixing. The beads were then washed several times with PBS, pH 7.5, and then incubated with 1.8 ml of PBS buffer containing 0.8 M NaCl for 1 h at 4°C with rotation. The beads were washed again in PBS, followed by two final washes with distilled water. Proteins were eluted from the heparin-Sepharose beads directly into Na_2DodSO_4 PAGE sample buffer, electrophoresed on Na_2DodSO_4-15% polyacrylamide gels, and transferred electrophoretically to BA-83 nitrocellulose paper (Transblot cell; Bio-Rad Laboratories) by described methods (Wadzinski et al., 1987). After incubation with anti-FGF antisera (1:1,000) for 24 h at 4°C, primary antibody binding was visualized by serial incubations with biotinylated goat anti-rabbit antibodies, peroxidase-conjugated streptavidin and 4-chloro-1-naphthol (Wadzinski et al., 1987). Additional antibodies to bFGF, raised to a synthetic peptide representing an internal portion of the molecule, amino acid residues 33–43 of bovine bFGF, were used in these studies.

FGF Sequence-specific Probes

The cDNA probe for bovine bFGF, given to us by Dr. John Fiddes (California Biotechnology) was pJJ11-1, which contains a 1.4-kb insert beginning ~100 bp upstream of the initial methionine, and extending to ~830 bp downstream of the translation stop codon (Abraham et al., 1986).

The probe for aFGF, provided by Dr. John Fiddes, was pCBI-100, which contains a 250-bp Alu fragment of bovine genomic DNA that encodes amino acids 1–41 of aFGF, plus a portion of an intron (Abraham et al., 1986). Before use in Northern analysis, the cDNA and genomic inserts for bFGF and aFGF were ^{32}P-labeled by random hexamer priming.

Northern Analysis

F9 stem cells were plated at 1.5×10^6 cells/dish, and after an overnight incubation at 37°C, RA, but$_2$ cAMP, and theophylline were added to some plates as previously described (Wang et al., 1985). Poly A$^+$ RNA was isolated from F9 stem cells and from F9 cells treated for 4 d with RA and but$_2$ cAMP to induce differentiation as described (Wang et al., 1985) and was analyzed by Northern blot hybridization as previously described (Wang et al., 1985). Poly A$^+$ RNA was also isolated from SK hepatoma cells. Hybridization conditions for the cDNA probes were: 6× SSC, 0.05% sodium pyrophosphate, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.1% SDS, 100 μg/ml denatured sonicated salmon sperm DNA, and 50% formamide at 42°C for 24 h. After washing the nitrocellulose blots in 2× SSC at 42°C for the bFGF and aFGF probes, or in 1× SSC for the laminin B1, J31, and actin probes (Wang et al., 1985), the blots were exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) at −70°C with an intensifying screen.

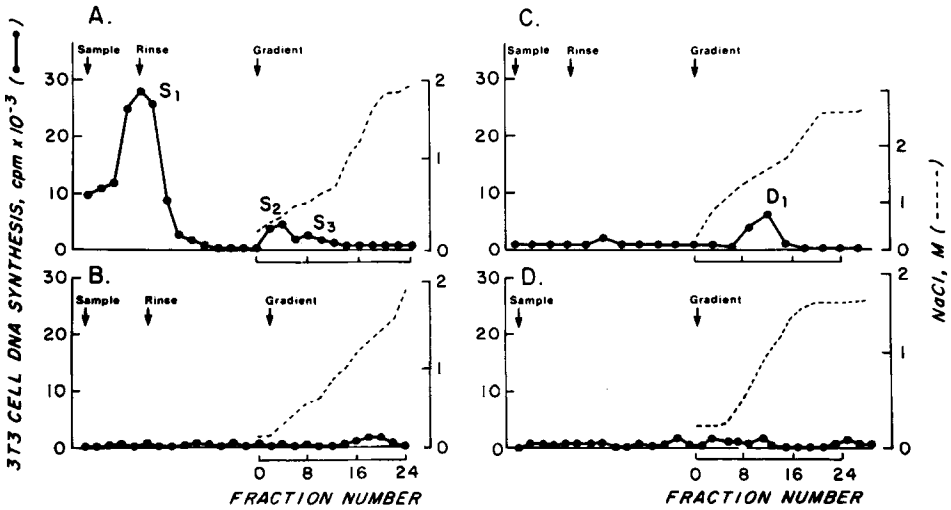

Figure 1. Heparin-Sepharose chromatography of (*A*) cell extracts of undifferentiated F9 stem cells; (*B*) cell extracts of F9 cells after 3-d treatment with RA and but$_2$ cAMP; (*C*) cell extracts; and (*D*) conditioned media from fully differentiated F9 parietal endoderm cells, treated for 5 d with RA and but$_2$ cAMP. The bound proteins were eluted by applying a linear sodium chloride gradient (- - -), and growth factor activity was monitored by the stimulation of DNA synthesis in 3T3 cells (-●-).

Results

Heparin-binding Mitogenic Activity in F9 Stem Cells

Over the course of 2–4 d after treatment with RA and but_2 cAMP, F9 mouse teratocarcinoma stem cells undergo differentiation to parietal endoderm. We used the affinity of immobilized heparin for FGF to assay for these proteins in cell extracts of F9 stem cells and differentiated F9 parietal endoderm cells. Heparin-Sepharose affinity chromatography of the extracts was performed (Shing et al., 1984), and fractions assayed for stimulation of DNA synthesis in BALB/c 3T3 cells (Klagsbrun et al., 1977). This assay system is widely used for the rapid screening of a variety of growth factors, including the FGFs.

The F9 stem cell extract fractionated over heparin-Sepharose exhibited three peaks of stimulatory activities, which we have designated S1, S2, and S3 (Fig. 1 *A*). The largest peak (S1) was present in the void volume and accounted for ~80% of the stimulatory activity in the starting material.

Two other peaks of 3T3 cell stimulatory activity, representing 10% of the total activity, eluted from heparin-Sepharose at 0.25 M and 0.5 M NaCl and are designated S2 and S3, respectively. These activities have not been fully characterized but, because PDGF is known to adhere to heparin and elute with 0.5 M NaCl (Shing et al., 1984), it is probable that S3 corresponds to the intracellular pool of a PDGF-like factor that one of us has reported in the conditioned media of F9 stem cells (Gudas et al., 1983). We recovered ~90% of the starting mitogenic activity in the F9 stem cell extract from the heparin-Sepharose column. Approximately 1×10^5 stem cells yielded one unit of 3T3 cell stimulatory activity. A unit is defined as the amount of mitogen required to achieve half-maximal stimulation of DNA synthesis in the BALB/c 3T3 cell assay; maximal stimulation is obtained with addition of 20% calf serum.

Heparin-binding Mitogenic Activity in Differentiated F9 Cells

3 d after treatment with RA and but_2 cAMP, F9 cells were morphologically changed; they were less compact, flatter, and more triangular. The extract of RA and but_2 cAMP day 3 F9 cells was subjected to heparin-Sepharose chromatography and was found to contain no 3T3 cell stimulatory activity (Fig. 1 *B*). Thus, the activities represented by peaks S1, S2, and S3 in the undifferentiated F9 stem cells are greatly reduced, or absent by 3 d after the induction of differentiation of F9 cells into parietal endoderm.

On day 5 after induction of differentiation, the extracts from differentiated F9 parietal endoderm cells contained a new 3T3 mitogenic activity (D1), that eluted from heparin-Sepharose as a broad peak at 1.5 M NaCl (Fig. 1 *C*). That 1.5 M NaCl is required to elute the activity from immobilized heparin strongly suggests that it is a bFGF-like protein.

Mitogenic Activity Associated with Differentiated F9 Cells Is Not Secreted

To determine whether the activity represented by peak D1 was secreted, media conditioned by a culture of differentiated F9 parietal endoderm cells were chromatographed on heparin-Sepharose (Fig. 1 *D*). Results revealed no 3T3 stimulatory activity in the conditioned media, even after 10-fold concentration by heparin-Sepharose chromatography. This is consistent with the recent reports that both basic and acidic FGF lack signal peptides (Abraham et al., 1986; Jaye et al., 1986) and that they are not secreted by cells that synthesize these factors in vitro (Vlodavsky et al., 1987*b*). Further, this finding distinguishes D1 from factors produced by other embryonal carcinoma cell lines, which are reportedly secreted (Heath and Isacke, 1984), and from the potential protein product of the *int-2* mRNA transcripts expressed by these cells (Yoshida et al., 1988).

Effects of F9 Cytosolic Proteins on Endothelial Cells

TGF-β does not adhere to heparin. We, therefore, suspected that S1 might be TGF-β. Because TGF-β is known to inhibit endothelial cell proliferation (Heimark et al., 1986), the S1 fractions were pooled and tested in vitro for their ability to modulate the proliferation of bovine capillary endothelial cells. S1 inhibited, in a dose-response fashion, the proliferation of capillary endothelial cells in response to recombinant bFGF (Takeda Chemical Corp, Inc., Osaka, Japan) (Table I). The inhibitory activity for bovine capillary endothelial cells was reduced by 60% by prior absorption of the cell extract with antibodies directed against TGF-β (Table I). Furthermore, prior acid treatment (Lawrence et al., 1984) of S1 increases the inhibitory activity, an indication that the latent form of TGF-β may also be present (data not shown). S1 may comprise several factors, but the above findings strongly suggest that TGF-β is at least one of the components in the S1 peak.

In contrast, the 3T3 cell mitogenic activity associated with the differentiated F9 cells eluted from the heparin-Sepharose column at a high molarity of salt, and was found to be mitogenic in a dose-response fashion for bovine capillary endothelial cells (Fig. 2). Fractions representing the D1 peak were pooled, dialyzed against PBS, tested in an endothelial cell proliferation assay (Fig. 2 *A*), and the results compared directly with the response of the cells to a range of doses of brain-derived heparin-purified aFGF (Fig. 2 *B*) and recombinant bFGF (Fig. 2 *C*). Each plate contained two internal controls: cells receiving PBS (Fig. 2, *stippled bars*) and cells receiving an identical dose, 10 mitogenic units, of recombinant bFGF (Fig. 2, *solid bars*). These studies were repeated several times with comparable results and a representative experiment is shown in Fig. 2.

The molarity at which D1 elutes from heparin-Sepharose

Table I. Effect of S1 on Bovine Capillary Endothelial Cell Response to bFGF

Addition	Cell No. ($\times 10^{-4}$)
PBS	1.19
Basic FGF (*3 ng/ml*)	13.19
+ S1 (*1 μl/ml*)	13.80
(*5 μl/ml*)	12.89
(*20 μl/ml*)	8.10
(*50 μl/ml*)	2.87
(*100 μl/ml*)	1.37
+ S1 (*50 μl/ml*) preabsorbed with anti-TGF-β antibody	8.49

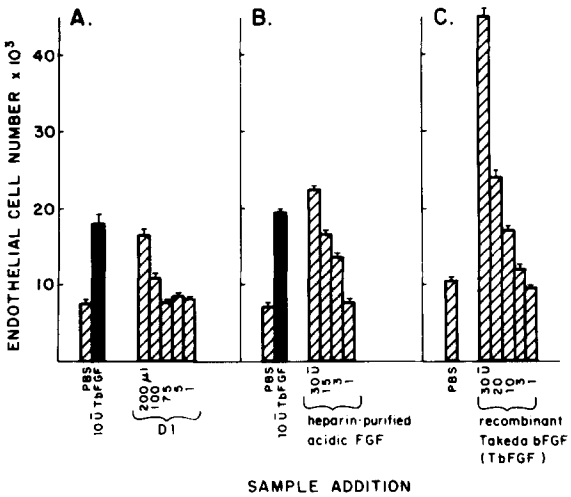

Figure 2. Effect of heparin affinity-purified F9 parietal endoderm cellular extract on the proliferation of adrenal capillary endothelial cells. Capillary endothelial cells were seeded (1×10^4 cells) into gelatinized 24-well tissue culture dishes and allowed to attach for 24 h in DME containing 5% calf serum. Aliquots from the pooled active fractions of the heparin-Sepharose column of F9 parietal endoderm lysates (Fig. 1, *D1*), dialysed against PBS, pH 7.5 (*A*); (*B*) heparin-purified brain-derived aFGF; and (*C*) recombinant bFGF were added at various doses to triplicate wells. Internal controls of PBS (*first stippled bars*) and 10-U doses of recombinant bFGF (*solid bars*) were included on each plate. After 72 h, cells were washed, trypsinized, and counted.

and its ability to stimulate endothelial cells are characteristics of the FGFs. The activity in peak D1 was present at ~3 U per 1×10^6 cells, and 50% of the starting activity was recovered from the heparin-Sepharose column. A 200-µl dose achieved a level of endothelial cell proliferation comparable to 15 U of highly purified aFGF or 10 U of recombinant bFGF. Therefore, the total activity derived from 1×10^8 differentiated F9 cells is 450 U of aFGF or 300 U of bFGF. Assuming that the biological activity we measure is a 50:50 mix of acidic and basic FGF and based on published specific activities of acidic and basic FGFs (~7 U/ng) (Klagsbrun and Shing, 1985; Burgess et al., 1985), the lysates of 1×10^8 differentiated cells contain 50–75 ng of FGF total.

Analysis of FGF mRNA Levels in Stem and Differentiated F9 Teratocarcinoma Cells

To determine whether the genes encoding basic and acidic FGF were differentially expressed in F9 stem cells vs. differentiated F9 parietal endoderm cells, cDNA probes were used to measure the mRNAs encoding FGFs from both cell types.

The bFGF cDNA probe hybridized to two RNAs of 3.8 and 2.2 kb in F9 stem cells (Fig. 3 *A*, lane *1*) and to the 2.2-kb RNA in differentiated F9 cells (Fig. 3 *A*, lane *2*), whereas in the human SK hepatoma line (Fig. 3 *A*, lane *3*), RNAs of 6.5–7.0, 5.3, 2.2, and ~1.0 kb were seen (Fig. 3 *A*). No change in the steady-state level of the major 2.2-kb RNA was observed, but a decrease in the 3.8-kb mRNA transcript was detected when F9 stem and F9 parietal endoderm cells were compared (Fig. 3 *A*). Actin mRNA (Fig. 3 *B*) is shown on the same blot to demonstrate that equal amounts of RNA were loaded from F9 stem cells and F9 parietal endoderm, as the actin mRNA steady-state level does not change during differentiation (Wang et al., 1985).

The F9 parietal endoderm cells expressed other differentiation-specific markers, such as the mRNAs for laminin B1 and J31 (Fig. 3 *C*, lane *2*), demonstrating that the treated cells had the properties of parietal endoderm cells. The laminin B1 and J31 mRNA expression was extremely low in SK hepatoma cells (Fig. 3 *C*, lane *3*).

A genomic probe specific for bovine aFGF hybridized to a 6.0–6.3-kb mRNA only in the differentiated F9 cells (Fig. 4 *A*). In addition, a 5.8–6.0-kb mRNA was observed in F9 stem and to a lesser extent in parietal endoderm and SK hepatoma cells with this genomic aFGF probe (Fig. 4 *A*). Similar results were obtained with an aFGF oligomer probe (not shown). Actin mRNA was also used to control for the amount of RNA loaded (Fig. 4 *B*).

Differentiated F9 Cells Contain FGF-like Molecules

Analysis of 3T3 cell and endothelial cell stimulatory activity derived from F9 stem cells and differentiated cells strongly indicated that FGFs were expressed only after differentiation. However, mRNAs that hybridized with probes for both acidic and basic FGF were detected in both stem and differentiated cells. To determine if these FGF messages were being translated into immunoreactive but biologically inactive

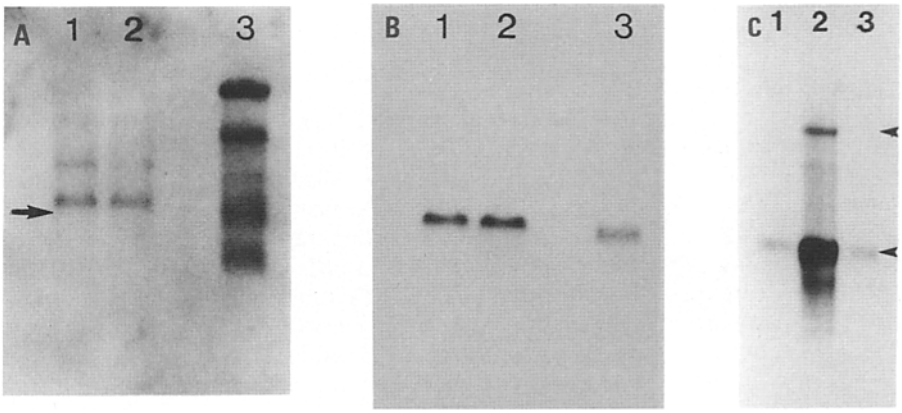

Figure 3. Expression of bFGF mRNAs. Poly A^+ RNA (5 µg/lane) from F9 stem cells (*A–C*, lane *1*); F9 cells treated with RA, but_2 cAMP, and theophylline for 4 d (*A–C*, lane *2*); and SK hepatoma cells (*A–C*, lane *3*). (*A*) bFGF cDNA probe; (*B*) actin cDNA probe, same blot as *A*; (*C*) laminin B1 and J31 cDNA probes. Exposure times were 3 d (bFGF), 1 h (actin), or 2 h (laminin B1 and J31). (*A*, *large arrow*) The position of the actin mRNA seen in *B* when this same blot was rehybridized using the actin probe. (*C*, *arrowheads*) Positions of laminin B1 mRNA at 6.2 kb (*top arrowhead*) and J31 mRNA at 2.2 kb (*bottom arrowhead*) in parietal endoderm (*C*, lane *2*).

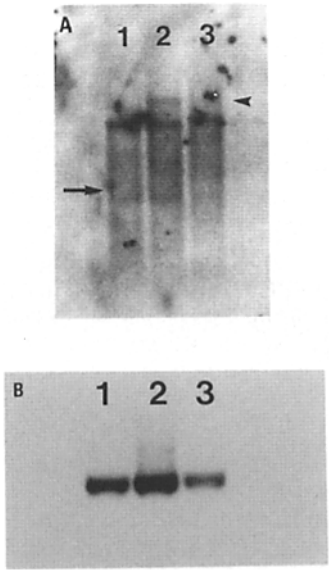

Figure 4. Expression of aFGF mRNAs. Poly A^+ RNA (5 μg/lane) from F9 stem cells (*A* and *B*, lane *1*); F9 cells treated with RA, but_2 cAMP, and theophylline for 4 d (*A* and *B*, lane *2*); and SK hepatoma cells (*A* and *B*, lane *3*). (*A*) aFGF cDNA probe, 1.5 × 10^6 cpm/ml; (*B*) actin cDNA probe. Exposure time was 1 d for aFGF and 1 h for actin. (*A*, *arrowhead*) Position of the aFGF mRNA in parietal endoderm cells (lane *2*). (*A*, *arrow*) Position of the actin probe binding to this blot when rehybridized using the actin probe (*B*).

proteins, both cell types were analyzed for FGF content by Western blot analysis and biosynthetic labeling studies.

Several attempts using heparin-Sepharose chromatography followed by dialysis and concentration of the sample for Western analysis failed to yield reproducible results, primarily because of protein loss. To circumvent these problems, we used a modified heparin-Sepharose batch technique for the isolation of the FGFs (see Materials and Methods). Proteins from the F9 stem and differentiated F9 cell extracts that remained bound to heparin-Sepharose after washing with buffer containing 0.8 M NaCl were dissociated from the resin by boiling in Laemmli's buffer (Laemmli, 1970), and processed for routine one-dimensional gel electrophoresis. Subsequent electrophoretic transfer (Western) blotting was performed and the nitrocellulose-bound proteins were reacted with polyclonal antibodies directed against acidic or basic FGF (Wadzinski et al., 1987) (Fig. 5).

Affinity-purified rabbit antibodies raised against peptide fragments corresponding to (*a*) an internal region of bFGF (amino acid residues 33–43) (Fig. 5 *a*, lanes *1–4*) and (*b*) the amino-terminal end (amino acid residues 1–12) of bFGF (Fig. 5 *A*, lanes *6–9*) reacted with a single protein band of 18.4 kD from differentiated F9 parietal endoderm cells (Fig. 5 *A*, lanes *4* and *9*). This band comigrated with bFGF derived from SK hepatoma cells, which was also recognized by these antibodies as a single, 18.4-kD protein species (Fig. 5 *A*, lanes *2* and *6*). No immunoreaction occurred with these antibodies tested in parallel against brain-derived aFGF (Fig. 5 *A*, lanes *3* and *8*) or proteins from F9 stem cells (Fig. 5 *A*, lanes *1* and *7*).

An affinity-purified rabbit antibody, raised against the carboxyl-terminal region of aFGF (amino acid residues 59–90), also reacted with differentiated F9 cell proteins, recognizing a band of ∼15 kD (Fig. 5 *B*, lane *4*). The molecular weight of this F9 protein, 15,000, corresponds to that of brain-derived aFGF recognized by this antibody as a doublet (Fig. 5 *B*, lane *2*). There was no cross-reactivity of this antibody with SK hepatoma-derived bFGF (Fig. 5 *B*, lane *3*) or with F9 stem cell extract tested in parallel (Fig. 5 *B*, lane *1*). The molecular weights of these proteins were calculated by comparison to Coomassie blue–prestained markers (Fig. 5 *A*, lane *5*). These studies clearly establish the presence of factors with antigenic determinants and molecular weights characteristic of both basic and acidic FGF in cell extracts of differentiated F9 cells, not detected in F9 stem cells.

FGFs Are Synthesized by Differentiated F9 Cells But Not by F9 Stem Cells

Our Western blot analysis indicated the presence of both aFGF and bFGF proteins in F9 differentiated but not F9 stem cell extracts. To demonstrate synthesis of these factors, 6 × 10^8 F9 stem and 1 × 10^8 differentiated cells were metabolically labeled and the FGFs were immunoprecipitated using the antibodies described above. After radiolabeling with [^{35}S]methionine for 4 h, the cells were harvested and the cell extract immunoprecipitated with (*a*) preimmune sera, (*b*) the affinity-purified polyclonal antibodies against the amino-terminal end of bFGF, or (*c*) the affinity-purified antibodies directed against the carboxyl-terminal region of aFGF (Wadzinski et al., 1987). The immunoprecipitated proteins were separated by one-dimensional Na_2DodSO_4 gel electrophoresis and autoradiographed (Fig. 6). No specific radiolabeled proteins were immunoprecipitated from F9 stem (Fig. 6, lanes *1–4*) or F9 differentiated (Fig. 6, lanes *5–8*) cell cytosol, after a 4-h labeling, by the bFGF antibodies (Fig. 6, lanes *2* and *6*) as compared with its preimmune sera (Fig. 6, lanes *1* and *5*). A single 20–21-kD radiolabeled protein was precipitated with the aFGF antibodies from the extract of differentiated cells (Fig. 6, lane *8*), which was not seen in the stem cell extract (Fig. 6, lane *4*) or in the preimmune sera immunoprecipitate (Fig. 6, lanes *3* and *7*). These studies indicate the presence of a newly synthesized protein, immunoreactive with an antibody specific to aFGF, only within F9 cells after differentiation.

The Western blot analysis demonstrated both acidic and basic FGF-like molecules to be present in F9 cells after differentiation. The absence of specific proteins immunoprecipitated by the antibody directed against the amino terminus of bFGF is probably due to the inability of these antibodies to effectively recognize rodent bFGF proteins in native form. One of us (T. Kurokawa) has recently determined that the amino-terminal sequence of rodent bFGF differs from that of bovine bFGF (Kurokawa et al., 1987). Rat bFGF has an amino acid deletion at residue position 9, within the region of the bovine sequence to which this antibody is raised. If mouse bFGF has the same deletion, it may be difficult to immunoprecipitate native mouse F9 cell-derived bFGF with this antibody.

Discussion

We have determined that F9 teratocarcinoma stem cells do not synthesize FGF, but do express this potent class of mitogens after the induction of differentiation with RA and but_2 cAMP. F9 stem cells do contain stimulatory activities for BALB/c 3T3 cells with characteristics different from FGF. The activity from the stem cells that does not bind heparin is increased by acid treatment, is inhibitory for endothelial cells (in the presence and absence of FGF) in vitro and is a potent stimulator of angiogenesis in vivo, (observations in the chick chorioallantoic membrane assay; unpublished data). The endothelial cell inhibitory activity can be partially

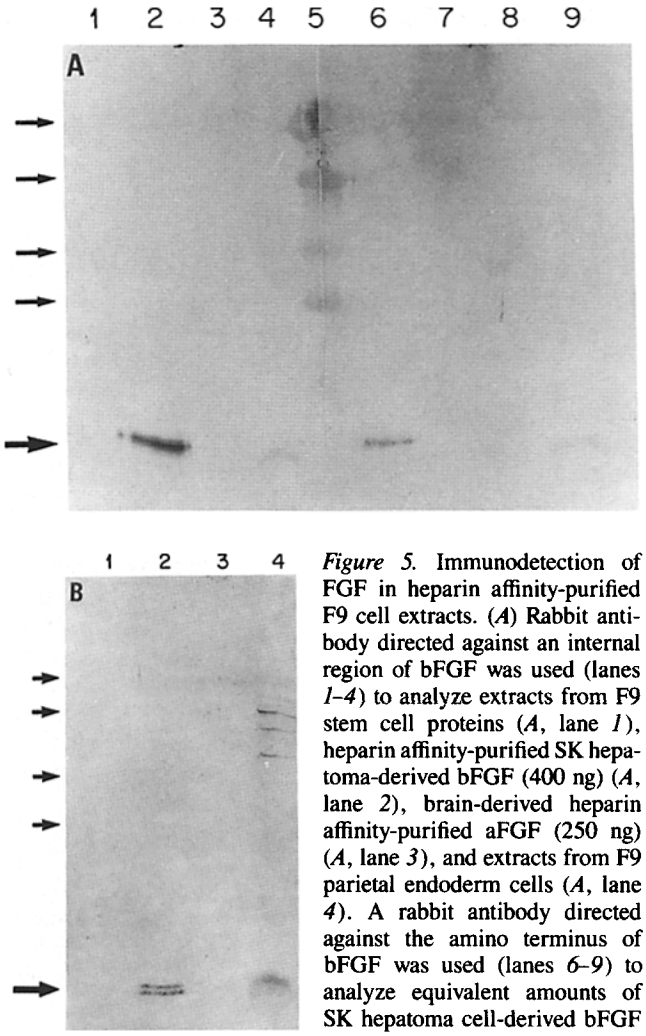

Figure 5. Immunodetection of FGF in heparin affinity-purified F9 cell extracts. (*A*) Rabbit antibody directed against an internal region of bFGF was used (lanes *1–4*) to analyze extracts from F9 stem cell proteins (*A*, lane *1*), heparin affinity-purified SK hepatoma-derived bFGF (400 ng) (*A*, lane *2*), brain-derived heparin affinity-purified aFGF (250 ng) (*A*, lane *3*), and extracts from F9 parietal endoderm cells (*A*, lane *4*). A rabbit antibody directed against the amino terminus of bFGF was used (lanes *6–9*) to analyze equivalent amounts of SK hepatoma cell-derived bFGF (lane *6*), F9 stem cell extracts (lane *7*), brain-derived heparin purified aFGF (lane *8*), and F9 parietal endoderm cell extracts (lane *9*). (*B*) A rabbit antibody directed against aFGF was used to analyze equivalent samples as above: extracts from F9 stem cells (*B*, lane *1*), highly purified brain-derived aFGF (*B*, lane *2*), SK hepatoma cell-derived bFGF (*B*, lane *3*), and F9 parietal endoderm cell extracts (*B*, lane *4*). The amount of stem or differentiated F9 proteins used was below the detection of a 280 or 260 wavelength absorbance reading or a microprotein assay (Bio-Rad Laboratories), indicating it is <1 μg per lane. The curvature of the bands visualized is sometimes observed in a Western analysis due to residual salt in association with the sample and is not due to protein overload. Coomassie blue–prestained molecular mass markers (*small arrows*) include proteins of 55, 43, 36, and 29 kD (lane *5*). *Large arrows*, positive reaction products.

removed by immunoadsorption with antibodies specific for TGF-β, suggesting that F9 stem cells synthesize a TGF-β-like activity before differentiation.

Teratocarcinoma stem cells, when placed in an aberrant position in the adult organism, form very large tumors that eventually kill their host (Martin, 1980). Folkman has proposed that all solid tumors must evoke angiogenesis, to grow beyond 1–2 mm^3 in diameter (Folkman, 1963). The activity in the stem cells, which is in part TGF-β, appears to greatly decrease or disappear upon differentiation of the F9 cells in response to RA. This may prove to be an important correlate of the in vivo tumorigenicity of the undifferentiated F9 stem cells. Alternatively, the expression of TGF-β by the stem cells may be unrelated to their transformed state but rather characteristic of undifferentiated embryonic stem cells. In support of this latter hypothesis, recent studies have shown that a maternal mRNA in *Xenopus* eggs encoding for a TGF-β-like protein is localized to the region that eventually gives rise to embryonic endoderm cells (Weeks and Melton, 1987).

The two other minor peaks of activity derived from the extract of F9 stem cells have not been conclusively identified. The peak that elutes with 0.5 M NaCl most likely represents the intracellular PDGF-like factor that is known to be secreted by undifferentiated F9 stem cells (Gudas et al., 1983; Rizzino and Bowen-Pope, 1985). Of primary importance is the observation that none of the three activities are detected in the cytosol 3 d after the induction of differentiation. 5 d after treatment with RA and but_2 cAMP, the differentiated F9 parietal endoderm cells synthesize an entirely new class of mitogens, the FGFs. In a preliminary study, we have examined extracts from F9 cells 7 d after treatment with RA and but_2 cAMP and found ~1.5–2-fold more FGF than on day 5. This may represent FGF that has accumulated over time, or an increased level of expression of FGF in the day 7 F9 parietal endoderm cells.

Significantly, the activity made by F9 parietal endoderm cells is not detected in conditioned media. Since lack of secretion also characterizes acidic and basic FGF this finding provides additional evidence that the differentiated F9 activity is FGF. That the F9 cell FGF-like activity is not secreted also serves to distinguish it from the *int-2* and *hst 1* proteins. These proteins are related to the FGFs but both have gene sequences that predict a leader signal peptide sequence that would facilitate secretion (Yoshida et al., 1987; Bovi et al., 1987).

The genes for acidic and basic FGF have been cloned (Abraham et al., 1986; Jaye et al., 1986) and a variety of cell types have been identified that synthesize bFGF (for review see Klagsbrun et al., 1986; Schweigerer et al., 1987*b*), but few cells have been identified that synthesize aFGF (Lobb et al., 1986). Using a bFGF cDNA probe, we detect a major mRNA of 2.2 kb in both stem and differentiated F9 cells and an additional mRNA in stem cells at 3.8 kb. Similar bFGF message sizes have been reported in human fibroblasts (Steinfeld et al., 1988). We do not know the significance of the multiple transcripts for bFGF mRNA in F9 stem cells. The genomic aFGF probe detected a 6.0–6.3-kb mRNA in parietal endoderm that was not detectable in F9 stem cells and a 5.8–6.0-kb transcript that was visible in both cell types (Fig. 4). That the size of aFGF mRNA from human brain stem (Jaye et al., 1986), differs from that in F9 cells may be due to tissue-specific differences in the processing, or species differences in mRNA size. Very few studies of aFGF mRNA are currently in the literature.

That there were few differences in the steady-state levels of the major species of bFGF mRNA between the stem and parietal endoderm cells was surprising in light of the fact that there was no evidence in stem cells of FGF-like material by biological assay, biosynthetic studies, or Western blot analysis. There are at least two possible explanations for this difference. First, the stem cell mRNA may not be translated into immunoreactive or biologically active protein. This is

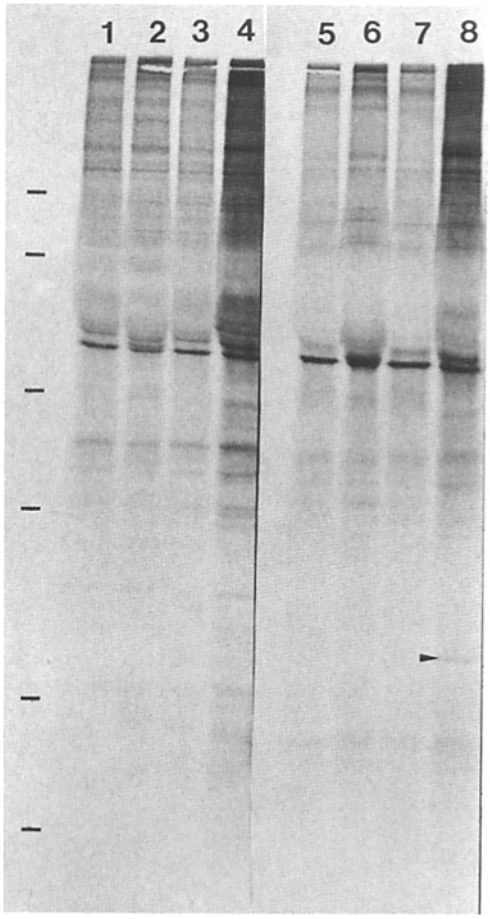

Figure 6. Autoradiogram of immunoprecipitated [^{35}S]methionine-labeled proteins from F9 stem and parietal endoderm cells electrophoresed on a 15% $NaDodSO_4$ polyacrylamide gel. Proteins from extracts of F9 undifferentiated stem (lanes *1–4*) and F9 parietal endoderm cells (lanes *5–8*) were immunoprecipitated with the preimmune serum of bFGF antibody (lanes *1* and *5*, respectively); or with the immune specific antibody directed against bFGF (amino acids 1–12) (lanes *2* and *6*, respectively); or immunoprecipitation with the preimmune serum of the aFGF antibody (lanes *3* and *7*); or with the immune sera to aFGF (amino acid residues 136–145) (lanes *4* and *8*, respectively). Antibodies were used at 1:300. Molecular weights were calculated by comparison to prestained molecular weight markers (*solid line*) including phosphorylase b (94,000), BSA (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,500) and alpha-lactalbumin (14,400). (*Arrowhead*) Radiolabeled proteins immunoprecipitated by specific antibodies and not recognized by the preimmune serum.

a reasonable postulate since differential translation of c-*myc* has been similarly reported in the F9 system (Dony et al., 1985). Second, FGF may be synthesized by both the stem and differentiated cells but may not be detectable in the stem cells because of immediate consumption or degradation, whereas the cytoplasmic constituents, i.e., heparan sulfate, synthesized only by the differentiated cells may better stabilize FGF protein. This explanation is argued against by the in vivo radiolabeling data (Fig. 6), which does not demonstrate synthesis of radiolabeled FGF in stem cells, even during a relatively short labeling period. It is also possible that the 6.0–6.3-kb aFGF mRNA seen exclusively in parietal endoderm cells encode the majority of FGF in these cells.

A finding of consequence in this report is that factors with properties of acidic and basic FGFs are synthesized by embryonic parietal endoderm cells. The F9 cell system serves as a model for the differentiation of the mouse blastocyst, and thus our results may indicate that FGFs are present in parietal endoderm in early embryogenesis. Recent studies by Kimmelman and Kirschner (1987) have identified a mRNA encoding a bFGF-like protein in *Xenopus* embryos that is present throughout development, an indication that FGF may be endogenous to the developing embryo. Notably, in this system, the message abruptly increases during the midblastula transition, just before gastrulation and the development of mesoderm.

What role might this class of mitogens serve in the developing embryo? In the adult, FGFs are thought to play a primary role in stimulating neovascularization during wound healing and ovulation and in association with a number of disease states. However, vascularization would not be occurring in the embryo at the time our studies indicate these FGFs would first appear. A more likely possibility is that other cell types such as epithelial cells, fibroblasts, myoblasts and chondrocytes are the targets for FGF at this point in embryonic development, as FGFs are known to stimulate the proliferation and/or differentiation of these cells.

Finally, the FGFs appear likely to function as other than mitogens. FGF has been shown to induce neurite outgrowth in PC12 cells, a morphologic change associated with the differentiation of these neural cells (Togari et al., 1985; Wagner and D'Amore, 1986). Slack et al. (1987) demonstrated that bFGF and aFGF induces the formation of mesoderm in early embryonic ectodermal explants of amphibians. bFGF was shown to act as a morphogen, causing the induction of mesoderm independent of a mitogenic response. Further, the dose of FGF was critical in determining the type of mesodermal cells that resulted: low doses (2–30 ng/ml) gave rise to mesothelial and blood cells, whereas higher doses (>30 ng/ml) resulted in the formation of muscle tissue. These studies strongly suggest that FGFs can function as inducers of mesodermal tissue. Our studies implicate the parietal endoderm of the mouse embryo as one potential source of these potent inducers of various mesodermal elements.

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