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Human fibroblast growth factor 1 gene expression in vascular smooth muscle cells is modulated via an alternate promoter in response to serum and phorbol ester

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ABSTRACT

We have previously isolated the human FGF-1 gene in order to elucidate the molecular basis of its gene expression. The gene spans over 100 kbp and encodes multiple transcripts expressed in a tissue- and cell-specific manner. Two variants of FGF-1 mRNA (designated FGF-1.A and 1.B), which differ in their 5′ untranslated region, were identified in our laboratory. Recently, two novel variants of FGF-1 mRNA (designated FGF-1.C and 1.D) have been isolated. In this study we used RNase protection assays to demonstrate expression of FGF-1.D mRNA in human fibroblasts and vascular smooth muscle cells and to show that promoter 1D has multiple transcription start sites. A single-strand nuclease-sensitive region has also been identified in the promoter 1D region that may have implications in chromatin conformation and transcriptional regulation of this promoter. Using Northern blot hybridization analyses, a previous study demonstrated a significant increase of FGF-1 mRNA levels in cultured saphenous vein smooth muscle cells in response to serum and phorbol ester. Here we confirm these results by RNase protection analysis and show that FGF-1.C mRNA is significantly increased in response to these stimuli. RNase protection assays indicate that promoter 1C has one major start site. The phorbol ester effect suggests that a protein kinase C-dependent signalling pathway may be involved in this phenomenon. Our results point to a dual promoter usage of the FGF-1 gene in vascular smooth muscle cells. Thus, normal growing cells primarily utilize promoter 1D. In contrast, quiescent cells, when exposed to serum or phorbol ester, utilize a different FGF-1 promoter, namely promoter 1C. Overall, these phenomena suggest mechanisms for increased production of FGF-1 that may play a role in inflammatory settings, wound healing, tissue repair, and neovascularization events and processes via autocrine and paracrine mechanisms. Our findings suggest that different FGF-1 promoters may respond to different physiological conditions and stimuli, in reference to the cell type or tissue milieu, resulting in ultimate production of the FGF-1 protein.

INTRODUCTION

Interest in understanding the process of new blood vessel formation, or angiogenesis, stems from the desire and need to understand the etiology of processes of wound healing, tissue development and repair, and especially pathological neovascularization events of solid tumor growth. The available models of angiogenesis that have been proposed (1–4) point to proliferative capability of endothelial cells, the principal cell type lining the inner walls of blood vessels, in forming capillary tubes in response to stimulus. This stimulus may be a chemoattractant which plays a pivotal role in proliferation and migration of endothelial cells (5,6). Essential to this migration process is secretion of proteases by endothelial cells which subsequently degrade the basement membrane (7,8). This process, the basis of angiogenic events, leads to sprouting of capillaries by outgrowth and invasion by endothelial cells. The release of angiogenic factors is an important stimulus in this process. In this context, the understanding of the regulation and control of expression of genes for angiogenic factors is crucial, i.e. under what physiological conditions are the promoters of these genes upregulated? What are the *cis*- and *trans*-acting regulatory elements involved in such a process? With this basic knowledge we can begin to identify the molecular events, particularly signal transduction pathways or transcriptional factors, that may subsequently play a critical role in angiogenesis.

Several angiogenic factors have been described (9,10). One of these angiogenic factors is acidic fibroblast growth factor (aFGF). This growth factor, originally called endothelial cell growth factor because of its mitogenic effect on these cells (11), is a single chain polypeptide of 155 amino acids and has a molecular mass

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of 17 kDa. aFGF belongs to the fibroblast growth factor (FGF) family (12), which now consists of nine members (12–16), with aFGF classified as fibroblast growth factor 1 (FGF-1). It is a mitogen for a variety of mesenchymal and neuroectodermal cells (12,17). Particularly, FGF-1 is a mitogen for cultured vascular endothelial cells and an angiogenic factor *in vivo* (18). It also has a role in heart development (19), vasodilation (20), tissue repair, wound healing and pathological angiogenic events, especially neovascularization of solid tumors (9,21).

The human FGF-1 gene spans over 100 kbp and contains three protein coding exons and four upstream untranslated exons designated -1A, -1B, -1C, and -1D (22–27). Splicing of -1A, -1B, -1C, or -1D to the first protein coding exon generates 1.A, 1.B, 1.C, and 1.D mRNAs respectively. These variant forms of FGF-1 mRNA are expressed in a tissue-specific manner. FGF-1.A mRNA is the predominant species in kidney, whereas FGF-1.B mRNA is the predominant species in brain (26). Furthermore, using RNase protection assays we demonstrated that the FGF-1 transcript expressed in U1242MG and D65MG glioblastoma cell lines is neither 1.A, 1.B nor 1.C, suggesting the existence of a fourth untranslated exon (27). We describe here the strategy employed to identify this fourth untranslated exon along with the distribution of this fourth variant of FGF-1 mRNA. The transcription start sites of this promoter have been mapped by RNase protection and a single-strand nuclease-sensitive site within this region has been localized.

FGF-1 and FGF-2 expression in human vascular smooth muscle cells cultured *in vitro* has been previously reported (28–31). Using Northern blot analyses, Winkles and Gay (32) previously reported significant induction of FGF-1 transcripts in vascular smooth muscle cells treated with fetal bovine serum (FBS) or phorbol 12-myristate 13-acetate (PMA). Here we confirm these previous results by a more sensitive RNAse protection assay and show that the promoter 1C is responsive to these stimuli. Moreover, the FGF-1.C transcription start site is determined by RNase protection analysis. Taken together, these results point to mechanisms of up-regulation of FGF-1 transcripts in vascular smooth muscle cells and have implications in understanding atherosclerosis and hypertension, wound healing and tissue repair processes, and also inflammatory responses.

MATERIALS AND METHODS

Genomic Southern blotting

Genomic DNA from mouse, rat, sheep, and rabbit was provided by Dr Yung Yu, and mouse NIH 3T3 DNA was provided by Dr Linda Ernst, both at The Ohio State University. DNA from other species was isolated by homogenizing tissue in digestion buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 25 mM EDTA, pH 8.0, 0.5% SDS). The homogenate was incubated for 18 h at 55°C in digestion buffer plus 0.1 mg/ml proteinase K. After ethanol precipitation, the high molecular weight DNA was spooled by a glass rod. Twenty μg of genomic DNA from various species was digested with appropriate restriction enzymes and separated on a 0.8% agarose gel. The DNA was transferred to a nylon filter (Hybond-N, Amersham) and processed for blotting (33). A 1.2 kbp *Eco*RI–*Bgl*II fragment, 6.6 kbp upstream from the first protein coding exon (22), was used to generate a ^{32}P-labeled probe (Multiprime DNA labeling system, Amersham). The filter was pre-hybridized in 40% formamide, 6 × SSPE (0.9 M NaCl, 60 mM NaH_2PO_4, 20 mM EDTA, pH 7.4), 3% SDS, 0.5% powdered milk for 12 h at 42°C. Hybridization was performed at 42°C with 1 × 10^6 c.p.m./ml probe in hybridization buffer (prehybridization buffer plus 10% dextran sulfate) for 40 h at 42°C. Reduced stringency washes were performed initially [2 × SSC (0.3 M NaCl, 30 mM Na citrate, pH 7.0), 0.5% SDS (2×, room temperature, 15 min); 2 × SSC, 0.5% SDS (2×, 55°C, 15 min)]. Subsequently, the filter was washed in 0.5 × SSC, 0.1% SDS (2×, 55°C, 15 min), and finally under high stringency in 0.1 × SSC, 0.1% SDS (2×, 55°C, 15 min). The results were visualized by autoradiography after each set of washes.

Tissues and cell lines

The human tissues used in this study were obtained from the Tissue Procurement Service of The Ohio State University. The cell lines used in this study were maintained under recommended conditions. The human glioblastoma cell lines U1240MG and U1242MG were provided by Drs Christer Betsholtz and Bengt Westermark, University of Uppsala, Sweden. The D65MG glioblastoma cell line was provided by Dr Yancey Gillespie, University of Alabama at Birmingham, AL. These glioblastoma cells were grown in modified Eagle's minimal essential medium (MEM) (Gibco-BRL), supplemented with 10% calf serum, antibiotics and antimycotic. The neonatal foreskin fibroblast cell line NFF-6 was obtained from Dr Gary Shipley, Oregon Health Sciences University, Portland, OR, and grown in Dulbecco's modified Eagle's medium (D-MEM) (Gibco-BRL), supplemented with 10% FBS, antibiotics and antimycotic. Cultured saphenous vein smooth muscle cells and iliac artery smooth muscle cells were provided by Dr Peter Libby, Brigham and Women's Hospital, Boston, MA. These cells were grown on flasks coated with human fibronectin (Gibco-BRL) at 2 μg/cm^2 in D-MEM (BioWhittaker Inc.) with low glucose, 2 mM L-glutamine, 25 mM HEPES buffer, antibiotics and antimycotic, and 10% FBS. The human embryo lung fibroblast cell line M426 was provided by Drs Stuart Aaronson and Steven Tronick, National Cancer Institute, Bethesda, MD, and was grown in D-MEM (Gibco-BRL) medium supplemented with 10% FBS, antibiotics and antimycotic. For mitogen stimulation, cells were cultured in medium containing 0.5% FBS for 72 h, then stimulated with either 10% FBS or 100 ng/ml PMA (Sigma, St Louis, MO). The cells were then harvested at indicated time intervals.

Reverse transcription and polymerase chain reaction (RT–PCR)

RNA was isolated from tissues, cell lines and cultured cells using the guanidinium thiocyanate and cesium chloride gradient method (34). Complementary DNA was synthesized from 1 μg of total cellular RNA using avian myeloblastosis virus (AMV) reverse transcriptase, oligo(dT)$_{12–18}$ and random hexamers. After heating at 95°C for 5 min, 3 μl of the cDNA reaction was amplified in a 50 μl PCR reaction for 35 cycles. The parameters were: denaturation at 94°C for 30 s, annealing at 48°C for 45 s, and extension at 72°C for 1 min. Primers (HBGF 1702 and HBGF 151 in ref. 26) used were derived from sequences of the first and second protein coding exons of human FGF-1 and unique sequences upstream of a putative splice donor sequence identified in the 1.2 kbp *Eco*RI–*Bgl*II fragment 6.6 kbp upstream from the first coding exon. The PCR product was analyzed on a 2% Nusieve and 1% Seakem (FMC BioProducts, Rockland, ME)

agarose gel and visualized by staining with ethidium bromide. The amplified fragment was cloned into *Eco*RV-digested, T-tailed pBluescript KS(+) as described by Marchuk *et al.* (35).

Generation of FGF-1.C and 1.D minigenes

The FGF-1.C minigene was generated by subcloning into pBluescript KS(+) a 184 bp *Pst*I–*Bam*HI fragment from a 299 bp RT–PCR-generated FGF-1.C cDNA clone (27). A 340 bp *Pst*I fragment isolated from cloned FGF-1 genomic DNA was then subcloned into the *Pst*I site to provide sequence 5′ to the -1C exon. The resultant minigene was then digested with *Eco*RI and used as a template to generate a riboprobe for use in RNase protection assays. Recombinant PCR was used to generate an FGF-1.D minigene (M. A. Chotani and I.-M. Chiu, manuscript submitted).

Plasmid constructs and nucleotide sequencing

FGF-1.A, -1.B, -1.C, and -1.D cDNA constructs have been described previously (25–27). The sequencing of the 1.2 kbp *Eco*RI–*Bgl*II fragment containing the exon -1D region was performed on sub-fragments, which were: 0.39 kbp *Hpa*I–*Xho*I fragment subcloned in pBluescript KS(+) digested with *Eco*RV/*Xho*I; 0.47 kbp *Xho*I–*Hpa*I fragment subcloned in pBluescript KS(+) digested with *Xho*I/*Eco*RV; 0.35 kbp *Xho*I–*Bgl*II fragment subcloned in *Xho*I/*Bam*HI sites of pBluescript KS(+); 0.74 kbp *Hpa*I–*Bgl*II fragment subcloned in *Hin*cII/*Bam*HI sites of pBluescript KS(+). Genomic fragments and cDNA clones were sequenced by Sanger's dideoxy nucleotide chain termination method (36) using double-stranded and single-stranded DNA templates (37), with T3 and T7 primers (United States Biochemicals, Cleveland, OH). A computer program (DNA Star Inc., Madison, WI) was used to search for the splice donor consensus sequence GTRAGT. FGF-1D homology searches were performed using the FASTA program (38).

RNase protection assays

RNA isolated from tissue and cell lines/cultured cells was checked for integrity on a formaldehyde-containing agarose gel and visualized by staining with ethidium bromide. A ^{32}P-labeled antisense riboprobe was derived from FGF-1.D cDNA by *in vitro* transcription. The probe included 39 nt of known FGF-1D sequence spliced to 203 bp of the first and 7 bp of the second protein coding exons (26). Twenty to forty μg of RNA and antisense riboprobe (5×10^5 c.p.m.) were denatured at 85°C for 5 min and hybridized in 30 μl of 80% formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM PIPES (pH 6.4) at 42°C for 18 h. Samples were digested with RNase A (7–20 μg/ml) and T1 (0.35–1 μg/ml) for 60 min at 30°C, and analyzed on a 6% denaturing polyacrylamide gel. RNA isolated from HeLa, Hep G2, and Nalm 6 cell lines was provided by Dr Lai chu Wu, The Ohio State University. U937 (± γ-interferon) and K562 RNA was provided by Dr Linda Ernst, The Ohio State University. A 780 bp *Pst*I–*Xba*I human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragment was subcloned in pBluescript KS(+) and used to generate an anti-sense riboprobe. Autoradiograms were scanned with an LKB-Bromma laser densitometer or a Betagen scanner.

Single-strand nuclease treatment of plasmid DNA

Supercoiled plasmid DNA containing the 1.2 kbp *Eco*RI–*Bgl*II fragment having the FGF-1 exon -1D region was treated with mung bean nuclease (Boehringer Mannheim) at 2 U/μg DNA in buffer containing 30 mM NaOAc, pH 5.0, 50 mM NaCl, 1 mM $ZnCl_2$, 5% glycerol v/v in 20 μl volume for 60 min at 37°C. Subsequently, phenol/chloroform/isoamyl alcohol (25:24:1) extraction was performed once, followed by chloroform/isoamyl alcohol (24:1) extraction, and ethanol precipitation. The DNA was digested with various restriction enzymes. Samples were separated by electrophoresis on a 1.2% agarose gel in TBE (100 mM Tris–HCl, 100 mM Na borate, 2 mM EDTA, pH 8.0) and visualized by ethidium bromide staining. As a control, supercoiled DNA was treated in the reverse order also, i.e. linearized with *Eco*RI first, and then treated with mung bean nuclease.

RESULTS

The FGF-1 exon -1D region shows a high degree of nucleotide sequence conservation in primates

To search for the fourth FGF-1 transcript, we used DNA fragments upstream from the first coding exon as probes to hybridize to different primate DNA. Conservation of DNA sequences would imply the presence of exon sequences. A 1.4 kbp *Eco*RI fragment, 10.7 kbp upstream of the first coding exon was previously shown to hybridize to primate DNA (24). Upon sequencing, no splicing donor sequences were detected. Subsequently, a 1.2 kbp *Eco*RI–*Bgl*II fragment, 6.6 kbp upstream of the first protein coding exon, was also shown to hybridize to primate DNA. Figure 1A shows a Southern blot analysis of human and rhesus monkey genomic DNA digested with various restriction enzymes and probed with the 1.2 kbp *Eco*RI–*Bgl*II fragment. The blot was washed under high stringency. The Southern blot in Figure 1B is a Zoo blot using the same probe as in Figure 1A. The blot was also washed under high stringency. When washed under reduced stringency, the weak bands detected in non-primate mammals are more easily detectable (data not shown). These data suggest a high degree of conservation of this region in primates, and conservation to a lesser degree in other mammals. Sequence analysis revealed a putative splice donor sequence in this fragment (GenBank accession no. L01488). RT–PCR was subsequently used to isolate a cDNA that was designated FGF-1.D (GenBank accession no. L01487).

The FGF-1.D mRNA is expressed in two glioblastoma cell lines, a prostate carcinoma cell line, two fibroblast cell lines and vascular smooth muscle cells

In order to identify different cell types expressing FGF-1.D mRNA, RNase protection assays were performed using an FGF-1.D cDNA-derived riboprobe. Representative RNase protection assay results (Fig. 2) indicate that FGF-1.D mRNA is the major FGF-1 transcript in a glioblastoma-derived cell line (U1242MG), an embryo lung fibroblast-derived cell line (M426), and iliac artery smooth muscle cells. The glioblastoma-derived cell line U1240MG which we previously showed to express predominantly FGF-1.B mRNA (26) was used as a negative control. RNase protection assays utilizing riboprobes specific for FGF-1.C and -1.D transcripts demonstrated that the prostate carcinoma-derived cell line PC-3, the glioblastoma-derived cell line D65MG, the neonatal foreskin fibroblast-derived cell line

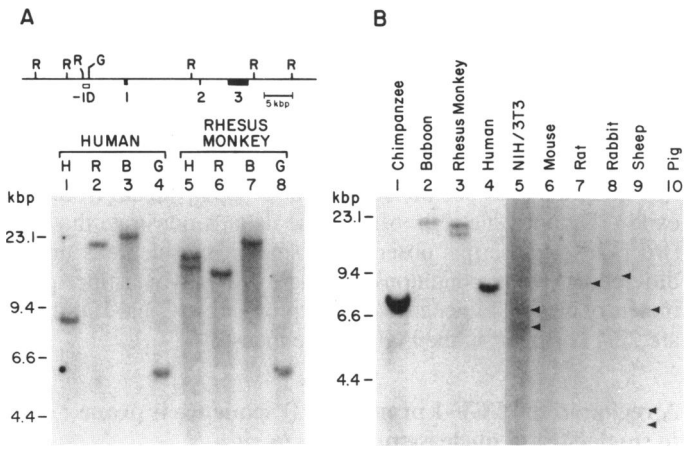

Figure 1. An *Eco*RI–*Bgl*II fragment in the human FGF-1 gene locus is highly conserved in primates. A 1.2 kbp *Eco*RI–*Bgl*II DNA fragment, 6.6 kbp upstream of the first protein exon of the human FGF-1 gene, was used as a probe (indicated by an open box) in Southern blot analysis. (**A**) Human and rhesus monkey genomic DNA was digested with various restriction enzymes: H, *Hin*dIII, R, *Eco*RI, B, *Bam*HI, G, *Bgl*II. The DNA size markers used were λ*Hin*dIII restriction fragments. (**B**) DNA from various species was digested with *Hin*dIII and hybridized under high stringency conditions. The hybridizing bands in non-primate mammals are indicated with arrowheads.

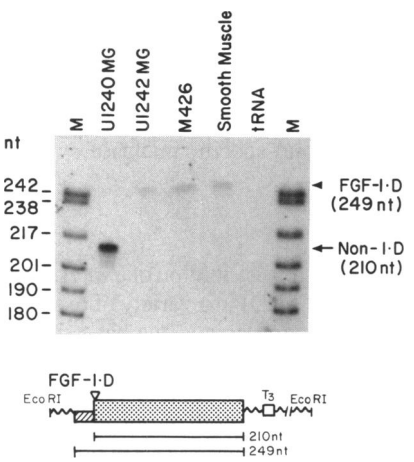

Figure 2. FGF-1.D transcript expression in various cell types. An anti-sense riboprobe derived from FGF-1.D cDNA, linearized with *Eco*RI, was used in an RNase protection assay. U1240MG expresses a non-1.D transcript corresponding to 210 nt of exons 1 and 2 (represented by the dotted box) The striped box represents the exon -1D-specific sequence. U1242MG, M426, and iliac artery-derived smooth muscle cells predominantly express FGF-1.D mRNA, seen at 249 nt. Yeast tRNA was used for background control. The DNA size marker is pBR322 digested with *Msp*I, and labeled with [α-^{32}P]dCTP and Klenow fragment. T3 represents the bacteriophage T3 RNA polymerase recognition site.

NFF-6, and saphenous vein smooth muscle cells predominantly express FGF-1.D mRNA. These results show that FGF-1.C and FGF-1.D mRNAs are co-expressed in a variety of tissue culture cells, with -1.D mRNA being the major species. Furthermore, ovary and a variety of cell lines, including U937, either in the presence or absence of γ-interferon, K562, HeLa, Hep G2,and Nalm 6, do not express detectable levels of FGF-1 mRNA, while prostate and endometrium express low levels of non-1.C, non-1.D mRNA (data not shown).

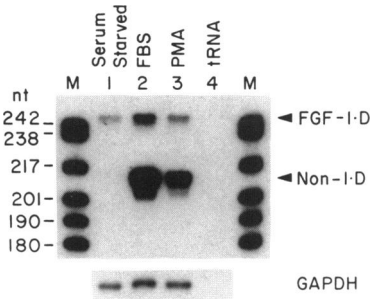

Figure 3. Significant increase of FGF-1 mRNA levels in response to serum or PMA in saphenous vein-derived smooth muscle cells. RNase protection assays were performed using FGF-1.D cDNA-derived riboprobe. Significant increase of non-1.D mRNA is observed in response to serum or PMA treatments, 6 h (lanes 2 and 3), compared to serum-starved cells, 72 h (lane 1). Yeast tRNA was used for background control (lane 4). A GAPDH cDNA-derived riboprobe was used to check for loadings of RNA in each lane. Marker used is pBR322 digested with *Msp*I, and labeled with [α-^{32}P]dCTP and Klenow fragment.

Serum or PMA treatment of saphenous vein smooth muscle cells leads to a significant increase of non-1.D mRNA levels

Saphenous vein smooth muscle cells were grown to sub-confluency and subsequently incubated for 72 h in medium containing 0.5% FBS. Serum-starved cells were treated with 10% serum or PMA (100 ng/ml) for 6 h, the time point which was previously shown to have peak FGF-1 mRNA levels in saphenous vein smooth muscle cells (32). RNA was isolated and analyzed by an RNase protection assay using an FGF-1.D cDNA-derived riboprobe. GAPDH was used to show the relative loadings of RNA in each lane. Normally growing sub-confluent cells express FGF-1.D mRNA predominantly (data not shown). Serum-starved cells show low levels of FGF-1.D mRNA (Fig. 3, lane 1), which is slightly increased (less than 2-fold) after treatment with serum (lane 2) or PMA (lane 3). Furthermore, a significant increase (at least 10-fold) of non-1.D mRNA is observed over FGF-1.D mRNA in the serum-starved state (compare lanes 2 and 3 with lane 1). These data provide evidence that the level of non-1.D mRNA increases significantly in response to extracellular stimuli such as serum and PMA. The response to PMA implies that a protein kinase C-dependent signalling pathway may be involved in the regulation of FGF-1 gene expression. In contrast, serum or PMA treatment for 6 h does not affect FGF-1 mRNA levels in iliac artery smooth muscles cells (data not shown). These data suggest heterogeneity in the cell types in regard to cell surface receptors or intracellular second messenger systems that may lead to increased levels of FGF-1 mRNA. Such variety may be considered a mechanism of regulation that provides specificity to certain cell types exhibiting FGF-1 gene inducibility.

Serum or PMA treatment of cultured saphenous vein smooth muscle cells leads to increased levels of FGF-1.C mRNA

To identify which FGF-1 transcript is increased in response to serum and PMA, RNase protection assays were performed using riboprobes derived from other FGF-1 cDNAs. There is a slight increase in the levels of FGF-1.A mRNA in response to serum and

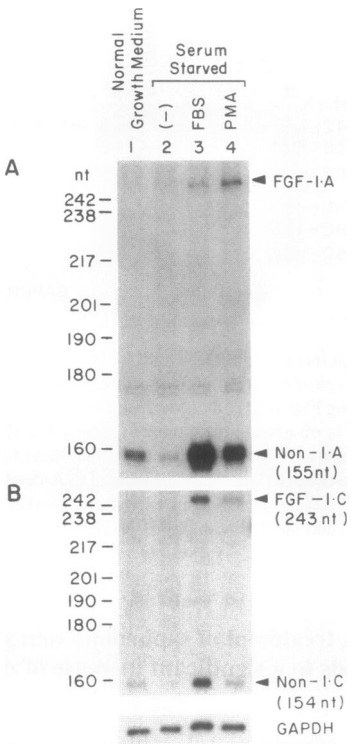

Figure 4. FGF-1.C mRNA is the major FGF-1 transcript increased in response to serum and PMA. RNase protection assays were performed to identify the FGF-1 non-1.D mRNA seen in Figure 3 as FGF-1.C mRNA. (**A**) a riboprobe derived from FGF-1.A, and (**B**) a riboprobe derived from FGF-1.C cDNA were used for the RNase protection assays. The expected size of the FGF-1.A protected fragment is 241 nt. The samples used (lanes 1–4) are the same as in Figure 3.

PMA (Fig. 4A, lanes 3 and 4), when compared overall to the non-1.A mRNA levels. Similarly, RNase protection analysis using FGF-1.B cDNA-derived riboprobe showed that the induced FGF-1 transcript is non-1.B (data not shown). In contrast, there is a significant increase in levels of FGF-1.C mRNA in response to serum and PMA (Fig. 4B, lanes 3 and 4), when compared to the normally growing and serum-starved cells (lanes 1and 2), and also to the levels of non-1.C mRNA. Densitometric scanning of these autoradiograms and normalizing the values to GAPDH suggest the possible existence of a fifth FGF-1 transcript with a different 5′ end. However, in all other experiments, we consistently observed FGF-1.C mRNA as being the major species observed (75–90%) in response to serum and PMA (data not shown; also see Fig. 5A). We therefore conclude that serum or PMA significantly up-regulate the level of FGF-1.C mRNA.

FGF-1.C mRNA has a unique start site, whereas FGF-1.D mRNA consists of multiple 5′ ends

High level expression of FGF-1.C mRNA in serum-stimulated saphenous vein-derived smooth muscle cells allowed us to use this RNA preparation to map the transcription start site for FGF-1.C. We constructed a minigene consisting of genomic DNA sequence adjacent to the cDNA sequence utilizing a common *Pst*I site. The riboprobe derived from this construct provides an internal control to distinguish FGF-1.C from non-1.C transcripts. As shown in Figure 5A, a single 260 nt fragment was protected, indicating that the FGF-1.C mRNA has a unique start site positioned 105 bp upstream of the exon -1C splice donor site (Fig. 6A). Similarly, a recombinant PCR approach was devised to generate a minigene consisting of FGF-1.D genomic sequences contiguous with the FGF-1.D cDNA. This minigene was used to generate an anti-sense riboprobe and used in an RNase protection assay. Results of this assay (Fig. 5B) suggest multiple transcription initiation sites positioned at 40 or 57 bp upstream from the exon -1D donor site (Fig. 6B). These data demonstrate that the two FGF-1 transcripts observed in smooth muscle cells under different growing conditions originate from two distinct promoters of the FGF-1 gene, i.e. FGF-1 promoters 1C and 1D which are 22.4 kbp apart in the FGF-1 gene locus.

A region in the FGF-1 promoter 1D sequence is prone to single-strand nuclease breakage *in vitro*

Having sequenced the 1.2 kbp *Eco*RI–*Bgl*II fragment, we noticed a purine stretch of 72 bp upstream from the FGF-1.D start site. The chromatin conformation may cause this region to become susceptible to single-strand nuclease digestion (39). Treatment of supercoiled plasmid DNA containing the FGF-1 promoter 1D region with mung bean nuclease and subsequent restriction digestion with various enzymes allowed mapping of this single-strand nuclease-sensitive region (Fig. 7). This region is ~950 bp 3′ to the *Eco*RI site, which is immediately upstream from the FGF-1.D start site. Treatment of linearized DNA (digested with *Eco*RI) with mung bean nuclease did not reveal additional bands other than the linear plasmid DNA. These data indicate an unusual DNA conformation in the 1D promoter region that leads to single-strand exposure which is susceptible to cleavage by single-strand-specific nuclease.

DISCUSSION

In this report we show the distribution of expression of two FGF-1 mRNAs (FGF-1.C and -1.D) in a variety of tissues and cell lines. By using RNase protection assays, we have shown that the two FGF-1 mRNAs are co-expressed, with FGF-1.D mRNA being the major species in glioblastoma U1242 MG cells, lung fibroblasts and iliac artery smooth muscle cells under logarithmically growing conditions (Fig. 2). The corresponding untranslated exons of the FGF-1 gene that are unique for FGF-1.C and -1.D mRNAs have been designated exons -1C and -1D, respectively. We further show here that each of these two mRNAs are under the control of its promoter respectively (Fig. 5) and that these two promoters are different from the two previously reported (26). Lastly, promoter 1C is the one that is responsive to stimulation by serum or PMA (Fig. 4). Our results, taken together, suggest a novel phenomenon observed in the regulation of the FGF-1 gene. In the system used, i.e. saphenous vein smooth muscle cells, normally growing cells utilize promoter 1D. Upon serum or PMA treatment of serum-starved cells, an alternative promoter, 1C, is utilized. Our present findings point to dual promoter usage of the FGF-1 gene in saphenous vein smooth muscle cells.

The nucleotide sequence homology between the human FGF-1 promoter 1C region and the corresponding Syrian hamster sequence has previously been described (27). Interspecies sequence comparison for the promoter 1D is not possible since the corresponding sequences in other species have not been cloned and sequenced. Comparison of the promoter 1D sequence

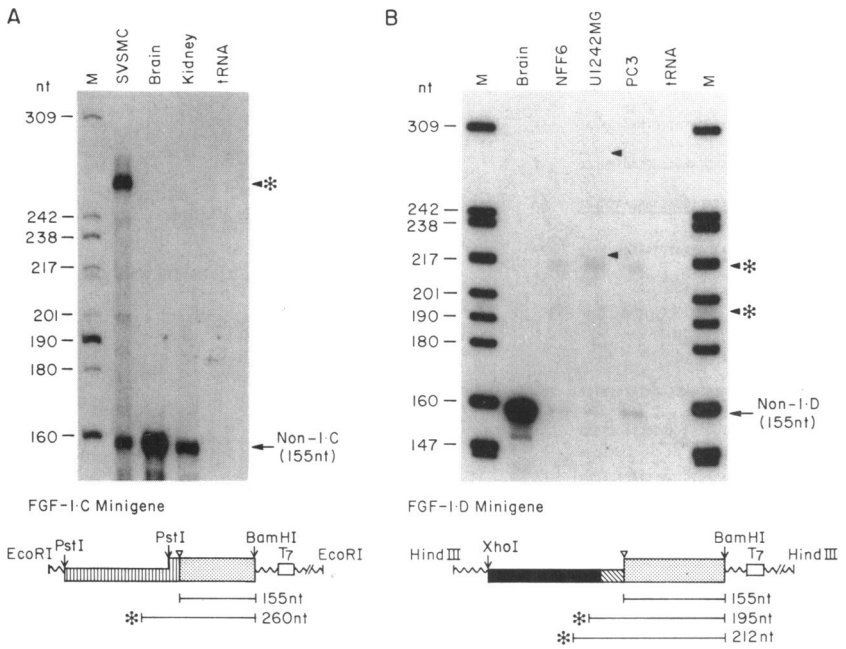

Figure 5. Determination of FGF-1.C and -1.D mRNA transcription start sites. RNase protection assays were used to map the start site for (**A**) FGF-1.C and (**B**) FGF-1.D transcripts. Minigenes for FGF-1.C and -1.D were constructed having the genomic sequences contiguous with the cDNA sequences. (A) Serum-treated saphenous vein smooth muscle cell RNA was used for FGF-1.C start site mapping, and shows one major band (SVSMC lane). Brain and kidney RNA were used as negative controls for FGF-1.C mRNA, and yeast tRNA was used as a general negative control. The dotted box represents exon 1, the tall and short striped boxes represent exon -1C and its immediate upstream genomic sequence, respectively. (B) NFF-6, U1242MG, and PC-3 cell RNAs were used for FGF-1.D mRNA start site mapping. Brain RNA was used as a negative control for FGF-1.D mRNA, whereas yeast tRNA was used as a general negative control. NFF-6, U1242MG, and PC-3 cells show multiple protected bands, indicating multiple start sites for FGF-1.D mRNA. Common bands for these samples are indicated by asterisks, whereas arrowheads indicate unique bands seen only in U1242MG. The bands that are unique in U1242MG cells are more discernible in the original autoradiogram. The 195 nt fragment appears to be a doublet. The dotted box represents exon 1; the striped box and closed box represent exon -1D and its immediate upstream genomic sequence, respectively. Marker used in (A) and (B) is pBR322 digested with *Msp*I, and labeled with [α-^{32}P]dCTP and Klenow fragment. T7 represents the bacteriophage T7 RNA polymerase recognition site.

and other published sequences using the FASTA program (38) revealed significant similarity between the 1D promoter and the following genes: 86.5% similarity over 74 bp to the spacer region upstream from the rat rRNA transcription unit (40), 77.3% similarity over 88 bp to human erythropoietin receptor gene (41), and 72.5% similarity over 109 bp to murine urokinase plasminogen activator gene (42). These similarities are essentially in the purine-rich region of these sequences, especially the promoter regions of the human erythropoietin receptor gene (–400 to –313) (41) and the murine urokinase plasminogen activator gene (–211 to –103) (42). The functional significance of any of these sequences remains to be determined.

There have been several reports of purine-rich regions prone to forming an unusual DNA conformation (39). Sensitivity of the FGF-1.D purine stretch to a single-strand-specific nuclease (Fig. 7) suggests it may have an unusual conformation with single-strand regions. Such a conformation may be a triple helical state whereby two pyrimidine strands share one common purine strand. This sharing of strands is possible for the FGF-1 promoter 1D region and is diagrammed in the lower part of Figure 7. By using monoclonal antibodies to triplex DNA, Burkholder *et al.* (43) have suggested such regions might be involved in chromatin condensation via interspersed homologous interaction of DNA sequences in chromatin. Furthermore, it was suggested that this triple helical state may be stabilized by non-nucleosomal single-strand DNA binding proteins (43,44). Such a region may form a transcriptionally competent complex. In fact, such protein binding has been shown to be involved in transcriptional regulation of the c-*myc* (45,46), epidermal growth factor receptor (47) and c-Ki-*ras* (48) genes. Additionally, such purine-rich regions can be targets for oligonucleotide-mediated repression of transcription, as shown for c-*myc* (49).

Upon searching for putative protein binding sites in the promoter 1D region (1.2 kbp *Eco*RI–*Bgl*II fragment, GenBank accession no. L01488), there appears to be an AP-1 site, a CArG box, and several PU.1 binding sites (27). The PU.1 binding protein, the product of the *Spi*-1 proto-oncogene (50–52) is expressed in macrophages and B cells exclusively. To test for FGF-1.D mRNA expression, a promonocytic U937 cell line that can be induced to terminal differentiation by γ-interferon (53) and a human erythroleukemia K562 cell line were analyzed by RNase protection assay. No expression of FGF-1.D or other FGF-1 mRNA was detected in γ-interferon-induced or non-induced U937 cells or in K562 cells. These data, however, do not exclude the possibility that cells of the hematopoietic system may express FGF-1. In fact, Brogi *et al.* detected immunoreactive FGF-1 in macrophage-rich regions of human atheroma (54). Whether this FGF-1 is produced by macrophages or is sequestered from neighboring vascular cells remains to be established by *in situ* hybridization.

The CArG box has been shown to be necessary for regulation of expression of cardiac and skeletal α-actin genes (55,56) and binds a CArG factor. Furthermore, Boxer *et al.* (57) have shown that the CArG binding factor is indistinguishable from the c-*fos* serum response factor. This raises the possibility that promoter 1D is activated via the serum response factor and CArG box

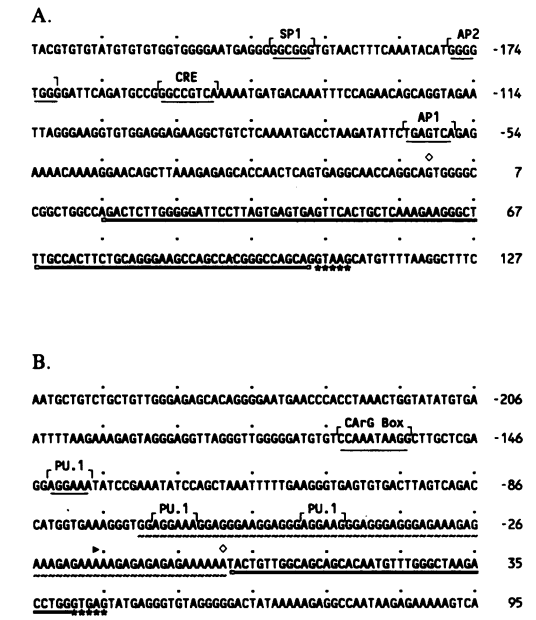

Figure 6. Nucleotide sequences of FGF-1 promoters 1C (**A**) and 1D (**B**). The major transcription start sites for FGF-1 promoters 1C and 1D as determined by RNase protection assays are denoted by a diamond symbol and are designated as +1 in the numbering of nucleotides. The second major start site for FGF-1.D is indicated by a filled-in triangle symbol. Consensus splice donor sequences are indicated by asterisks. Putative *cis*-acting DNA regulatory elements, including AP1, AP2, Sp1 and CRE (cAMP response element), are present in the immediate upstream region of promoter 1C (A). A CArG box (–164 to –155) and an AP1 site (–745 to –739) reside upstream of the transcription start site of promoter 1D. A 72 bp homopurine stretch, which contains multiple PU.1 binding sites, is indicated by a wavy line (B). The sequences contained in the cDNA are double underlined. The GenBank accession no. for (A) is L01486, for (B) L01488.

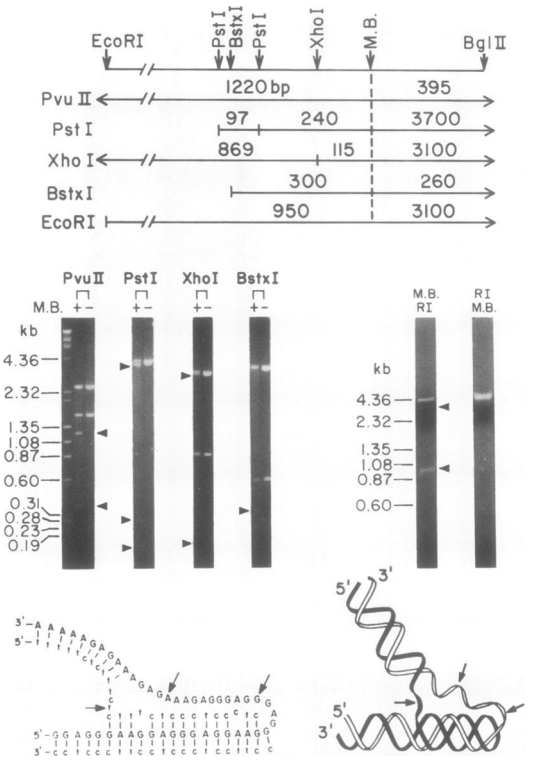

Figure 7. The FGF-1 promoter 1D region is single-strand nuclease-sensitive. Supercoiled plasmid DNA containing the 1.2 kbp *Eco*RI–*Bgl*II fragment that harbors the FGF-1 promoter 1D was treated with mung bean nuclease and then digested by various restriction enzymes. This allowed determination of the mung bean nuclease-sensitive region denoted by M.B. The sizes of fragments observed in the agarose gels are shown. The (+) and (–) denote restriction enzyme patterns with and without mung bean nuclease treatment. The template was also treated with restriction enzyme *Eco*RI first and then mung bean nuclease (RI/M.B.) to compare the pattern with treatment in the reverse order (M.B./RI). The bands smaller than 300 bp were clearly visible in the original stained gels, and the positions are indicated by arrowheads. The DNA size markers used were λ*Hin*dIII and ϕ*Hae*III restriction fragments. A postulated triple helical DNA conformation for a region of FGF-1D promoter, that may be sensitive to single-strand nuclease (indicated by arrows), is shown in the lower part (adapted from 66–68).

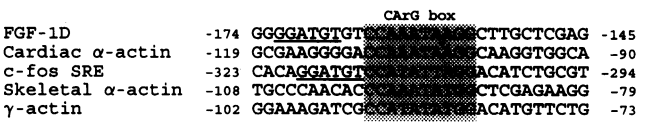

Figure 8. The promoter 1D CArG box is the same as the CArG box of human cardiac α-actin gene, and similar to CArG boxes found in the human c-*fos* serum response element, skeletal α-actin gene, and γ-actin gene. The adjacent sequence GGATG to the left of the FGF-1D CArG box is the same as the human c-*fos* SRE (underlined) that has been shown to bind a ternary complex protein (58).

interaction. Shaw *et al.* (58) have demonstrated that ternary complex formation is necessary for induction of the c-*fos* gene in response to serum. A comparison of promoter 1D CArG box and CArG boxes of c-*fos*, α-actin and γ-actin genes is shown in Figure 8. The ternary complex factor (TCF) binding region is present in 1D CArG box flanking sequences. It is possible that expression of FGF-1.D mRNA seen in logarithmically growing cells depends on the presence of this TCF. Electrophoretic mobility shift assays as well as functional analysis of the CArG-like box of the 1D promoter may elucidate the role of these sequences in gene regulation.

Atherosclerosis and hypertension are pathological conditions that result from smooth muscle cell hyperplasia and/or hypertrophy (59). FGF-1 is a mitogen for vascular endothelial cells and smooth muscle cells *in vitro* and *in vivo*. Thus, the precise regulation of FGF-1 gene expression within the vessel wall may be critical for vascular cell growth control. Furthermore, Hoover *et al.* (60) and Clowes *et al.* (61) have reported the anti-proliferative property of heparin on vascular smooth muscle cells. Heparan sulfate is produced by endothelial cells and has been suggested to regulate vascular smooth muscle cell growth *in vivo* (62,63). This regulation may be via selective inhibition of protein kinase C-dependent signalling pathways involved in gene expression (63,64), particularly of growth related genes. It can, therefore, be speculated that deregulation of this anti-proliferative activity of heparin at sites of trauma, for example balloon catheter angioplasty, may affect FGF-1 gene expression. This may subsequently lead to proliferation of vascular smooth muscle cells, leading to a lesion. Such aberrant regulation of proliferation may be the hallmark of the pathological conditions. This hypothesis is consistent with the findings that FGF-1 promotes intimal hyperplasia and angiogenesis in arteries *in vivo* (65) and

the findings that show an increased association of FGF-1 with atherosclerotic plaque microvessels (54). Using the vascular smooth muscle cells as a model system, it is now possible to test for transcriptional regulation of FGF-1 promoters by heparin. Additionally, *in situ* hybridizations using FGF-1.C- and -1.D-specific probes on affected tissues in these diseases may define which promoters are activated in such pathological sites.

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