

Cloning of two novel forms of human acidic fibroblast growth factor (aFGF) mRNA

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ABSTRACT

We have previously isolated two different aFGF cDNA clones from kidney and brain. The two corresponding mRNA, designated aFGF 1.A and 1.B, are the predominant species in kidney and brain, respectively. During the characterization of aFGF mRNA in glioblastoma cells, we demonstrated that aFGF mRNA in U1242MG and D65MG glioblastoma cells contain 5'-untranslated sequences different from those of 1.A and 1.B. Through a strategy combining chromosome walking, identification and sequencing of evolutionarily conserved DNA regions, and a reverse transcription and polymerase chain reaction (RT-PCR)-based assay for RNA expression, we have isolated two novel aFGF cDNA clones. The cDNA clone representing aFGF mRNA 1.C was isolated from U1242MG cells; another aFGF cDNA, designated 1.D, was isolated from D65MG cells. Promoter 1C has extensive sequence homology to the hamster aFGF gene promoter that was shown to respond to testosterone stimulation by chloramphenicol acetyltransferase reporter gene assays. Using RT-PCR, we showed that normal, benign and cancerous prostate tissues do not express aFGF 1.C mRNA. In contrast, a prostate carcinoma cell line (PC-3) expresses 1.C mRNA. RT-PCR using 1.D-specific primers showed that kidney, brain and prostate do not express 1.D mRNA even though kidney and brain are the most abundant source for aFGF protein. RNase protection analysis further showed that 1.D mRNA is the predominant aFGF transcript in D65MG glioblastoma cells and in NFF-6 neonatal foreskin fibroblast cells. The genomic DNA corresponding to these two cDNA clones and the 5'-flanking regions were also isolated and their sequences determined. These DNA clones will provide important reagents for studying the regulatory elements of aFGF gene expression.

INTRODUCTION

Expression patterns of growth factors and their receptors are highly complex, reflecting their crucial need in normal development processes as well as responses to wounding and infection. Multigene families of related ligands and their receptors, often with overlapping specificities, allow for greater flexibility in the timing and tissue distribution of these proteins. Perturbation of these balances could create an autocrine transforming loop, leading to abnormal cell growth and regulation, resulting in tumorigenesis (1). One distinct family is that of the fibroblast growth factors (FGF) and their receptors (FGFR), which include seven growth factors (2, 3) and four receptors (4–7) each encoded by a distinct gene. Another level of complexity has been demonstrated recently in that variants of the same receptor are encoded as alternative transcripts of the same gene (8). In the case of FGFR-2, an alternative 49 amino acid stretch at the second half of the third immunoglobulin-like domain confers specific ligand binding capacity to basic FGF (bFGF) and keratinocyte growth factor (KGF), respectively. Remarkably, acidic FGF (aFGF) binds to both variants of FGFR-2 with equal affinity (9, 10).

Acidic FGF was originally identified as a mitogen for endothelial cells (11) and subsequently for a variety of mesenchymal- and neuroectodermal-derived cells (3, 12). It was also shown to be a mitogen for prostatic epithelial cells (13). In contrast, the mitogenic activity of KGF is tightly restricted to epithelial cells (14). A gene designated K-SAM was identified as an amplified sequence in a human stomach carcinoma cell line (15); and its cDNA encodes a KGFR variant of the FGFR-2 (16). KGFR/K-SAM is expressed exclusively in keratinocytes (17) and has been shown to transform NIH/3T3 cells (18). Thus, aFGF or KGF may be important in human epithelial tumors. This is consistent with the reports that showed ectopic expression following transfection of the cDNA of either wild type aFGF (19) or a truncated form of aFGF ($\Delta 1-21$) (20, 21) confers a transformed phenotype to the recipient cells. Thus, over-expression of the aFGF gene may be important in certain malignancies.

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The aFGF mRNA levels are up-regulated by a variety of biological response modifiers including phorbol esters, serum and combinations of growth factors (22–24). Of particular interest is the up-regulation of aFGF mRNA by androgens in a ductus deferens derived smooth muscle tumor cell line, DDT1 cells (25) as well as in a metastasized human prostate tumor cell line, LNCaP (26). Characterization of the androgen response element(s) in the aFGF gene promoter will likely shed light on the role of aFGF in the etiology or pathogenesis of prostate cancer. We have previously isolated two different human aFGF cDNA clones from kidney (27) and brain (28). The two corresponding mRNA, designated aFGF 1.A and 1.B, are the predominant species in kidney and brain, respectively. A Syrian hamster aFGF cDNA was originally isolated from DDT1 cells (25). The 5'-untranslated sequences of the two human aFGF transcripts bear no homology to that of the hamster aFGF cDNA sequences, although the brain-specific sequence is homologous to the aFGF cDNA isolated from bovine retina (29). During the characterization of aFGF mRNA in glioblastoma cells, we demonstrated that aFGF mRNA in U1242MG and D65MG glioblastoma cells contain 5'-untranslated sequences different from the previously published sequences. Here we report the isolation of two novel aFGF cDNA clones; one of them (aFGF 1.C) is homologous to the hamster aFGF cDNA clone while the other (aFGF 1.D) has not been previously reported. The genomic DNA corresponding to these two cDNA clones was also isolated and their sequences determined. These DNA clones will provide important reagents for studying the regulatory elements of aFGF gene expression.

MATERIALS AND METHODS

Cell culture

The human glioblastoma cell line U1242MG was provided by Dr. Christer Betsholtz and Dr. Bengt Westermarck, University of Uppsala, Sweden. The D65MG human glioblastoma cell line was provided by Dr. Yancey Gillespie, University of Alabama at Birmingham. These glioblastoma cells were maintained in modified Eagle's minimal essential media (MEM) (Gibco, Grand Island, NY) supplemented with 10% calf serum. PC-3, a prostate carcinoma cell line, was grown in RPMI medium plus 10% fetal bovine serum. The NFF-6 cells (human neonatal foreskin fibroblasts strain 6) were obtained from Dr. Gary Shipley, Oregon Health Sciences University, Portland, Oregon and maintained as described (23). Human tissues were obtained from the Tissue Procurement Service of The Ohio State University Comprehensive Cancer Center.

Library screening, Southern blotting and hybridization

A chromosome 5 specific library was constructed by partial digestion of DNA with Sau3AI and cloned into the BamHI site of Charon 40. One million independent recombinants were screened with appropriate Alu-free probes. Plaque lift filters were hybridized overnight at 62°C with 1×10^6 cpm/ml of [α -³²P]dATP-labeled probe in $6 \times$ SSC, $1 \times$ Denhardt's solution, 0.5% SDS containing 100 μ g/ml herring sperm DNA. The probes were labeled using the random-primer labeling method (30). The filters were washed at 62°C in $0.1 \times$ SSC/0.1% SDS prior to autoradiography. Phage DNA was prepared from 25 ml of bacterial lysate as described (31). Phage DNA digested with appropriate restriction enzymes was followed by Southern blot hybridization analysis according to standard procedures (32).

Nucleotide sequencing and sequence comparison

Human DNA fragments shown to be homologous to the hamster aFGF promoter region were subcloned into pBluescript vectors (Stratagene) and sequenced by the dideoxynucleotide chain termination method (33) on double-stranded DNA templates. Occasionally single-stranded DNA was isolated following superinfection with f1 helper phage and used as a template for sequence analysis. The DOTPLOT and ALIGN programs (DNA Star, Inc., Madison, WI) were used to compare the aFGF sequences of human and hamster.

Reverse transcription and polymerase chain reaction (RT-PCR)

RNA was isolated from tissues or cell lines using the guanidinium thiocyanate and CsCl gradient method (34). Complementary DNAs were synthesized from 1 μ g of total cellular RNA by murine leukemia virus reverse transcriptase using oligo(dT), random hexamers or gene-specific oligonucleotides as primers. After heating at 95°C for 5 min, 5 μ l of the cDNA reaction was amplified using a GeneAmp kit (Perkin-Elmer) in a 100 μ l PCR reaction for 35 cycles (35) with appropriate annealing and extension parameters. Primers used were derived from sequences of the first coding exon for human aFGF and the unique upstream sequences of aFGF cDNA 1.C or 1.D. The PCR products were analyzed on a 2% Nusieve and 1% Seakem (FMC BioProducts, Rockland, ME) agarose gel and visualized by staining with ethidium bromide. Restriction enzyme digestion was performed to confirm the identity of the amplified products. The amplified fragments were cloned into pCR1000 using the TA cloning kit (Invitrogen, San Diego, CA) or cloned into the T-tailed, EcoRV-digested pBluescript KS(+) as described (36).

RNase protection analysis

RNase protection analysis was performed as outlined in Current Protocols in Molecular Biology (31) with minor modifications. A [α -³²P]UTP-labeled antisense riboprobe specific for 1.D

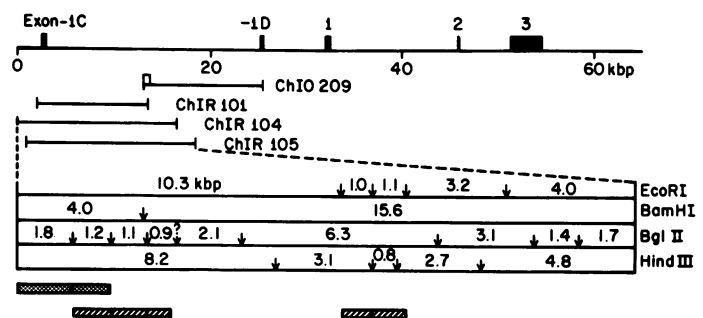


Figure 1. Restriction enzyme map of genomic clones ChIR 101, 104 and 105. A human genomic DNA library was screened using the 0.52 kbp XbaI-BglII fragment derived from the 5'-end of our most 5' clone, ChIO 209. Genomic clones downstream from ChIO 209 have been previously described (37). The restriction enzyme maps of each of the three new clones were determined and the topographic relationship of these three clones was established by aligning the common restriction sites. Restriction digests of ChIR 104 was hybridized to total human genomic DNA to reveal regions that do not contain Alu-repetitive sequences (hatched boxes). The open box on clone ChIO209 indicates the probe used for library screening. The stippled boxes indicate sequences homologous to the 1.6 kbp EcoRI fragment (see Fig. 4) of hamster genomic DNA which is adjacent to the 5'-untranslated sequences of a hamster aFGF cDNA clone (39). Vertical bars indicate NotI sites flanking the insert of each ChIR phage clone. Closed boxes indicate the position of each exon.

aFGF cDNA was generated by *in vitro* transcription of a PCR fragment cloned into pBluescript vector, which contains the entire 203 bp first coding and 7 bp of the second coding exons spliced to 39 bp of known -1D exon sequence. Forty μg of RNA and antisense riboprobe (5×10^5 cpm) were denatured at 85°C for 5 min and hybridized in $30 \mu\text{l}$ of 80% formamide, 0.4M NaCl, 1 mM EDTA and 40 mM PIPES (pH 6.4) at 42°C for 18 hr. Samples were digested with RNase A ($20 \mu\text{g}/\text{ml}$) and T1 ($1 \mu\text{g}/\text{ml}$) for 60 min at 30°C , and analyzed on a 6% polyacrylamide gel.

RESULTS

We have previously isolated a continuous stretch of 54 kbp of human genomic DNA containing the three coding exons of aFGF (37). Since the 5'-end of the first coding exon (exon 1) does not

represent a transcription start site (38) we isolated upstream clones to identify the aFGF gene promoter(s). Three positive clones, designated ChIR 101, 104 and 105, were mapped and regions not containing repetitive sequences were determined (Fig. 1). The phage DNA from ChIR104 was hybridized to a 1.6 kbp EcoRI fragment that contains the promoter region and part of the 5'-untranslated sequence of the hamster aFGF mRNA (39). The results showed hybridization to the 1.8 kbp NotI-BglII and 1.2 kbp BglII-BglII fragments indicating sequences homologous to the hamster DNA (Fig. 1). Comparison of the DNA sequence from the 1.2 kbp BglII-BglII fragment with the hamster cDNA sequence showed a stretch of 21 nucleotides of human sequence with 85.7% (18 out of 21) sequence similarity to the last 21 nucleotides of the hamster aFGF 5'-untranslated exon (Fig. 2). Moreover, this sequence was followed by GTAAG, a consensus splice donor recognition sequence.

We designed an oligonucleotide (HBGF1601), 31 nucleotides long, whose 5'-end is 89 bp upstream from a putative splice donor site (Fig. 2). This oligonucleotide in combination with HBGF151 (downstream oligonucleotide) was used in reverse transcription and polymerase chain reactions (RT-PCR). Oligonucleotide HBGF151 contains the last 16 nucleotides of exon 1 and the first 7 nucleotides of exon 2. The RNA templates used were from two glioblastoma cell lines D65MG and U1242MG which expressed aFGF but did not express either of the two previously identified transcripts (aFGF 1.A or 1.B). In the specimens of both glioblastoma cell lines, a band of the predicted size of 299 bp was detected. Upon digestion with BamHI, an expected 241 bp fragment was also observed in both samples of D65MG and U1242MG. Cloning and sequence analysis (accession no. L01485) of the 299 bp RT-PCR product from U1242MG showed that the first 89 bp is identical to nucleotides 1-89 shown in Fig. 2 while the remaining 210 bp is identical to the sequences



Figure 2. Alignment of the hamster aFGF gene sequence with the homologous human DNA sequence. The human sequence was derived from the 1.2 kbp BglII-BglII fragment of ChIR104. The hamster sequence represents the exon that contains the 5'-untranslated sequence of aFGF mRNA. Vertical lines indicate identity between the two sequences. Asterisks indicate the consensus splicing donor sequence. Oligonucleotide HBGF1601 was used in RT-PCR for amplification of the 1.C-specific cDNA.



Figure 3. Nucleotide sequence of the 0.8 kbp SacI-BglII fragment and the adjacent 1.2 kbp BglII-BglII fragment and localization of exon -1C. The nucleotide sequences of the 0.8 kbp SacI-BglII fragment and the 1.2 kbp BglII-BglII fragment that hybridized to the hamster 1.6 kbp EcoRI DNA were determined. The sequence present in our aFGF 1.C cDNA clone is double underlined. The consensus splice donor sequence, GTAAG, is indicated by asterisks. Several candidate regulatory DNA elements (50) including AP1, AP2, ARE, CRE (cAMP response element) and Sp1, are indicated. The diamond symbol indicates the transcription start site for aFGF 1.C mRNA assuming the human and hamster genes have similar transcription start sites. The accession no. is L01486.

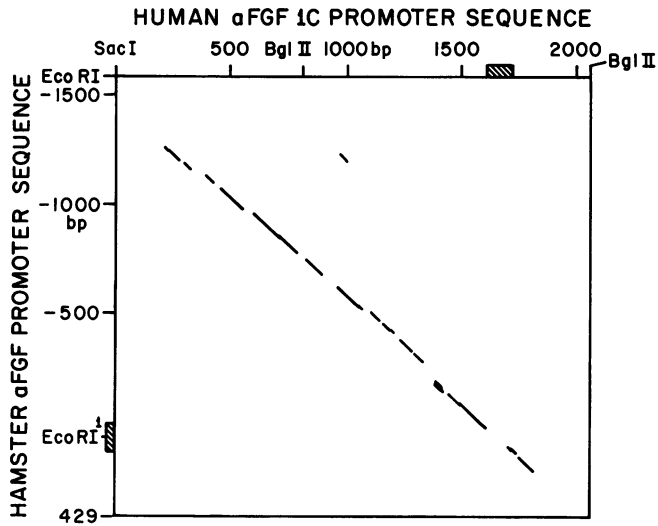


Figure 4. Homology between the human (the 2.06 kbp SacI-BglII fragment) and the hamster genomic DNA sequences. The 2.01 kbp hamster genomic DNA sequence used starts from EcoRI (nucleotide -1584) and ends at the 3' end (nucleotide 429) of available 5'-flanking sequence of the hamster aFGF gene (39). Nucleotide 1 is the transcription start site of hamster aFGF gene. The human sequence is the 2.06 kbp DNA shown in Fig. 3. Hatched boxes represent the sequences present in the human aFGF 1.C and in the hamster aFGF cDNA clone. The parameters used for the DOTPLOT program were 70% sequence similarity with a window of 30 nucleotides.

derived from the coding exons of aFGF 1.A and 1.B mRNA except one nucleotide. These results established that the 299 bp fragment represents an aFGF mRNA variant (designated aFGF 1.C) resulting from the splicing of an upstream untranslated exon (hereafter referred to as exon -1C) to the first coding exon. RNase protection analyses showed that 1.C mRNA is not expressed in kidney and brain (data not shown).

Upon further restriction digestion and Southern hybridization using the hamster 1.6 kbp EcoRI fragment as a probe, the hybridizing region in the 1.8 kbp NotI-BglII fragment derived from ChIR104 was further localized to its 3'-portion represented by a 0.8 kbp SacI-BglII fragment (Fig. 1; data not shown). Having established that exon -1C represents the corresponding human DNA to the hamster aFGF cDNA, we determined the sequence of the 0.8 kbp SacI-BglII fragment and the adjacent 1.2 kbp BglII-BglII fragment for comparative analysis (Fig. 3). Exon -1C localizes to 29.3 kbp upstream from exon 1. Using DOTPLOT analysis, we were able to show that a stretch of more than 1.5 kbp of human DNA surrounding exon -1C has greater than 70% sequence similarity with the corresponding hamster sequence (Fig. 4). Since the hamster sequence was shown to be activated by testosterone and aFGF itself in chloramphenicol acetyltransferase (CAT) reporter gene assays (39), and aFGF mRNA is up-regulated by testosterone in a ductus deferens derived smooth muscle cell line (25), we speculated that 1.C mRNA may be expressed in human prostate tissues. We screened one normal and three benign hypertrophic prostate tissues and four primary prostate carcinomas using RT-PCR and all of them were negative for 1.C mRNA. In contrast, PC-3, a prostate carcinoma cell line, expresses the aFGF 1.C mRNA (Fig. 5).

When a riboprobe derived from the aFGF 1.C cDNA was used to analyze the aFGF mRNA in D65MG and U1242MG cells,

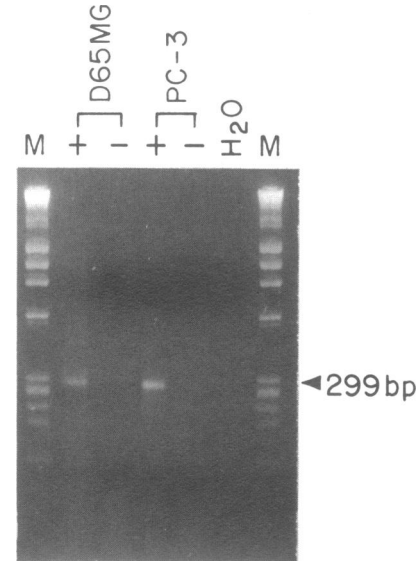


Figure 5. Expression of aFGF 1.C mRNA in D65MG and PC-3 cells. RT-PCR was utilized to detect 1.C- specific transcripts in D65MG and PC-3 cells. One μ g of RNA was used to generate cDNA using random hexamers as primers. PCR was carried out using primers HBGF1601 and HBGF151 and the following cycling conditions: 95°C for 2 minutes, 35 cycles of 1 minute at 94°C, 1 minute at 58°C, and 2 minutes at 72°C, followed by a 15 minute extension at 72°C. Plus and minus signs indicate reverse transcription in the presence or absence of reverse transcriptase. Lane H₂O was a negative control with no DNA template in the PCR reaction. An expected 299 bp amplicon as indicated by an arrowhead is seen only in plus RT lanes for both cell lines. Lane M contains λ DNA digested with HindIII and ϕ X174-RF DNA digested with HaeIII.

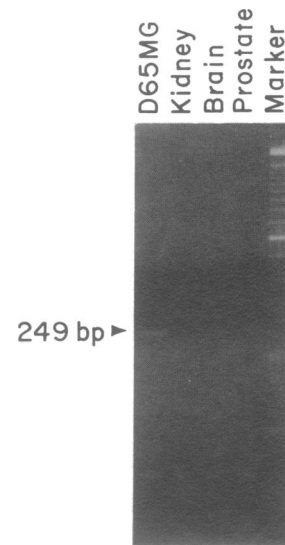


Figure 6. RT-PCR of aFGF 1.D mRNA. RNA was isolated from human glioblastoma cell line D65MG, kidney, brain and prostate. One μ g of RNA was reverse transcribed using both oligo(dT)₁₂₋₁₈ and random hexamers as primers. The cDNA was then amplified by PCR with primers HBGF1702 and HBGF151. The PCR parameters were denaturation at 94°C for 30 sec, annealing at 48°C for 45 sec and extension at 72°C for 1 min for 35 cycles. An expected 249 bp product, indicated by arrowhead, is seen only in the D65MG lane. The PCR product was cloned into EcoRV-digested, T-tailed pBluescript and sequenced (accession no. L01487). Marker is a 100 bp DNA ladder (Gibco/BRL).

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EcoRI
GAATTCACAGGACACTAATAATGCATAGGATTTGCTCAATGCTAGATACAGAAAGAAAGGCATAGTTTCTGTCCAGGGTCTCAGAGACTAATGAAGGAGTCAGTGAAGTAAACGTA* 120
TTAAATTCACATTCATTTGCCATTCAACAGATGTTTATCAATGCCATCAGGTGTCAGGCCATTTTGTAGGCCACTGGGATACCATTTGTGAGCAAGACCAATTGGTTTGTACTATCATGG 240
AGCTGACAGTCCAGTCATGAAAGAGACATTAATAATCACAAGAGTGAATGTGTAATTAACACTGGTAGTAAAGGCCATGAAGAAATGTTAAATGGTCTATGAGAACTGGAGAA* 360
AP1 HpeI
TGACTCAGTTTCAGGGAAGACAGGAAAGGCTTTCATGAGGAATTGAGAGTTGAACATAAAATTTGAAGAAAATTAGGAGTTAACAGGGGAGTAGGGTAGAGAGAAAGAGAGGA* 480
TCCAGAGGGAACAGCATGGGCAAGGCTCTAGGGTGGGGGAATCAGAAAATGGAAGGCAAGTTTGGAGGGAGACGTGATGGGAAATGAGGCAGAGGGGGAGTAAGGGCTGAACCTCCCA 600
GAGTCCTGCAGGCCACAGCAAGGCTTTTGTGTTTGTCCAGAGCAATGGGAAATCACTGAAGAGTTTAGGCAGGGGGATATAGATGGGAAATACACCCAGCGCTGCAGTGAAGTAAAGGA 720
AATGCTGTCTGCTGTTGGGAGAGCACAGGGGAATGAACCCACCTAAACTGGTATATGTGAATTTAAGAAAGAGTAGGGAGTTAGGGTTGGGGATGTGTC* 840
CARG Box XhoI
CGAGGAATATCCGAAATATCCAGCTAAATTTTGAAGGGTGAAGTGAAGTGTAGTCAAGCATTGGTGAAGGGTGGAGGAAGGAGGGAGGAAATGGAGGGGAGGAGAAAGAG 960
AAAGAGAAAAAGAGAGAGAAAAAATACTGTTGGCAGCAGCAATGTTTGGGCTAAGACCTGGGTCAGTATGAGGGTGTAGGGGGACTATAAAAAGAGCCCAATAAGAGAAAAAGTCA 1080
BglII
CAGGGTTAAGAAGGGGTCAAATGATGAGAGCCTCACAGGTCATGCCAGAGTCTGGGTTTATCTGTAAGACAACGGGAAATCAATGACCCAGTCAAAGATCT 1184

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Figure 7. Nucleotide sequence of the 1.2 kbp EcoRI-BglII fragment and localization of exon -1D. The 1.2 kbp EcoRI-BglII fragment was subcloned into pBluescript KS(+). The 39 bp sequence present in our aFGF 1.D cDNA clone is double underlined. The consensus splice donor sequence, GTGAG, is indicated by asterisks. The 72 bp homopurine stretch is indicated by a wavy line. Several *trans*-acting transcription factor binding sequences (50), including AP1, CARG Box and PU.1, are indicated. The accession no. is L01488.

it was shown that 1.C mRNA is not the predominant aFGF transcript in these cells (unpublished results). To identify the 5'-untranslated exon that is used predominantly in these glioblastoma cells, we used the 'exon-connection' strategy (40). We have previously identified a highly conserved DNA fragment, 10.7 kbp upstream from the first coding exon of human aFGF, which hybridized to distinct DNA fragments in primate and rodent genomes (37). DNA sequences of this fragment and the surrounding region were determined. We then searched for the consensus mammalian splice donor sequences, GT G/A AG, as a guideline to design oligonucleotides for the upstream exon. If the putative upstream exon was present in the same RNA transcript, then, with the use of the downstream oligonucleotide HBGF151, it would be possible to amplify a cDNA product linking these two regions. One oligonucleotide (HBGF1702), 39 bp upstream of a consensus splicing donor sequence, together with HBGF151 generated a discrete 249 bp RT-PCR product from the D65MG RNA but not from kidney, brain or prostate RNA (Fig. 6).

The 249 bp PCR product from D65MG was cloned into pBluescript KS(+), sequenced (accession no. L01487) and found to be the product (designated aFGF mRNA 1.D) of splicing the predicted exon from the upstream fragment to exon 1 of the aFGF gene. This upstream exon (designated exon -1D) was localized to a 1.2 kbp EcoRI-BglII fragment in our previously isolated clone, ChIK 117 (37,38). The D65MG-specific cDNA sequence of the RT-PCR product is present in nucleotides 987-1025 of the genomic DNA sequence (Fig. 7). This sequence is immediately followed by the consensus splice donor sequence, GTGAG. The size of the intron between exon -1D and exon 1 is 6.9 kbp (Fig. 1). It is also noted that exon -1D is preceded by 72 nucleotides of purine stretch, a sequence prone to triplex formation.

A riboprobe derived from aFGF 1.D was used in RNase protection analysis to confirm the authenticity of this clone. The result showed that 1.D mRNA is the predominant aFGF transcript in the D65MG cell line (Fig. 8). It was previously shown that a human neonatal fibroblast cell line expresses aFGF mRNA (23). We also included a neonatal foreskin fibroblast cell line, NFF-6, in the RNase protection assay, and we were able to demonstrate that aFGF 1.D is also the major aFGF transcript in NFF-6 cells (Fig. 8).

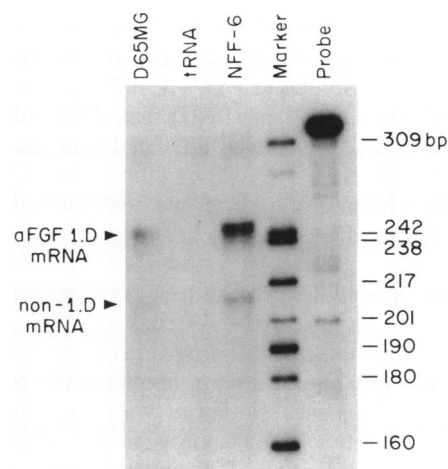


Figure 8. RNase protection analysis using aFGF 1.D cDNA derived riboprobe. The aFGF 1.D cDNA was linearized with EcoRI and used as template to generate an anti-sense riboprobe (314 nucleotides) by *in vitro* transcription. Forty μ g of tRNA was used as a negative control. Protected fragments of 249 nucleotides correspond to aFGF 1.D mRNA while protected fragments of 210 nucleotides correspond to aFGF mRNA that contains an upstream untranslated exon other than exon -1D. Marker is pBR322 DNA digested with MspI.

DISCUSSION

The human aFGF gene extends > 100 kbp and contains at least 7 exons (37, unpublished results). Determination of the exon-intron organization of the coding region revealed a gene structure very similar to other members of the FGF gene family (3). In each case, the gene is comprised of three protein-coding exons and the second coding exon is invariably 104 bp. The similarity in primary amino acid sequence, the common domain structure, and common modes of action through membrane receptors are additional proof that the various FGF proteins are related evolutionarily. The first four exons of the aFGF gene, which are untranslated, are spliced alternatively to a common protein-coding exon (27, 28, Fig. 3, Fig. 7). Thus, in contrast to the conservation of gene structure in the coding region, the promoter organization of the aFGF gene seems to be highly complex and is diverged from other FGF genes.

Unlike bFGF which is ubiquitously expressed, aFGF is expressed in a more restricted manner. Using the aFGF coding region as a probe, we have shown that aFGF is expressed in kidney, brain and heart but not in intestine, liver and lung (28). In addition to the two aFGF transcripts reported previously (27, 28) we have identified two novel aFGF transcripts through a strategy combining chromosome walking, identification and sequencing of evolutionarily conserved DNA regions, and RT-PCR based assays for RNA expression. Transcripts arising from the four promoters differ in their expression in various tissues and cell lines. Promoter 1A is active in kidney (27); promoter 1B is active in brain (28); while promoter 1D is active in two glioblastoma cell lines and a neonatal foreskin fibroblast cell line (Fig. 8). We also showed that kidney, brain and prostate do not express 1.D mRNA (Fig. 6) even though kidney and brain are the two most abundant sources for aFGF (27, 28). Thus, alternate promoter sampling and utilization may be an indication of the potential diversified function of the aFGF protein in various organs and tissues.

The transcript for aFGF mRNA 1.C is expressed in low levels in both U1242MG and D65MG cells as demonstrated by RNase protection analysis. Similar to 1.D, 1.C mRNA is not expressed in kidney and brain. We have found that aFGF mRNA levels are up-regulated in LNCaP cells stimulated with androgens (26) and we have isolated a 1.C cDNA clone from LNCaP cells cultured in the presence of testosterone (unpublished results). A 1.7 kbp BglII-BanI hamster sequence, 5'-flanking exon -1C, was shown to be activated by testosterone and aFGF itself in chloramphenicol acetyltransferase (CAT) reporter gene assays (39). The hamster promoter 1C-CAT constructs when transfected into DDT1 cells demonstrated androgen responsiveness in a cell-specific manner (39). Furthermore, human aFGF promoter 1C shares extensive sequence similarity with the hamster aFGF promoter that is activated by androgens and aFGF (Fig. 4). Remarkably, nucleotides 668–720 and nucleotides 1471–1500 share greater than 90% sequence similarity with their hamster counterpart. A half androgen response element (ARE), AGAA-CA (41) was found in nucleotides 1485–1490. These results imply that the putative ARE may be functional in human promoter 1C. However, our studies on one normal and three benign hypertrophic prostate tissues and four primary prostate carcinomas showed no expression of 1.C mRNA. It remains possible that the 1.C mRNA is restricted to certain cell types within the prostate. Expression of 1.C mRNA in both PC-3 (Fig. 5) and LNCaP (26) prostate epithelial cells emphasizes the significance of *in situ* hybridization of normal and cancerous prostate tissues in order to identify the source of aFGF mRNA synthesis. Whether the activity of this promoter is connected with androgen-induced tumorigenesis requires further study. In other cell types, aFGF mRNA levels are up-regulated by phorbol esters, serum, and combinations of growth factors (22–24). It would be of interest to identify which promoter(s) is under the control of these regulatory factors.

A homopurine stretch of 72 bp (Fig. 7, nucleotides 915–986) was observed upstream of the 39 bp sequence present in exon -1D. Homopurine stretches are known to favor transition to a triplex state *in vitro* (42–44). This triplex state involves strand looping that exposes a single stranded region of DNA which forms a substrate for single strand specific nucleases such as mung bean nuclease. When supercoiled plasmid containing this region (EcoRI-BglII, Fig. 7) was treated with mung bean nuclease, a sensitive region around the homopurine stretch was observed

(unpublished results). The same plasmid, when linearized with EcoRI prior to incubation with mung bean nuclease, did not show this sensitivity. Several actively transcribed eukaryotic genes have 5' flanking homopurine-homopyrimidine sequences that are S1 nuclease hypersensitive (45–48). We believe that these regions may assume a triple-helical DNA structure, and may be involved in modulation of gene expression. It has been proposed that transcription could be regulated via triplex formation and that the triplex structure may be stabilized by cell-specific protein(s) (49). It is tempting to speculate a similar mechanism of control for expression of aFGF 1.D mRNA.

An understanding of cell differentiation requires the elucidation of the molecular basis for the regulation of tissue-specific gene expression. A more detailed investigation of the human aFGF gene with its remarkable regulation of expression is imperative. Such studies may lead to the identification of development-specific transcription factors and the elucidation of various processes involved in development and differentiation. Characterization of the four distinct promoters of the aFGF gene may provide new insights concerning the molecular mechanisms regarding aFGF-induced tumorigenesis.

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