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Characterization of the Promoter for the Human Antisense Fibroblast Growth Factor-2 Gene; Regulation by Ets in Jurkat T Cells

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Abstract Human lymphoid cells were found to synthesize predominantly antisense, and not sense, fibroblast growth factor-2 (FGF-2) mRNA. Two cDNAs corresponding to human 1069- and 1173-nucleotide antisense FGF-2 mRNAs were cloned from Jurkat T cells. The two cDNAs each possess a unique exon 1 and common exon 2, 3, 4, and 5 sequences. Exon 4 and 5 sequences overlap in the 3′ untranslated region of FGF-2 cDNA, but not in the FGF-2 open reading frame. This is unlike the *Xenopus* antisense FGF-2 homologue, which overlaps with parts of both the FGF-2 3′ untranslated region and its open reading frame. To investigate the regulation of human antisense FGF-2 gene expression, a 2.5-kilobase (kb) promoter region was isolated and characterized. Transient transfection of promoter-luciferase constructs demonstrated the antisense FGF-2 promoter to be active in Jurkat cells. Using transient transfection and in vitro binding assays, specific mutations within the promoter sequence have implicated that Ets-like transcription factors are significant in regulating the human antisense FGF-2 gene in Jurkat cells. J. Cell. Biochem. 72:492–506, 1999. © 1999 Wiley-Liss, Inc.

Key words: FGF-2; antisense RNA; Jurkat; promoter; Ets

Basic fibroblast growth factor (FGF-2) is a multifunctional heparin-binding growth factor that plays important roles in embryonic mesodermal and neuroectodermal development, angiogenesis, and wound healing [Baird and Bohlen, 1990; Basilico and Moscatelli, 1992; Abraham and Klagsbrun, 1996; Bikfalvi et al., 1997]. It is a potent autocrine and paracrine mitogen for numerous cell types, including vascular smooth muscle cells, endothelial cells, and fibroblasts [Baird and Bohlen, 1990]. Basic FGF also acts as a cell migration [Sato et al., 1991] and survival factor [Tamm et al., 1991; Haimovitz-Friedman et al., 1994; Fox and Shanley, 1996] and is active in the stimulation of neurite outgrowth [Walike et al., 1986]. The regulation of FGF-2 gene expression has been observed in various differentiation processes. For example, FGF-2 synthesis is down-regulated during myogenic differentiation of cultured myoblasts [Moore et al., 1991]. An increase in FGF-2 synthesis is associated with several pathological events, such as smooth muscle cell proliferation in atherosclerosis and vascular restenosis [Ross, 1993]. In addition, the proliferation of a number of tumors has been shown to be dependent on endogenous expression of FGF-2, including human melanomas and gliomas [Becker et al., 1989; Morrison, 1991].

The regulation of FGF-2 gene expression has been shown to occur at the levels of transcription, mRNA stability and translation [Prats et al., 1989; Murphy et al., 1990; Winkles and Gay, 1991; Stachowiak et al., 1994; Peifley et al., 1996; Wang et al., 1997]. Post-transcriptional regulation of FGF-2 expression may also occur through the synthesis of an endogenous anti-

Abbreviations used: FGF-2, fibroblast growth factor-2; AS-FGF-2, antisense FGF-2; RT-PCR, reverse transcription-polymerase chain reaction; EMSA, electrophoretic mobility shift assay; kb, kilobase; bp, base pair; aa, amino acid; ORF, open reading frame; UTR, untranslated region.

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sense mRNA to FGF-2, resulting from transcription of the FGF-2 gene in the opposite direction. A 1.5-kb antisense FGF-2 (AS-FGF-2) mRNA was first identified in the amphibian *Xenopus laevis*, where it is present in the oocyte in a 20-fold excess over the FGF-2-encoding 4.5-kb sense mRNA [Kimelman and Kirschner, 1989; Volk et al., 1989]. These two mRNAs overlap in their 3′ ends, including parts of the FGF-2 open reading frame (ORF) and 3′ untranslated region. During *Xenopus* oocyte maturation, it was proposed that the hybridization of sense and antisense mRNAs resulted in the rapid degradation of both mRNAs, suggesting a regulatory role for the antisense mRNA. Natural AS-FGF-2 mRNAs have since been identified in chicken, rat, and human [Borja et al., 1993; Knee et al., 1994, 1997; Murphy and Knee, 1994; Li et al., 1996a].

To date, the regulation of AS-FGF-2 mRNA synthesis has not been investigated, and there has been no identification of a promoter region responsible for the expression of the AS-FGF-2 gene in any species. In this report, we have cloned two AS-FGF-2 cDNAs from human Jurkat T cells and shown their mRNAs to be the predominant form of FGF-2 mRNA expressed in lymphoid cells. We also describe the human AS-FGF-2 cDNAs and for the first time identify and characterize an AS-FGF-2 promoter. A 332-bp promoter region of the human AS-FGF-2 gene was found to be functional in a transient transfection assay in Jurkat cells. One or more members of the Ets-like family of transcription factors were implicated in the positive regulation of AS-FGF-2 gene expression in Jurkat cells in transient transfection and in vitro binding assays, with two specific Ets binding site DNA–protein complexes being identified.

MATERIALS AND METHODS

Materials

Minimum essential media (MEM), RPMI 1640, and all oligonucleotide primers were purchased from Life Technologies (Gaithersburg, MD). A mixture of glutamine, penicillin, and streptomycin (GPS) was purchased from Irvine Scientific (Santa Ana, CA). Fetal calf serum (FCS) was obtained from Intergen (Purchase, NY). Radioactive isotopes and hybridization membranes were purchased from NEN Life Science Products (Boston, MA).

Cell Culture

The human leukemic T-lymphocyte clones Jurkat (clone E6–1) and MOLT-4, and the human histiocytic lymphoma cell clone U-937 were obtained from American Type Culture Collection (ATCC, Rockville, MD) and cultured in RPMI 1640/10% FCS/GPS. Human SK-HEP-1 hepatoma cells (ATCC) were cultured in MEM/10% FCS/GPS with 1 mM sodium pyruvate. Human peripheral T lymphocytes were isolated as previously described [Blotnick et al., 1994].

RNA Isolation and Northern Blot Analysis

Total RNA was prepared from exponentially growing cells using RNAzol B (Tel Test, Friendswoods, TX). PolyA$^+$ RNA was prepared from total RNA using the PolyATtract mRNA Isolation Systems III kit (Promega, Madison, WI); 2 μg of polyA$^+$ RNA was electrophoresed on a 1.2% formaldehyde-agarose gel and transferred to GeneScreen Plus membrane. Hybridization probes were made from cDNA fragments using [α-^{32}P]-dCTP and the Rediprime kit (Amersham, Arlington Heights, IL). The FGF-2 probe was made from a 1290-bp *Pst*I-*Eco*RI fragment of the human FGF-2 cDNA clone which contains the entire ORF, as well as sequences 41-bp and 784-bp 5′ and 3′ of the ORF, respectively [Prats et al., 1989]. The β-actin probe was made from a reverse transcription-polyacrylamide chain reaction (RT-PCR) product, using the specific primers described under Reverse Transcription and Polymerase Chain Reaction Analysis. Northern blots were hybridized in 1 M NaCl, 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, 0.1 mg/ml salmon sperm DNA, 10^6 cpm/ml probe, at 65°C overnight. Filter blots were washed in 2× SSC/0.1% SDS at 65°C and exposed to Hyperfilm (Amersham). RNA sizes were determined using the 0.24- to 9.5-kb RNA Ladder (Gibco-BRL).

Isolation of cDNA Clones

An oligo-dT primed cDNA library was made from 5 μg Jurkat mRNA using the SuperScript Choice System for cDNA Synthesis (Gibco BRL, Gaithersburg, MD). The cDNAs were modified with *Eco*RI (*Not*I) adapters and ligated into the *Eco*RI site of Bluescript KSII($^+$) vector (Stratagene, La Jolla, CA). The cDNA library was transformed into electrocompetent DH5α bacteria, and colonies were transferred to Colony/Plaque Screen nylon membrane. Membranes

were hybridized with the FGF-2 probe (described above) in Rapid-hyb buffer (Amersham) for 2 h at 65°C and washed in 2× SSC/0.1% SDS at 65°C. After exposure to Hyperfilm, positively labeled colonies were identified and isolated. Inserts of clones were sequenced from alkaline-denatured double-stranded plasmid templates by the dideoxy chain termination method [Sanger et al., 1977] with Sequenase 2.0 DNA polymerase (Amersham), using T7 and T3 primers, as well as internally designed primers. The longest cDNA clone consisted of 1023 bp, in addition to a poly-dA tail of 21 bp. A 5′ rapid amplification of cDNA ends (RACE) was used to clone the 5′ end of the cDNA. First-strand cDNA was synthesized from 3 μg Jurkat mRNA with Superscript II RNase H^- reverse transcriptase (Gibco-BRL) and 50 pmol primer A (TTCTGATTTTATACCAGTCTC), which is complementary to nucleotides 30–50 of exon 5 (see Fig. 2). The cDNA reaction mixture was purified and the 3′ end of the single-stranded cDNA was extended with dATP using terminal deoxynucleotidyl transferase (Gibco-BRL). The reaction mixture was purified, and polymerase chain reaction (PCR) was performed on the cDNA using primer B (CCCTTCGGATCCTAACCTTTTTTTTTTTTTTTTT) and primer C (CCTACTTGATGTGAAGCATAT), which are complementary to the poly-dA tail and nucleotides 166–196 of exon 2 (Fig. 2), respectively. The PCR reaction contained 10% of the cDNA, 20 pmol each primer, 0.2 mM dNTPs, and 1 U Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN). Two PCR products of approximately 500 and 400 bp were gel purified, phosphorylated with T4 DNA polynucleotide kinase (New England Biolabs, Beverly, MA), and ligated into a blunt-ended Bluescript KSII(+) vector. Nucleotide and amino acid homologies between cDNAs of different species were determined using the MacDNAsis program.

Reverse Transcription and Polymerase Chain Reaction Analysis

Reverse transcription of 2.5 μg total RNA used Superscript II RNase H^- reverse transcriptase and 200 ng random hexamers (Gibco-BRL). Primer pairs used in PCR analysis were: 5′β-actin (GACCTTCAACACCCCAGCC), 3′β-actin (GCATACCCCTCGTAGATGG), 5′Antisense(AS)Ex2 (CTGCAGTACAGCAATGG), 3′ASEx2 (CCTACTTGATGTGAAGCATAT), 5′FGF-2 (AGTGTGTGCTAACCGTTAC), and 3′FGF-2 (TCTAGGTAAGCTTCACTGG). Primer pairings and expected product sizes are as follows: 5′β-actin+3′β-actin=130 bp; 5′ASEx2+3′ASEx2=195 bp; 5′FGF-2+3′FGF-2=481 bp, including 241 bp ORF and 240 bp 3′UTR. PCR in 30 μl contained 10% of the RT reaction, H_2O or 1 ng of control plasmid as template, 20 pmol of each primer, 0.2 mM dNTPs, and 1 U Taq DNA polymerase, using 25 (β-actin) or 30 (FGF-2, ASEx2) PCR cycles. A total of 5 μl of the completed PCRs was electrophoresed in agarose gels and stained with ethidium bromide.

Isolation of 5′ Genomic Flanking Clones

Two genomic clones containing the 5′ flanking region of the human AS-FGF-2 gene were isolated from size-fractionated human genomic libraries as previously described [White et al., 1988]. A probe of the entire 168-bp exon 1A cDNA sequence labeled a 2.6-kb band in Southern blot analysis of *Pst*I-digested human genomic DNA (data not shown). *Pst*I-digested human genomic DNA (100 μg) was electrophoresed and stained in ethidium bromide; 10 fractions of DNA ranging in size from 2.0 to 3.0 kb were purified from gel slices. Southern blot analysis of 10% of each DNA fraction, using the probe of exon 1A cDNA sequence, identified the genomic DNA fraction, which contained AS-FGF-2 exon 1A sequence. This positive fraction DNA was ligated to *Pst*I-digested Bluescript KSII(+) vector, transformed into bacteria and screened using the exon 1A cDNA probe as described under Isolation of cDNA Clones. Sequencing showed this genomic clone to contain 2639 bp, located between two *Pst*I sites at −2357 and +282 (relative to the exon 1A transcription initiation site). A second genomic clone which contains exon 1B sequence was similarly cloned. This 1.7-kb *Kpn*I-*Xba*I genomic fragment spanned from the *Kpn*I site located at −151 to an *Xba*I site in intron sequence located 0.9 kb downstream the 3′ end of exon 1B. The AS-FGF-2 5′ genomic flanking sequence was analyzed for potential transcription factor binding sites using the TFSearch program in the GenomeNet Database Service.

Primer Extension Analysis

Primer extension analysis was carried out as previously described [Chen et al., 1995]. Primers complementary to specific sequences in exons 1A and 1B were used to map the initiation site for each 5′ exon. Primer D (TTATTTTGTTTGGTAGTTTAA) corresponded to nucleo-

tides 148–168 of exon 1A and primer E (TAACCCTGTGCGCCCGAGGCCCAGCGGT AACCCGCCGAAG) corresponded to nucleotides 99–139 of exon 1B. Each primer was end-labeled with [γ-^{32}P]ATP and hybridized with 30 μg of Jurkat total RNA or yeast tRNA and then subjected to reverse transcription. Primer extension products were analyzed by 6% PAGE. To map the initiation sites of transcription, a sequencing reaction of the promoter clone using primer D, or an unrelated sequencing reaction, was run adjacent to the extension products on the same polyacrylamide gel.

AS-FGF-2 Promoter-Luciferase Activity

All constructs were made using the promoterless luciferase vector pGL2-Basic (Promega). The −1871/+181, −1315/+181, −151/+181, and +189/+647 AS-FGF-2 genomic fragments were isolated from the 2.6-kb *Pst*I-*Pst*I and 1.7-kb *Kpn*I-*Xba*I genomic clones through restriction enzyme digest and PCR manipulations. All genomic fragments were ligated into pGL2-Basic between the *Kpn*I and *Hin*dIII sites upstream of the luciferase gene. Three mutations were made within the −151/+181 construct, using PCR with primers spanning the site of each mutation that possesses altered sequence, as well as primers flanking the ends of the −151/+181 sequence. Sequence mutations are as follows: Ets (−90/−83) ACTTCCGG to AGAATTCC; E-box (+30/+35) CAGCTG to GGATCC; GATA (+55/+60) AGATAG to ATTCCG. The 2.0-kb FGF-2 promoter-luciferase construct was previously described [Chen et al., 1995]. PGK-*lac*Z, a β-galactosidase reporter plasmid, was used to correct for transfection efficiency. Endotoxin-free promoter-luciferase construct DNAs were made using the EndoFree Plasmid Maxi Kit (Qiagen, Santa Clarita, CA) and multiple plasmid preparations of each construct were used in transient transfection assays with the SuperFect Transfection Reagent (Qiagen). For each individual transfection, 2×10^6 exponentially growing Jurkat cells were washed once in PBS and seeded in 3 ml RPMI/10% FCS/1% GPS in a 6-well dish. A promoter-luciferase construct plasmid (4-μg) and PGK-*lac*Z (0.4 μg) were brought to a total volume of 150 μl in RPMI without FCS or GPS. The DNA was mixed with 10 μl SuperFect Reagent, incubated 15 min at room temperature, and added to the cells. Cells were harvested 48 h later and analyzed for luciferase activity using the Luciferase Assay System (Promega) and a luminometer (BioOrbit 1251; Pharmacia Biotech, Piscataway, NJ). The β-galactosidase activity was measured using the β-Galactosidase Enzyme Assay System (Promega) to correct for transfection efficiency.

Nuclear Extract Preparation

Nuclear extract preparation and electrophoretic mobility shift assay (EMSA) was performed as previously described [Lambert et al., 1997] with the following modifications. Exponentially growing Jurkat cells (5×10^7) were washed 3× in ice-cold phosphate-buffered saline (PBS), resuspended in 0.4 ml Buffer A (10 mM Hepes pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, and protease inhibitors), and incubated 15 min on ice. After the addition of 25 μl Nonidet-P-40 (NP-40) and vortexing for 10 s, nuclei were pelleted for 2 min at 4,000 rpm, 4°C, and washed once in Buffer A without NP-40. The nuclear pellet was resuspended in 150 μl Buffer C (50 mM Hepes pH 7.8, 50 mM KCl, 300 mM NaCl, 1 mM DTT, 10% glycerol, and protease inhibitors) and incubated for 30 min at 4°C while rocking. Nuclear debris was pelleted 5 min at 14,000 rpm, 4°C and the nuclear extract supernatant was collected, aliquoted and stored at −80°C. The protein concentration of the extract was determined with the BioRad Protein Assay.

Electrophoretic Mobility Shift Assay

Three pairs of complementary strands of oligonucleotides were synthesized to form DNA hybrids which possess 5′ overhangs on each end as described in Figure 7A. Each DNA hybrid consisted of AS-FGF-2 promoter sequence from −103 to −74, with Ets WT DNA representing unaltered promoter sequence, and Ets MutA and MutB DNAs containing mutations in the Ets consensus binding site. Oligonucleotide pairs were annealed to form DNA hybrids and were desalted. DNA probes were labeled with [α^{32}P]dCTP using Klenow DNA polymerase in a fill-in reaction. Unlabeled competitor DNA hybrids in 1 μl were mixed with 17 μl of the following mix: 10 mM Hepes pH 7.9, 2.5 mM DTT, 50 mM KCl, 0.2 mM EDTA, 0.05% NP40, 10% glycerol, 50 μg/ml polydI-dC, and 2 μg Jurkat nuclear extract. After a 10-min incubation at 25°C, 2 μl of probe (2×10^4 cpm) was added, and the reaction was incubated for another 30 min at 25°C. Samples were placed on ice, 2 μl of 40% glycerol/dye solution was added

and the samples separated by 6% native PAGE at 180 V for 2.5 h at 4°C. The gel was dried and exposed to Hyperfilm.

RESULTS

Cloning of Human Antisense FGF-2 cDNA

Northern blot analysis of FGF-2 mRNA synthesis in the human Jurkat T-cell line, using a probe which contains ORF and 3′UTR cDNA sequences, identified a single mRNA of approximately 1.1 kb (Fig. 1A). By contrast, human SK-HEP cells expressed four FGF-2 mRNAs of 7.0, 3.5, 1.8, and 0.9 kb, which have been reported previously for these cells [Prats et al., 1989]. The size of the FGF-2 mRNA synthesized by Jurkat cells is similar to the reported size of the human antisense FGF-2 (AS-FGF-2) mRNA of 1.5 kb [Knee et al., 1994; Murphy and Knee, 1994]. The identity of the 1.1-kb Jurkat mRNA as AS-FGF-2 mRNA was confirmed through the cloning of its cDNA (Fig. 2). A 1044-bp cDNA, including a poly-dA tail of 21 bp, was isolated after the screening of a Jurkat polyA$^+$ cDNA library with the FGF-2 probe. A 5′ rapid amplification of cDNA ends (5′RACE) yielded two cDNA products of different sizes (data not shown), and sequence of these clones indicated the existence of two 5′ exons, 1A and 1B (Fig. 2). The original 1044-bp cDNA clone included nucleotides 47–168 of exon 1A. A 3′RACE identified a single 3′ end for this mRNA (data not shown). Thus, two AS-FGF-2 mRNAs are synthesized in Jurkat cells as a result of alternative splicing, each consisting of five exons. Both share exons 2–5 and differ only in the first exon, either 1A or 1B, resulting in cDNAs of 1069 and 1173 bp, respectively. Exons 1A and 1B are separated in the AS-FGF-2 gene by 174 bp (sequence shown in Fig. 4). Exon 4 and 5 sequences are complementary to previously described sequences in the 3′UTR of the sense FGF-2 cDNA, which are separated by 4395 bp [Prats et al., 1989], suggesting the 4395-bp sequence is an intron of the AS-FGF-2 gene.

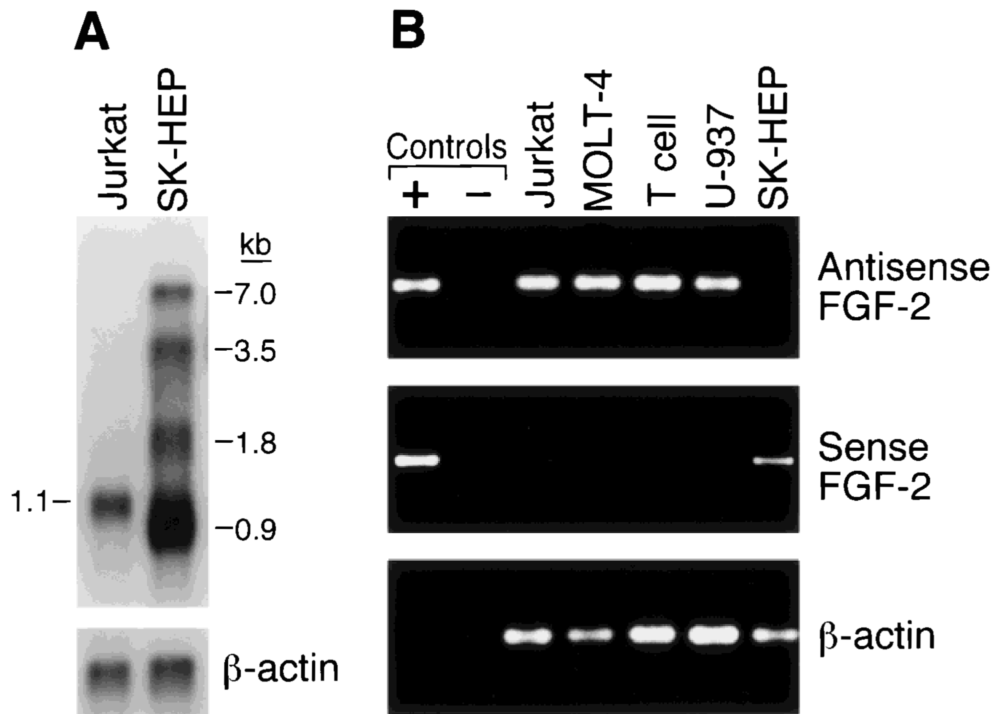

Fig. 1. Synthesis of FGF-2 sense and antisense mRNAs in lymphoid and SK-HEP cells. **A:** Northern blot analysis of polyA$^+$ RNA isolated from Jurkat and SK-HEP cells. The probe was synthesized from an FGF-2 cDNA sequence containing the human FGF-2 open reading frame (ORF) and a partial 3′ untranslated region (UTR). To control for equal RNA loading, the blots were stripped and reprobed with a β-actin cDNA probe (bottom). **B:** RT-PCR analysis of total RNA isolated from Jurkat, MOLT-4, peripheral blood T cells, U-937, and SK-HEP cells. PCR was performed with primers specific for AS-FGF-2, FGF-2, and β-actin cDNAs. AS-FGF-2 primers generate a product of 195 bp within exon 2, thus amplifying cDNAs possessing either exons 1A or 1B. (+) control indicates the template was a plasmid containing either FGF-2 or AS-FGF-2 cDNA sequence instead of RT product. No β-actin plasmid was available, therefore its (+) lane was empty. (−) control indicates no template was present in the PCR reaction.

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Ex 1A  AGCAGTCTTTGAAACAGCTGTAACGGCATCTGTGAAAGAAGATAGGTTCCAGGAACGGAA   60
       CTGCCACTTAGATTGTAAATTCCTGAAAAACAGGACGTTTTTGCATCTCCTCCCGGCTTC  120
       CCCATCCCTAAACCAACGTCTGTTGAATTAAACTACCAAACAAAATAA              168

Ex 1B  CATTTGGGCAACGGACGAATCAAGCGGTGTGGAGATGCGGCAGCCGCTGAGCTGGGGCCG   60
       CTGGCGCGCGATGCTTGCCCGAACCTACGGCCCCGGGCCTTCGGCGGGTTACCGCTGGGC  120
       CTCGGGCGCACAGGGTTACGTGCGGAATCCGCCAGTTGGAGCGTGCGATCTGCAGGGCGA  180
       GCTGGACAGATTCGGGGGCATCTCGGTGCGCCTGGCGCGGCTCGATGCGCTGGACCGCCT  240
       GGACGCTGCCGCCTTCCAGAAGGGCTTGCAGG|CTGCAGTACAGCAATGGCGATCAGAAGG  300
Ex 2   TAGAACAGCTGTATGGCTGCACATTCCCATCCTCCAAAGCCGATTTATTGCCCCTGCTGC  360
       TTCCCTGGGCTTCTGCTTTCACCACGCAGAATCGGATTCATCAACGTTGACTCTGTGGCT  420
       GAGAGAAGGGCCCAGCAGATTACCAGGATATGCTTCACATCAAGTAGGAGTTGCAG|GAGC  480
Ex 3   TGTATTTGATGAAAGTACTAGAAAAATACTGGTTGTACAAGATCGAAATAAA|TTGAAAAA  540
Ex 4   TATGTGGAAGTTTCCAGGAGGCCTGTCAGAGCCTGAAGAAGATATTG|GAGACACAGCGGT  600
Ex 5   TCGAGAAGTTTTTGAAGAGACTGGTATAAAATCAGAATTCAGGTCCGTCCTGAGTATTCG  660
       GCAACAGCACACAAATCCTGGAGCTTTTGGGAAGTCAGATATGTATATCATCTGCCGCCT  720
       AAAGCCATATTCATTCACCATAAATTTTTGCCAGGAAGAATGCTTAAGATGTGAGTGGAT  780
       GGATCTCAATGACCTGGCGAAGACTGAAAATACAACTCCCATCACCAGCAGAGTTGCTAG  840
       GCTGCTGCTGTATGGGTACAGAGAAGGGTTTGACAAAATTGACCTGACTGTGGAAGAACT  900
       TCCAGCAGTTTACACAGGACTGTTTTATAAACTCTATCATAAGGAACTGCCAGAGAATTA  960
       TAAAACTATGAAAGGAATTGATTAAATTCACATTTATATGTTTAGAAACATGTAGACTAA 1020
       CGAATGACATAAGAAATAGTGGACATTTTGGATTGATTGATTAAACATCTGACTGTGATT 1080
       TTCTAATGTATATGATTTCCATGAAGAAATTTTGTTTCTAAACATGCACATTTTAAAAGC 1140
       CTCTTTTCGAATAAAGCAAATGCGTGAAAAAGA                            1173
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Fig. 2. Human antisense FGF-2 cDNA sequence. Individual exons are separated by boxes. Two cDNAs of 1069 bp and 1173 bp were isolated, which possess either exon 1A or exon 1B sequences, respectively. Both cDNAs share exons 2, 3, 4, and 5. The identities of exons 1A, 1B, and 2 as individual exons were verified through sequencing of genomic clones (data not shown). Translation start (ATG) and stop codons (TAA) of an ORF encoding a potential 35-kDa peptide are underlined. The cleavage/polyadenylation site (AATAAA) is indicated with a double underline. The polyA tail in the cDNA product is not included as exon sequence.

Differential Synthesis of Sense and Antisense FGF-2 mRNAs in T Cells and Other Cell Lines

The cloning of AS-FGF-2 cDNAs from Jurkat cells suggests that the 1.1-kb mRNA identified in Northern blot analysis (Fig. 1A) is the AS-FGF-2 mRNA(s) and that these cells do not accumulate FGF-2 mRNA. In order to confirm this possibility, we used RT-PCR analysis to investigate the synthesis of both AS-FGF-2 and FGF-2 mRNAs in these and other lymphoid cells (Fig. 1B). Primer pairs used in the PCR were specific for either AS-FGF-2 or FGF-2 cDNA sequences. AS-FGF-2 specific primers could amplify only AS-FGF-2 exon 2 sequence, thereby amplifying both forms of AS-FGF-2 cDNA. Human peripheral blood T lymphocytes, Jurkat and MOLT-4 T cells, and U-937 macrophage-like cells were found to synthesize AS-FGF-2 mRNA, whereas FGF-2 mRNA was undetectable (Fig. 1B). By contrast, RT-PCR analysis of SK-HEP cells demonstrated relatively high levels of FGF-2 mRNA and barely detectable levels of AS-FGF-2 mRNA (Fig. 1B). These patterns are consistent with attributing

the Northern blot analysis in Figure 1A to AS-FGF-2 mRNA alone accumulating in Jurkat cells and to FGF-2 mRNA alone accumulating in SK-HEP cells. PCR analysis using primers that amplify AS-FGF-2 cDNA containing either exon 1A or 1B sequence produced the same RT-PCR profile as when using the AS-FGF-2 exon 2-specific primers, suggesting that both forms of AS-FGF-2 mRNA are synthesized in these lymphoid cells (data not shown).

Comparison of Human and *Xenopus* AS-FGF-2 cDNAs

Transcription of the overlapping human AS-FGF-2 and FGF-2 genes results in the synthesis of mRNAs which overlap in their 3′ ends (Fig. 3). There are two regions of overlap between the AS-FGF-2 and FGF-2 cDNAs, which occur only in the 3′UTR but not in the ORF of the FGF-2 cDNA. The 3′ end of the *Xenopus* AS-FGF-2 cDNA is also complementary to two regions of the 3′UTR of the *Xenopus* FGF-2 cDNA [Volk et al., 1989] (Fig. 3). In *Xenopus*, however, the region of AS-FGF-2 exon 4 overlap includes exon 3 of the FGF-2 ORF. The *Xenopus* cDNA is reported to possess a single exon 1, which is homologous with human exon 1B, and both exons 1B and 2 are homologous with the *Xenopus* exon 1 sequence. The human AS-FGF-2 cDNA shares 69% and 80% nucleotide identity with the *Xenopus* and rat homologues, respectively. The *Xenopus* and rat AS-FGF-2 cDNAs possess ORFs that predict translation products of 24 and 35 kDa, respectively [Kimelman and Kirschner, 1989; Volk et al., 1989; Li et al., 1996b]. The human AS-FGF-2 ORF of 316 amino acids initiates within exon 1B sequence, predicting a translation product of 35 kDa, which is 75 and 83% homologous with *Xenopus* and rat clones. No potential ORFs are initiated within exon 1A. Both *Xenopus* and rat ORFs contain sequence homologous to the MutT domain of dNTP hydrolases [Li et al., 1996b; Knee et al., 1997], and this domain is conserved in the human ORF.

Isolation and Characterization of the Human AS-FGF-2 Promoter

The differential synthesis of AS-FGF-2 and FGF-2 mRNAs may be regulated at transcriptional or post-transcriptional levels. We therefore initiated a study of the transcriptional regulation of the human AS-FGF-2 gene. A 2.5-kb fragment of 5′ flanking genomic region, which contains exons 1A and 1B, and upstream sequence, was isolated and sequenced (Fig. 4). The initiation sites of transcription for exons 1A and 1B were determined in primer extension analyses using primers specific for either exon 1A or exon 1B (Fig. 5). Using a primer specific for exon 1A, a single product was present when Jurkat total RNA was used as template. This indicated a single transcription start site located 15 bp upstream of exon 1A (Fig. 4, double underlined). Likewise, a primer specific for exon 1B indicated a single transcription start site in Jurkat cells located 84 bp upstream of 1B exon (Fig. 4, double underlined).

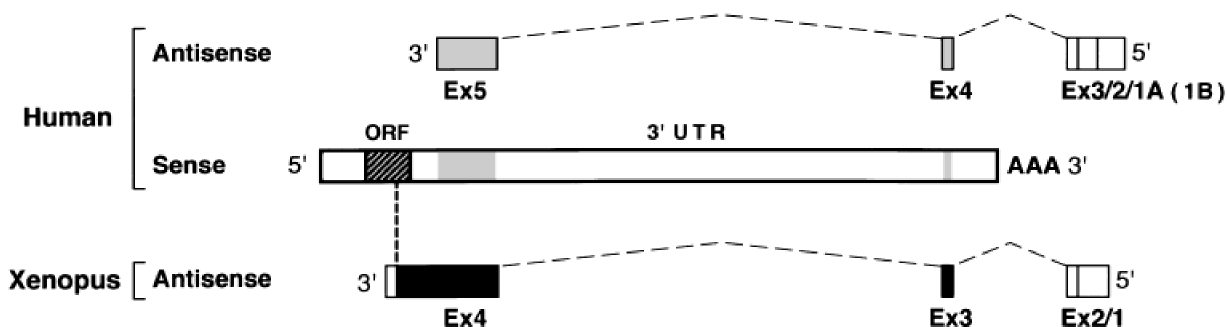

Fig. 3. Regions of overlap between AS-FGF-2 and FGF-2 cDNAs in human and *Xenopus*. The 6.75-kb human FGF-2 cDNA is shown in a 5′ to 3′ orientation. The 1.1-kb human and 1.5-kb *Xenopus* AS-FGF-2 cDNAs are shown above and below, respectively, in the opposite orientation with exons 4 and 5 of the human cDNA and exons 3 and 4 of the *Xenopus* cDNA aligned with complementary regions in the FGF-2 cDNA. The open reading frame (ORF) of FGF-2 cDNA is indicated with diagonal lines. Exons 4 and 5 of human AS-FGF-2 cDNA and complementary regions of FGF-2 cDNA are shaded. The 586-bp human AS-FGF-2 exon 5 overlaps in the FGF-2 3′UTR, with its 3′ end located 247 bp from the 3′ end of the FGF-2 ORF. Regions of *Xenopus* AS-FGF-2 exons 3 and 4, which are complementary with FGF-2 cDNA sequence are solid. *Xenopus* AS-FGF-2 exon 4 overlaps with 716 bp of 3′UTR and 180 bp of ORF of the FGF-2 cDNA, as well as 114 bp of intron 2 sequence of the FGF-2 gene. Dashed lines between antisense exons indicate splicing of intron RNA.

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-1871 TCTAGAAAATCATTTTTACT GTATTTTAGAAAAGTGCCGA TCTGTGACCTATTATAAATT TTAAAGTCTTCACCACAGAG AGCTTGAGAAGCATTTGGCT
                                 SRY
-1771 AAAAGGCAACCACATCAAGG CGCAAAAACAAAAGATTTAC AAATATAAATCTGACACTGA AGAGACTATAGATGGGAAAC TCTGGGCACTGGATGAACTT
-1671 GCCAAGGAGACAGGATTTAT TGAGAGGAAAAAAAAGGTTT TGGACTAAGAATTGACGAAC TTCAACATTTAATGGTCAAG GGAAGAGGACGAGCCTGCAA
                                                                       Nkx-2.5                          Sox-5
-1571 AGAAAAATGATGAAACATCA ACAAAGATGAAGAAAACCAG GCAAGTTTGACAACACATAA GAAGTTAAGTGTTTCGAGAA GGAGGGAATGGTCAACAATA
-1471 CCAAAAGTTGCTGTGGTCAC GTAAGGTTTATCAACACGGA GATGCTCGGAGACCACACCA GGAACAGTTTCCATGGAATG ATGGGGTGGAAGTCAAAGAG
-1371 GAATAGGCTAGGGAGAGTAT GTAAGTGGTGAGGCGGCAGA GACCACTCCATTTAAGAAGC TTGGCAATGGATAGAGAGAC GGTTGCAGGAAGAAAATGTG
-1271 GAGCTTTTGTTCTGATTTAA GATGGAAGAGAAATGAGCAT GTTTACATGTCAATGGGAAG GATCTAGTTCAAAAACAAGT TGAACCTTCAGGACAGAACA
-1171 AGTTGTCCTCTGATACCTAA GGCAGAGTGAAAATACCATG TTAACTCTAAATTGTCTTTA AGGTCTGATTAGACATAAAA GTTTTTGCCACTAGGTCTGG
-1071 TCCATTTTTCAAAGCTATTA CACATCAAATATAAATTTTC AATTTTTAATTCAATTTATA AGACTTTTCAGTATAAATAC GTTCTCCCAAATCAAAGGAG
                                                                        E-box                LyF-1
 -971 CTGTCATCTAGGAATGGCTT TTAAGTCAAACACATCTGGA CTCAAATATCGGCTCTGCCA CTTAGCACCTGTGTAGCCTG AATGGAATCACGTTAACTTT
 -871 CCTCCCTCTATAAAACTGTC ATTACTATACCCATCGGTCA CGGCTTCTGTAGAAATTAGA TGAGCTATATATACTGCTAA CACTAGTGTGCAGTTACTAG
 -771 CAGTTATTAAATATCATAAC CTGCGTAGTACTGGAAGAAA ATGCTATCATCACTCCTAAG GCAGAGTGATCCACATTTTT ACTGAATTGGTTATTTAGTT
          GATA                        Sox-5   C/EBP                                          Nkx-2.5        SRY
 -671 TTTTAGGATAAGTATCCCAT GTTCGCTAGTTATGAACAAT TTTGCAATTAAATGTGTCGT TAATGTTAAATGAGACAAGC GTTTCAAGTGCAACTTAAAA
 -571 ATTCAAAATAATTTACAAAA GAGAAACCTTGGACTCGGAT AAACCATTTCAGCCTAACTT CTCCAGTGCAGGCGCGGCTA CGTTTGCATGCTTCTTACAT
                                                                                                        Sp1
 -471 ACAGAAGTCTTACACGCTTC TGTGCCGCTCTCAGACCCAT GCCACGCCCAACTTTCAAAA GTCTCTGCACCCCAAGTCAG TATCACTGAGTCTCCCGCCC
 -371 CTCAGGTTGCGCCCCCTCGG CCTCTAGTCTCCCACCCGGA ATTCTTTACCCTTTTCTAAT AAGTTGGTCACCGTCAGAGT CCCGGAGGTTGCCGCGAAGT
 -271 CTGATCCAGCAGAAGGAGCC CGTGCCTCCGCACAAGAGGA AGCAGCAGAGGGCAAGGACG AACCATTTTCCGCGCTTTGG TTCAACCGCTTTCTATTCTT
                                          Ets                                                   Ets
 -171 CTTGGAAGACATGGTCACAA GGTACCCTAGCCGAAGCAGT AGAAAAGCCGACTCAATGTG CGCGCTTCAACTGAGAGAAA AACTTCCGGGGCAGAAGTCA
          Sp1
  -71 GCGAGGGTCCGCCCCTGCGC CGTAATCCCCTGAGTGGAGC GCAGCAGTGCACAGCGTGGT GGGAGGGACTGAGCGTTTTC AAAACCAGCAGTCTTTGAAA  Exon
  +30 CAGCTGTAACGGCATCTGTG AAAGAAGATAGGTTCCAGGA ACAACTGCCACTTAGATTGT AAATTCCTGAAAAACAGGAC GTTTTTGCATCTCCTCCCGG   1A
       E-box                   GATA      SRY        SRY
 +130 CTTCCCCATCCCTAAACCAA CGTCTGTTGAATTAAACTAC CAAACAAAATAAGTGAGTGG CGGTGGGGGAGGAGGTTTTC CCCGCTTAACTGGAGCGGGG
 +230 CAAATTGCTGAGAAGGGCTG GTGGACCACTTCGCGGTCTC CTTGGCATAGCTCTGCAGAG TGGTAACCCCAGCTCAACTC TGAGGGTATTGATGGCACTC
 +330 TTCTAAAATGTACGGCCGCG ATCTCCGCTCTGGAGCGCGG AGGGGTCATTTGGGCAACGG ACGAATCAAGCGGCGTGGAG ATGCGGCAGCCGCTGAGCTG
 +430 GGGCCGCTGGCGCGCGATGC TTGCCCGAACCTACGGCCCC GGGCCTTCGGCGGGTTACCG CTGGGCCTCGGGCGCACAGG GTTACGTGCGGAATCCGCCA  Exon
 +530 GTTGGAGCGTGCGATCTGCA GGGCGAGCTGGACAGATTCG GGGCATCTCGGTGCGCCTG GCGCGGCTCGATGCGCTGGA CCGCCTGGACGCTGCCGCCT   1B
 +630 TCCAGAAGGGCTTGCAGG
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Fig. 4. Nucleotide sequence of the 2518 bp human genomic AS-FGF-2 5′ flanking region. Exon 1A and 1B cDNA sequences are shaded. Transcription initiation sites upstream of exons 1A and 1B are double underlined. Nucleotide sequences are numbered on the left and are relative to the transcription initiation site upstream of exon 1A. Potential transcription factor binding sites are indicated with a line above or below the sequence and labeled.

The sequence of the 2.5-kb promoter region was analyzed for the presence of specific sequences known to be binding sites for transcription factors (Fig. 4). No consensus TATA boxes or CCAAT elements were located near either transcription start site, although two Sp1 binding sites were found at −372 and −58, relative to the exon 1A transcription initiation site. Potential binding sites for transcription factors known to be important for the regulation of lymphocyte-specific genes and for T-cell development were found within this promoter region. These included binding site sequences for Ets (−229, −83), GATA (−662, +56), and Lyf-1 (−981) transcription factors [Lo et al., 1991; Nye et al., 1992; Merika and Orkin, 1993]. Other potential DNA regulatory sites that may be involved in expression in various other cell types were identified in this promoter region. There are two consensus E-boxes (−901, +30), which are binding sites for members of the MyoD family of skeletal muscle transcription factors [Blackwell and Weintraub, 1990]. In addition, consensus binding sites for the cardiac transcription factor Nkx-2.5 (−1501, −582), the liver/adipose tissue-specific C/EBP factor (−624), the testis determination factor SRY (−1740, -671, +163, +171) and Sox-5 (−1472, −632), which is involved in spermatogenesis were found [Denny et al., 1992; Harley et al., 1994; Johnson and Williams, 1994; Chen and Schwartz, 1995].

Transcriptional Activity of the Human Antisense FGF-2 Promoter in Jurkat Cells

To determine whether the 5′ flanking region of the AS-FGF-2 gene is active as a promoter, promoter-luciferase constructs were prepared and used in transient transfection assays in Jurkat cells (Fig. 6). A series of 5′ deletion promoter fragments, which terminate at the 3′ end of exon 1A, were fused upstream of the luciferase reporter gene. Construct −151/+181 had nearly full promoter activity, as active as the full-length −1871/+181 construct, both about 13-fold more active than a promoterless construct. A negative regulatory element (NRE) was identified between −1871 and −1315, as the −1315/+181 construct exhibited 50% more promoter activity than the −1871/+181 construct. In contrast to the −151/+181 promoter-

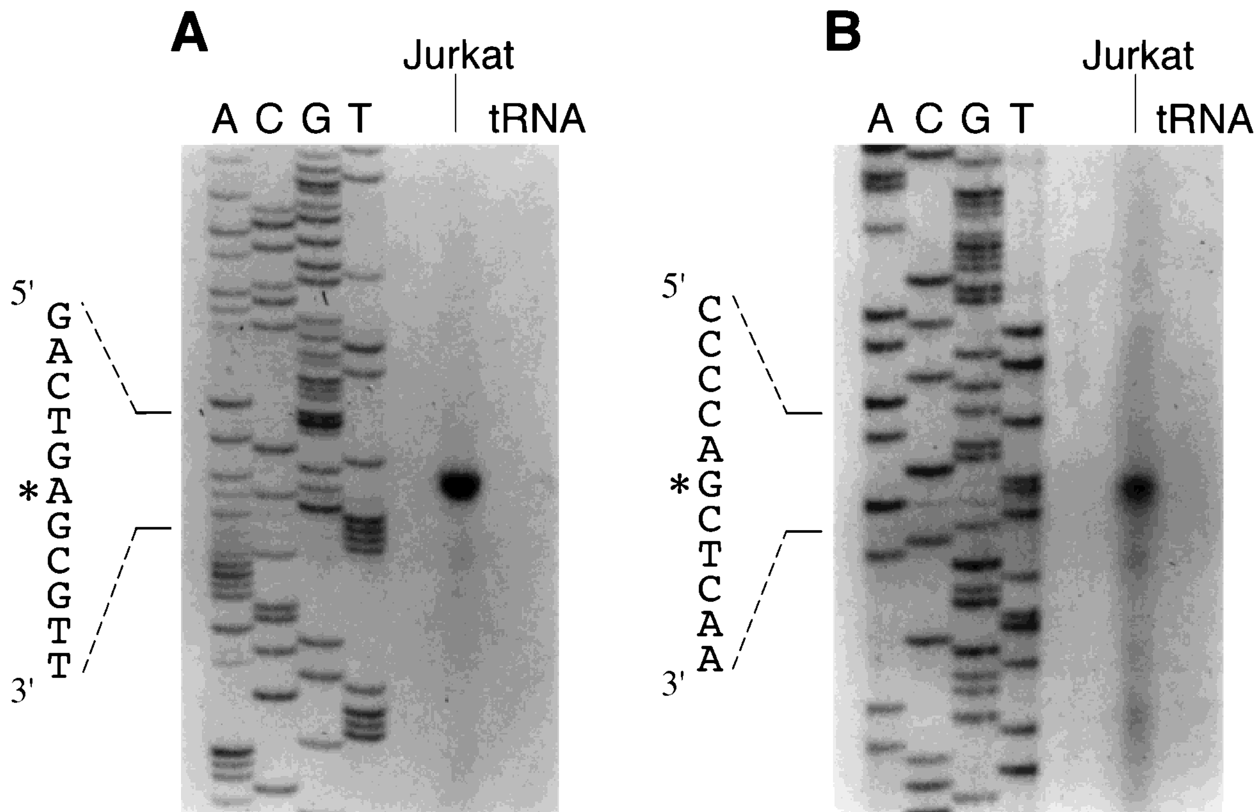

Fig. 5. Primer extension analysis of the human AS-FGF-2 mRNAs. Total RNAs from Jurkat cells or yeast tRNA were analyzed using synthetic oligonucleotides specific for exons 1A **(A)** or 1B **(B)**. Sequence reactions of human AS-FGF-2 genomic DNA using the same exon 1A primer were run in parallel. Unrelated sequence in B is used to determine the size of the exon 1B primer extension product. Asterisks, transcription initiation sites for exons 1A and 1B.

luciferase construct, the +189/+647 construct that includes exon 1B, as well as 194 bp of the upstream sequence, had no promoter activity in Jurkat cells. A 2.0-kb FGF-2 promoter-luciferase construct was only 20% as active as the −151/+181 AS-FGF-2 construct in these cells. Within the active 332-bp AS-FGF-2 promoter fragment located between −151 and +181 are potential transcription factor binding sites for Ets, GATA, and MyoD. To determine whether any of these sites are active in Jurkat cells, point mutations were introduced into these sequences to inhibit transcription factor binding activity (Fig. 6). The ΔEts, ΔGATA, and ΔE-box binding site mutations resulted in 76%, 33% and 17% inhibitions, respectively, in the promoter activity of the wild-type 151/+181 construct. These results suggest a prominent role for Ets-like transcription factors in the regulation of the human AS-FGF-2 gene in Jurkat cells.

The Proximal Ets DNA Binding Site Forms Two Specific Complexes With Jurkat Nuclear Extract

The presence of proteins in Jurkat nuclear extract that can bind the proximal putative Ets binding site (−90/−83) was investigated. EMSA using a 30-bp double-stranded DNA probe containing sequence from −103 to −74, which includes the wild-type Ets binding site (Ets WT, Fig. 7A) formed two complexes with Jurkat nuclear extract (Fig. 7B, lane 2). The presence of 10- and 50-fold molar excess of unlabeled Ets WT DNA in the binding reaction inhibited the formation of both complexes (Fig. 7B, lanes 3 and 4, respectively). Two DNA probes spanning the same promoter region but containing different point mutations (Fig. 7A) were constructed to analyze the contribution of the Ets binding site sequence in the formation of both complexes. Both Ets MutA and MutB probes failed to form any complex with Jurkat nuclear extract (Fig. 7B, lanes 9 and 10, respectively). In

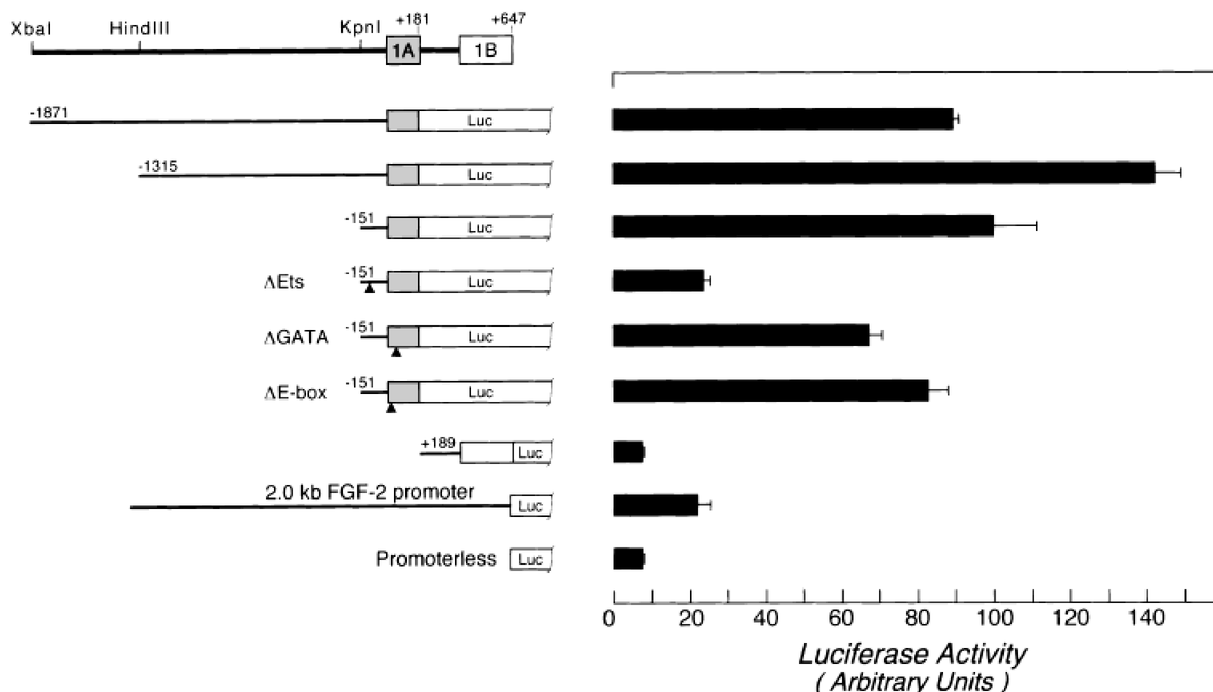

Fig. 6. Expression of human AS-FGF-2 promoter-luciferase constructs in Jurkat cells. The 5′ ends of the promoter constructs correspond to *Xba*I (−1871), *Hin*dIII (−1315), and *Kpn*I (−151) sites. The AS-FGF-2 promoter fragments terminate at the very 3′ end of exon 1A (+181) or exon 1B (+647). The 5′ end of the +189/+647 construct begins 5 bp downstream from the end of exon 1A. Positions of point mutations within the −151/+181 construct indicated by solid triangles are Ets (−90/−83), GATA (+55/+60) and E-box (+30/+35). Luciferase activities were normalized to β-galactosidase activity in each extract, and the activity of the −151/+181 construct was established as 100. Each value is the average of either 4 (−1871/+181; +189/+647; 2.0 kb FGF-2 promoter) or 7 (−1315/+181; −151/+181; ΔEts; ΔGATA; ΔE-box; Promoterless) independent transfections, and standard deviations were determined.

addition, an excess of unlabeled Ets MutA and MutB DNAs would not compete with the formation of the two complexes with the Ets WT probe (Fig. 7B, lanes 5–8).

DISCUSSION

We have demonstrated that human T cells synthesize 1.1-kb and 1.2-kb antisense FGF-2 mRNAs that differ in their 5′ ends and have cloned their cDNAs from Jurkat cells. In addition, we have isolated and characterized its promoter region. This is the first description of an AS-FGF-2 promoter in any species. Although a number of endogenous eukaryotic antisense mRNAs have been described [reviewed in Knee and Murphy, 1997], to our knowledge the identification of antisense promoters has been limited to the human eIF-2α, human WT1, and mouse c-*myc* genes [Silverman et al., 1992; Spicer and Sonenshein, 1992; Noguchi et al., 1994; Malik et al., 1995]. The human FGF-2 gene and promoter were previously characterized [Shibata et al., 1991], and the FGF-2 and AS-FGF-2 promoters are separated by at least 44 kb. This relatively large distance of separation is quite unlike the other eukaryotic antisense promoters located in introns of the sense gene and often overlap with sense promoters [Silverman et al., 1992; Spicer and Sonenshein, 1992; Noguchi et al., 1994; Malik et al., 1995].

The FGF-2 and AS-FGF-2 promoters are similar in that they are both TATA-less, contain a negative regulatory element (NRE), as well as Sp1 and Ets binding site sequences [Shibata et al., 1991; Erdos et al., 1995]. By contrast, we have identified potential binding sites for transcription factors in the AS-FGF-2 promoter that have not been reported in the FGF-2 promoter. These include proteins expressed in T cells such as Lyf-1 and GATA factors [Lo et al., 1991; Merika and Orkin, 1993], as well as factors active in other cells, including C/EBP, MyoD, Nkx-2.5, Sox-5, and SRY [Blackwell and Weintraub, 1990; Denny et al., 1992; Harley et al., 1994; Johnson and Williams, 1994; Chen and

A

Ets WT CTGAGAGAAAAACTTCCGGGGCAGA
TCTTTTTGAAGGCCCCGTCTTCAGT

Ets MutA CTGAGAGAAAAAGAATTCCGGCAGA
TCTTTTTCTTAAGGCCGTCTTCAGT

Ets MutB CTGAGAGAAAAACTTGGGGGGCAGA
TCTTTTTGAACCCCCCGTCTTCAGT

B

Probe: Ets WT MutA MutB

Competitor: (−) WT MutA MutB (−)

Complex 1

Complex 2

1 2 3 4 5 6 7 8 9 10

Fig. 7. Ets-like binding site forms two complexes with Jurkat nuclear extract. **A:** Oligonucleotide sequences used to generate ^{32}P-labeled double-stranded DNA probes and unlabeled competitor DNAs in EMSA. Hybridized oligonucleotide pairs form 30-bp DNAs, which span the AS-FGF-2 promoter sequence from −103 to −74. The GGAA Ets-like binding site core in the wild-type (WT) sequence, in the reverse orientation, is overlined. Nucleotides mutated from wild-type sequence in the Ets MutA and MutB oligonucleotides are shaded. The Ets MutA sequence is identical to the ΔEts binding site mutation used in the promoter-luciferase assay (Fig. 6). **B:** EMSA analysis of Ets WT, MutA, and MutB DNA probes with Jurkat nuclear extract. **Lane 1**, no extract. Unlabeled competitor DNAs were added in 10-fold (**lanes 3**, **5**, **7**) and 50-fold (**lanes 4**, **6**, **8**) molar excess over probe DNAs. Two specific complexes formed with the Ets WT probe are labeled.

Schwartz, 1995]. Furthermore, while the FGF-2 promoter is regulated by AP-1 and Egr-1 transcription factors [Wang et al., 1997; Shibata et al., 1991; Erdos et al., 1995; Biesiada et al., 1996], their binding sites were not identified within the AS-FGF-2 promoter region.

The AS-FGF-2 promoter region was found to be active in Jurkat cells using transient transfection assays with promoter-luciferase constructs. Of the characterized 2.5 kb genomic fragment, the 332-bp genomic sequence between −151 and +181, relative to the exon 1A transcription initiation site, was found to be sufficient for nearly full promoter activity. A 2.0 kb FGF-2 promoter-luciferase construct was 20% as active as the −151/+181 AS-FGF-2 construct, consistent with the undetectable FGF-2 mRNA accumulation in Jurkat cells.

The proximal Ets binding site was shown directly to be involved in Jurkat AS-FGF-2 gene regulation by making point mutations in this binding site. This mutation resulted in 76% lower transcriptional activity compared with the unaltered promoter sequence, suggesting a prominent role for one of the Ets-like proteins in the regulation of the AS-FGF-2 gene. The

loss of AS-FGF-2 promoter activity with the ΔEts point mutation correlated with a loss of formation of two specific complexes when the identical (Ets MutA) or alternative (Ets MutB) mutation was used in an EMSA. Many Ets-like transcription factors are expressed in T cells, are important in the regulation of T-cell-expressed genes, and are involved in T-cell development, activation, and proliferation [Wasylyk et al., 1993; Tymms and Kola, 1994; Bories et al., 1995; Muthusamy et al., 1995]. Ets-like proteins can bind DNA alone or in conjunction with a number of accessory factors, including TFIID. The proximal Ets binding site (−90/−83) in the AS-FGF-2 promoter may recruit TFIID in the formation of the transcription initiation complex, as Ets binding sites have been found near the transcription initiation site in many TATA-less promoters [Jolliff et al., 1991]. The proteins involved in the formation of the two specific complexes with the Ets binding site have not been identified, but may include accessory factors associated with the Ets-like transcription factor(s).

Antisense mRNA to FGF-2 was first identified in *Xenopus* and subsequently in chicken, rat, and human [Kimelman and Kirschner, 1989; Volk et al., 1989; Borja et al., 1993; Knee et al., 1994, 1997; Murphy and Knee, 1994; Li et al., 1996a]. A partial human AS-FGF-2 cDNA sequence has been previously reported that is different from our sequence, but upon examination appears to be in actuality the rat AS-FGF-2 homologue [Murphy and Knee, 1994; Knee et al., 1997]. There are several important differences between the structure of AS-FGF-2 cDNA in human and other species. Two human AS-FGF-2 cDNAs differ in having either an exon 1A or 1B sequence. By contrast, in *Xenopus* and rat [Knee et al., 1997] only a single AS-FGF-2 cDNA has been reported with sequence homology to human exon 1B. In human, as well as in rat, AS-FGF-2 cDNA is complementary to sequences in the FGF-2 3′UTR, but not in the FGF-2 ORF, unlike *Xenopus* AS-FGF-2, which is complementary to sequences in both FGF-2 cDNA regions [Volk et al., 1989]. Like the *Xenopus* and rat cDNAs, the human AS-FGF-2 cDNA possesses an ORF, and the ORFs of all three species have homology to the MutT family of the antimutator NTPases. In fact, the rat AS-FGF-2 protein has been detected *in vivo* and has been shown to possess antimutator activity [Li et al., 1996b, 1997]. It is unknown whether any of the aforementioned species-specific differences affect AS-FGF-2 function.

The finding that the AS-FGF-2 gene has two 5′ exons, 1A and 1B, which are found mutually exclusive in the two cDNAs suggests that two individual promoters may regulate their expression. Other genes that express mRNAs with multiple 5′ exons have been shown to be transcribed from separate promoters. For example, the acidic FGF (FGF-1) gene contains four individual promoters that result in the transcription of four mRNA transcripts, each with a unique exon 1 [Payson et al., 1993]. The +189/+647 AS-FGF-2 construct, however, which contains exon 1B as well as 194 bp of upstream sequence, did not possess any promoter activity in the transient transfection assay. If two promoters operate in the AS-FGF-2 5′ flanking genomic region, transcription of exon 1B may require regulatory elements located either 5′ or 3′ to sequences found in the +189/+647 construct. Two such promoters may also share common transcription regulatory elements.

Transfection of antisense FGF-2 constructs into cells down-regulates FGF-2 synthesis and function in rat vascular smooth muscle cells [Fox and Shanley, 1996] and in tumors [Graeven et al., 1992; Maret et al., 1995]. However, the biological function of endogenous AS-FGF-2 has not been clearly demonstrated. In *Xenopus*, it was suggested that AS-FGF-2 mRNA regulates FGF-2 expression through the direct hybridization of the two complementary mRNAs [Kimelman and Kirschner, 1989]. This hybridization was proposed to result in the modification of RNA nucleotides in the complementary region by a dsRNA-specific adenosine deaminase (DRADA) which could result in destabilization of the mRNAs or synthesis of a nonfunctional FGF-2 protein with altered amino acid sequence. Other mechanisms of an AS-FGF-2 mRNA-mediated regulation could occur through inhibition at the levels of transcription, splicing, and nucleocytoplasmic transport [Denhardt, 1992]. There is some evidence in developing systems of reciprocity in expression of sense and antisense FGF-2 mRNA. For example, in the developing rat brain, AS-FGF-2 gene expression is relatively high in the embryo and diminishes in the adult, the opposite of FGF-2 gene expression [Li et al., 1996a]. An earlier study demonstrated that a sole 1.8-kb FGF-2 mRNA

species expressed in embryonic rat brain, possibly AS-FGF-2, correlated with relatively low FGF-2 protein synthesis and that after birth there was an appearance of the typical 6.0-kb FGF-2 mRNA transcript accompanied by a relatively high level of FGF-2 protein, while the 1.8-kb mRNA was no longer synthesized [Powell et al., 1991].

In our limited analysis, we have found an apparent inverse relationship in the expression of sense and antisense FGF-2 mRNAs in various cell types. AS-FGF-2 mRNA is the predominant mRNA species expressed in a number of lymphoid cells, including peripheral blood T cells, Jurkat, MOLT-4, and U-937 cells. Little if any FGF-2 mRNA is expressed in these cells. Unlike T cells, the SK-HEP tumor cell line, which is a well-characterized FGF-2-producing cell [Prats et al., 1989], expresses four FGF-2 mRNA species and only trace levels of AS-FGF-2 mRNA. Human T47D breast carcinoma cells have been previously shown to express predominantly AS-FGF-2 mRNA and no FGF-2 mRNA, whereas human U87-MG glioma cells show the inverse expression pattern [Murphy and Knee, 1994]. By contrast, analysis of human tissues often shows accumulation of both AS-FGF-2 and FGF-2 mRNAs, for example, in heart, liver, skeletal muscle, and testis [Knee et al., 1994]. However, it may be that distinct cell types within the tissues show differential antisense and sense FGF-2 expression.

In summary, we have cloned two AS-FGF-2 cDNAs and characterized the human AS-FGF-2 gene promoter. We have analyzed the transcriptional activity of its promoter, and found a prominent role for Ets-like proteins in its regulation. Current studies are aimed at elucidating the transcriptional regulation of AS-FGF-2 gene expression in various cell types. Also, the proposed role of human AS-FGF-2 mRNA, which overlaps the FGF-2 3′UTR but not the ORF, in the down-regulation of endogenous FGF-2 function is being investigated.

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REFERENCES

Abraham JA, Klagsbrun M. 1996. Modulation of wound repair by members of the fibroblast growth factor family. In: Clark RAF, editor. The molecular and cellular biology of wound repair. New York: Plenum. p 195–248.

Baird A, Bohlen P. 1990. Fibroblast growth factors. In: Sporn MB, Roberts AB, editors. Handbook of experimental pharmacology. Vol 95: Peptide growth factors and their receptors. I. Berlin: Springer-Verlag. p 369–418.

Basilico C, Moscatelli D. 1992. The FGF family of growth factors and oncogenes. Adv Cancer Res 59:115–165.

Becker D, Meier CB, Herlyn M. 1989. Proliferation of human malignant melanomas is inhibited by antisense oligodeoxynucleotides targeted against basic fibroblast growth factor. EMBO 8:3685–3691.

Biesiada E, Razandi M, Levin ER. 1996. Egr-1 activates basic fibroblast growth factor transcription. Mechanistic implications for astrocyte proliferation. J Biol Chem 271: 18576–18581.

Bikfalvi A, Klein S, Pintucci G, Rifkin DB. 1997. Biological roles of fibroblast growth factor-2. Endocr Rev 18:26–45.

Blackwell TK, Weintraub H. 1990. Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. Science 250:1104–1110.

Blotnick S, Peoples GE, Freeman MR, Eberlein TJ, Klagsbrun M. 1994. T lymphocytes synthesize and export heparin-binding epidermal growth factor-like growth factor and basic fibroblast growth factor, mitogens for vascular cells and fibroblasts: differential production and release by $CD4^+$ and $CD8^+$ T cells. Proc Natl Acad Sci USA 91:2890–2894.

Bories J-C, Willerford DM, Grevin D, Davidson L, Camus A, Martin P, Stehelin D, Alt FW. 1995. Increased T-cell apoptosis and terminal B-cell differentiation induced by inactivation of the Ets-1 proto-oncogene. Nature 377:635–638.

Borja AZM, Meigers C, Zeller R. 1993. Expression of alternatively spliced bFGF first coding exons and antisense mRNAs during chicken embryogenesis. Dev Biol 157:110–118.

Chen CY, Schwartz RJ. 1995. Identification of novel DNA binding targets and regulatory domains of a murine tinman homeodomain factor, nkx-2.5. J Biol Chem 270: 15628–15633.

Chen X, Raab G, Deutsch U, Zhang J, Ezzell RM , Klagsbrun M. 1995. Induction of heparin-binding EGF-like growth factor expression during myogenesis. J Biol Chem 270:18285–18294.

Denhardt DT. 1992. Mechanism of action of antisense RNA. Sometime inhibition of transcription, processing, transport, or translation. Ann NY Acad Sci 660:70–76.

Denny P, Swift S, Connor F, Ashworth A. 1992. An SRY-related gene expressed during spermatogenesis in the mouse encodes a sequence-specific DNA-binding protein. EMBO 11:3705–3712.

Erdos G, Lee YJ, Cho JM , Corry PM. 1995. Heat-induced bFGF gene expression in the absence of heat shock element correlates with enhanced AP-1 binding activity. J Cell Physiol 164:404–413.

Fox JC, Shanley JR. 1996. Antisense inhibition of basic fibroblast growth factor induces apoptosis in vascular smooth muscle cells. J Biol Chem 271:12578–12584.

Graeven U, Rodeck U, Weinmann R, Herlyn M. 1992. Stable transfection of human malignant melanoma cells with basic fibroblast growth factor antisense cDNA. Ann NY Acad Sci 660:293–294.

Haimovitz-Friedman A, Balaban N, McLoughlin M, Ehleiter D, Michaeli J, Vlodavsky I, Fuks Z. 1994. Protein kinase C mediates basic fibroblast growth factor protection of endothelial cells against radiation-induced apoptosis. Cancer Res 54:2591–2597.

Harley VR, Lovell-Badge R, Goodfellow PN. 1994. Definition of a consensus DNA binding site for SRY. Nucleic Acids Res 22:1500–1501.

Johnson PF, Williams SC. 1994. CCAAT/enhancer binding (C/EBP) proteins. In: Tronce F, Yaniz M, editors. Liver gene expression. Austin: RG Landes. p 231–258.

Jolliff K, Li Y, Johnson LF. 1991. Multiple protein–DNA interactions in the TATAA-less mouse thymidylate synthase promoter. Nucleic Acids Res 19:2267–2274.

Kimelman D, Kirschner MW. 1989. An antisense mRNA directs the covalent modification of the transcript encoding fibroblast growth factor in *Xenopus* oocytes. Cell 59:687–696.

Knee R, Li AW, Murphy PR. 1997. Characterization and tissue-specific expression of the rat basic fibroblast growth factor antisense mRNA and protein. Proc Natl Acad Sci USA 94:4943–4947.

Knee R, Murphy PR. 1997. Regulation of gene expression by natural antisense RNA transcripts. Neurochem Int 31:379–392.

Knee RS, Pitcher SE, Murphy PR. 1994. Basic fibroblast growth factor sense (FGF) and antisense (GFG) RNA transcripts are expressed in unfertilized human oocytes and in differentiated adult tissues. Biochem Biophys Res Commun 205:577–583.

Lambert PF, Ludford-Menting MJ, Deacon NJ, Kola I, Doherty RR. 1997. The nfkb1 promoter is controlled by proteins of the Ets family. Mol Biol Cell 8:313–323.

Li AW, Seyoum G, Shiu RPC, Murphy PR. 1996a. Expression of the rat BFGF antisense RNA transcript is tissue-specific and developmentally regulated. Mol Cell Endocrinol 118:113–123.

Li AW, Too CK, Knee R, Wilkinson M, Murphy PR. 1997. FGF-2 antisense RNA encodes a nuclear protein with MutT-like antimutator activity. Mol Cell Endocrinol 133: 177–182.

Li AW, Too CKL, Murphy PR. 1996b. The basic fibroblast growth factor (FGF-2) antisense RNA (GFG) is translated into a MutT-related protein in vivo. Biochem and Biophys Res Commun 223:19–23.

Lo K, Landau NR, Smale ST. 1991. Lyf-1, a transcriptional regulator that interacts with a novel class of promoters for lymphocyte-specific genes. Mol Cell Biol 11:5229–5243.

Malik KTA, Wallace JI, Ivins SM, Brown KW. 1995. Identification of an antisense WT1 promoter in intron 1: Implications for WT1 gene regulation. Oncogene 1:1589–1595.

Maret A, Galy B, Arnaud E, Bayard F, Prats H. 1995. Inhibition of fibroblast growth factor 2 expression by antisense RNA induced a loss of the transformed phenotype in a human hepatoma cell line. Cancer Res 55:5075–5079.

Merika M , Orkin SH. 1993. DNA-binding specificity of GATA family transcription factors. Mol Cell Biol 13:3999–4010.

Moore JW, Dionne C, Jaye M, Swain JL. 1991. The mRNAs encoding acidic FGF, basic FGF and FGF receptor are coordinately downregulated during myogenic differentiation. Development 111:741–748.

Morrison RS. 1991. Suppression of basic fibroblast growth factor expression by antisense oligodeoxynucleotides inhibits the growth of transformed human astrocytes. J Biol Chem 266:728–734.

Murphy PR, Guo JZ, Friesen HG. 1990. Messenger RNA stabilization accounts for elevated basic fibroblast growth factor transcript levels in a human astrocytoma cell line. Mol Endocinol 4:196–200.

Murphy PR, Knee RS. 1994. Identification and characterization of an antisense RNA transcript (gfg) from the human basic fibroblast growth factor gene. Mol Endocrinol 8:852–859.

Muthusamy N, Barton K, Leiden JM. 1995. Defective activation and survival of T cells lacking the Ets-1 transcription factor. Nature 377:639–642.

Noguchi M, Miyamoto S, Silverman TA, Safer B. 1994. Characterization of an antisense Inr element in the eIF-2a gene. J Biol Chem 269:29161–29167.

Nye JA, Petersen JM, Gunther CV, Jonsen MD, Graves BJ. 1992. Interaction of murine Ets-1 with GGA-binding sites establishes the Ets domain as a new DNA-binding motif. Genes Dev 6:975–990.

Payson RA, Canatan H, Chotani MA, Wang W-P, Harris SE, Myers RL, Chiu I-M. 1993. Cloning of two novel forms of human acidic fibroblast growth factor (aFGF) mRNA. Nucleic Acids Res 21:489–495.

Peifley KA, Alberts GF, Hsu DKW, Feng S-LY, Winkles JA. 1996. Heparin-binding epidermal growth factor-like growth factor regulates fibroblast growth factor-2 expression in aortic smooth muscle cells. Circ Res 79:263–270.

Powell PP, Finklestein SP, Dionne CA, Jaye M, Klagsbrun M. 1991. Regulation of basic fibroblast growth factor mRNA expression in rat C6 glioma cells. Mol Brain Res 11:71–77.

Prats H, Kaghad M, Prats AC, Klagsbrun M, Lelias JM, Liauzun P, Chalon P, Tauber JP, Amalric F, Smith JA, Caput D. 1989. High molecular mass forms of basic fibroblast growth factor are initiated by alternative CUG codons. Proc Natl Acad Sci USA 86:1836–1840.

Ross R. 1993. The pathogenesis of atherosclerosis: A perspective for the 1990s. Nature 362:801–809.

Sanger F, Nicklen S, Coulson AR. 1977. DNA sequencing with chain-termination inhibitors. Proc Natl Acad Sci USA 74:5463–5467.

Sato Y, Hamanaka R, Ono J, Kuwano M, Rifkin DB, Takaki R. 1991. The stimulatory effect of PDGF on vascular smooth muscle cell migration is mediated by the induction of endogenous basic FGF. Biochem Biophys Res Commun 174:1260–1266.

Savage MP, Fallon JF. 1995. FGF-2 mRNA and its antisense message are expressed in a developmentally specific manner in the chick limb bud and mesonephros. Dev Dynam 202:343–353.

Shibata F, Baird A, Florkiewicz RZ. 1991. Functional characterization of the human basic fibroblast growth factor gene promoter. Growth Factors 4:277–287.

Silverman TA, Noguchi M, Safer B. 1992. Role of sequences within the first intron in the regulation of expression of eukaryotic initiation factor 2a. J Biol Chem 267:9738–9742.

Spicer DB, Sonenshein GE. 1992. An antisense promoter of the murine c-myc gene is localized within intron 2. Mol Cell Biol 12:1324–1329.

Stachowiak MK, Moffett J, Joy A, Puchacz E, Florkiewicz R, Stachowiak EW. 1994. Regulation of bFGF gene expression and subcellular distribution of bFGF protein in adrenal medullary cells. J Cell Biol 127:203–223.

Tamm I, Kikuchi T, Zychlinsky A. 1991. Acidic and basic fibroblast growth factors are survival factors with distinctive activity in quiescent BALB/c 3T3 murine fibroblasts. Proc Natl Acad Sci USA 88:3372–3376.

Tymms MJ, Kola I. 1994. Regulation of gene expression by transcription factors Ets-1 and Ets-2. Mol Reprod Dev 39:208–214.

Volk R, Koster M, Poting A, Hartmann L, Knochel W. 1989. An antisense transcript from the *Xenopus laevis* bFGF gene coding for an evolutionarily conserved 24 kd protein. EMBO 8:2983–2988.

Walike PA, Cowan WM, Ueno N, Baird A, Guillemin R. 1986. Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. Proc Natl Acad Sci USA 83:3012–3016.

Wang D, Mayo MW, Baldwin Jr AS. 1997. Basic fibroblast growth factor transcriptional autoregulation requires EGR-1. Oncogene 14:2291–2299.

Wasylyk B, Hahn SL, Giovane A. 1993. The Ets family of transcription factors. Eur J Biochem 211:7–18.

White EM, Shapiro DL, Allis CD, Gorovsky MA. 1988. Sequence and properties of the message encoding *Tetrahymena* hv1, a highly evolutionarily conserved histone H2A variant that is associated with active genes. Nucleic Acids Res 16:179–198.

Winkles JA, Gay CG. 1991. Serum, phorbol ester, and polypeptide mitogens increase class 1 and 2 heparin-binding (acidic and basic fibroblast) growth factor gene expression in human vascular smooth muscle cells. Cell Growth Diff 2:531–540.