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The Human FGF-5 Oncogene Encodes a Novel Protein Related to Fibroblast Growth Factors

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We previously described the isolation of a human oncogene which had acquired transforming potential by a DNA rearrangement accompanying transfection of NIH 3T3 cells with human tumor DNA (X. Zhan, A. Culpepper, M. Reddy, J. Loveless, and M. Goldfarb, Oncogene 1:369–376, 1987). We now term this oncogene the FGF-5 gene, since it specifies the fifth documented protein related to fibroblast growth factors (FGFs). Two regions of the FGF-5 sequence, containing 122 of its 267 amino acid residues, were 40 to 50% homologous to the sequences of acidic and basic FGFs as well as to the sequences of the FGF-related oncoproteins *int-2* and *hst*/KS3. The FGF-5 gene bears the three exon structures typical for members of this family. FGF-5 was found to be expressed in the neonatal brain and in 3 of the 13 human tumor cell lines examined. Several experiments strongly suggested that FGF-5 is a growth factor with properties common to those of acidic and basic FGFs. The rearrangement which activated the FGF-5 gene during DNA transfection had juxtaposed a retrovirus transcriptional enhancer just upstream from the native promoter of the gene.

Acidic and basic fibroblast growth factors (FGFs) are structurally related mitogens which stimulate growth or differentiation of a broad spectrum of mesoderm- and neuroectoderm-derived cell types (for a review, see reference 32). Mitogenicity of FGFs towards vascular endothelial cells also accounts for the potent angiogenic activity of these factors (9, 19, 34). FGFs were originally detected and purified from extracts of whole tissues, most notably from the brain and pituitary gland (5, 20, 33). FGFs have also been found to be associated with certain other tissues, with the extracellular matrix underlying the vascular endothelium (3, 36), and within vascular endothelial cells and vascular smooth muscle cells (26, 37). Tissue repair following injury may require the actions of FGFs released from damaged cells. Basic FGF is also synthesized by certain tumor cell lines (16, 28), suggesting a role for FGFs in tumor angiogenesis as well as in autocrine stimulation of tumor growth.

FGFs are monomeric proteins with molecular weights of 16,000 to 18,000. They bear 55% amino acid sequence identity (9, 13) and are encoded by distinct genes (1, 2, 15). Two other cellular genes have recently been characterized which specify proteins related to FGFs. One of these genes is *int-2*, which has been implicated in virus-induced mouse mammary carcinogenesis (8). The other gene, alternatively called *hst* or KS3, was detected as a gene in tumor DNAs which could transform NIH 3T3 murine fibroblasts (7, 30). The *hst*/KS3 protein has been shown to stimulate growth of cultured fibroblasts (7).

We have recently reported the molecular cloning of two potentially novel human oncogenes. These genes were detected by the transfer of DNAs from human tumor cell lines into NIH 3T3 cells, followed by selection for transformed cells using a defined medium lacking FGFs or platelet-derived growth factor (39). Here we report that one of these oncogenes encodes a protein related to FGFs. This novel protein, which we term FGF-5, is distinct from acidic and basic FGFs as well as from the *int-2* and *hst*/KS3 gene products. We had previously found that during transfection of 3T3 cells with human DNA, this oncogene had recombined with a cotransfected plasmid carrying a murine retrovirus promoter-enhancer element (39). Here we show that the DNA rearrangement which activated the FGF-5 gene had juxtaposed a murine retrovirus transcriptional enhancer next to the native promoter of the FGF-5 gene. We also present preliminary data regarding FGF-5 gene expression and FGF-5 mitogenic activities.

MATERIALS AND METHODS

Cell lines. BALB/c 3T3 cells, NIH 3T3 cells, and NIH 3T3 cells transformed by various oncogenes were previously described (39, 40). VMCUB2-1 are NIH 3T3 cells transformed with the rearranged human FGF-5 gene activated by the long terminal repeat (LTR); 3T3-LTR122 are NIH 3T3 cells bearing pLTR122, a plasmid containing 1-2-2 FGF-5 cDNA situated between two Moloney murine leukemia virus (MLV) LTR elements; 3T3-*src* and 3T3-*ras* are NIH 3T3 lines transformed by plasmids bearing the v-*src* and the mutant human c-H-*ras* (valine 12) oncogenes. Human tumor cell lines described in several references (10, 11, 12) were obtained from James Loveless at the Memorial Sloan Kettering Cancer Institute; VMCUB1, VMCUB2, 639V, and 253J are from bladder carcinomas; Calu4 and KNS62 are from lung carcinomas; BT20, MCF-7, and MDAMB-469 are from breast carcinomas; HT29 is from a colon carcinoma; SH-1 is from a melanoma; SKHEP-1 is from a hepatoma; and HEC-1A is from an endometrial carcinoma. Fetal bovine heart endothelial cells (14) were obtained from the American Tissue Culture Collection.

FGF-5 genomic and cDNA clones. NIH 3T3 cells transformed by the rearranged FGF-5 gene (the VMCUB2-1 cell line) had been used to clone the FGF-5 gene in lambda vector EMBL4, and these cells were also used to derive the biologically active cDNA clone 1-2-2 (39). An FGF-5 cDNA

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clone was also obtained by screening a lambda gt11 cDNA library derived from RNA of human brain stem (1-day-old, autopsy) (library kindly provided by R. Lazzarini). Both cDNAs were subcloned into plasmid pUC8 by *Eco*RI digestion, which cleaved the cDNAs at a native *Eco*RI cleavage site 3′ to the coding sequences; hence, the cDNAs lack their 3′ ends and poly(A) tails. For the purpose of DNA sequencing, fragments of cDNA and genomic clones were subcloned into pUC8 after restriction enzyme digestion or mild DNase I treatment.

DNA sequencing. Plasmid inserts were sequenced by modification of the standard DNA polymerase dideoxynucleotide chain termination method (25). Plasmids (1 to 2 μg) were denatured with alkali, neutralized in the presence of 25 ng of oligonucleotide primer, and ethanol precipitated. Pellets were dissolved and sequenced with T7 DNA polymerase (Sequenase; U.S. Biochemical) by using α-^{35}S-labeled dATP as the labeled nucleotide. The primers most often used were 17-mers complementary to pUC8 sequences flanking the inserts. The oligonucleotides 5′-CCTAAGTGCATCTT GTA-3′ and 5′-ACTTGCATGGAGTTTTC-3′, complementary to bases 105 to 121 and 579 to 595 of the 1-2-2 FGF-5 cDNA clone, were used in certain experiments. The cDNAs were fully sequenced on both strands, as was the 5′ region of the FGF-5 gene. For the purpose of mapping the exon-intron boundaries, certain genomic clones were sequenced on one strand only.

Analysis of FGF-5 RNA. Cytoplasmic RNAs were extracted from cultured cells (27) and in some cases enriched for polyadenylated RNA by oligo(dT) cellulose chromatography. RNAs were assayed for FGF-5 transcripts by formaldehyde agarose gel electrophoresis and filter blot hybridization by using the standard procedure (35). Ethidium bromide (50 ng/ml) was included in the gels to allow visualization of rRNAs before the blot.

Determinations of FGF-5 RNA 5′ ends were made by the standard primer extension procedure (24). The oligonucleotide 5′-CCTAAGTGCAGCTTGTA-3′, complementary to bases 105 to 121 of the 1-2-2 cDNA clone, was 5′-end-labeled with [γ-^{32}P]ATP and polynucleotide kinase, and 15 ng of primer was annealed with 10 μg of total cytoplasmic RNAs. The mixture was precipitated, redissolved, incubated with deoxynucleotide triphosphates and avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals), and analyzed by electrophoresis through a 8M urea–12% polyacrylamide gel, followed by autoradiography. DNA sequencing reactions using the same oligonucleotide primer and FGF-5 genomic DNA template were run alongside the samples to provide size markers.

Mitogenic assays. Transformed NIH 3T3 cells were maintained at high density in a defined medium containing insulin but lacking platelet-derived growth factor or FGFs (40). After 2 to 3 days of conditioning, the medium was harvested for testing of mitogenic activity towards BALB/c 3T3 fibroblasts. To test for activity towards endothelial cells, transformed cells were maintained in DME supplemented with 10 μg of heparin per ml.

For the fibroblast mitogenic assay, culture wells were seeded to 10% confluence with BALB/c 3T3 murine fibroblasts and were rendered quiescent by maintenance without refeeding for 5 days, as originally described (23). Cultures were refed with dilutions of conditioned medium (diluted with defined medium containing insulin), 4 μCi of [^{3}H] thymidine per ml was added 15 h later, and thymidine incorporation into DNA was measured 3 h later by fixing the cultures in 15% trichloroacetic acid, washing with water, dissolving in 0.5 M NaOH, and counting by liquid scintillation.

For the endothelial cell mitogenic assay, fetal bovine heart endothelial cells were plated at 40,000 cells per 60-mm dish in DME plus 3% calf serum. The next day, an equal volume of DME plus 10 μg of heparin per ml, containing various dilutions of conditioned medium, was added. The cell number was determined 6 days later by trypsinization and counting by hemacytometer.

Heparin affinity chromatography. NIH 3T3 cells transformed with the plasmid pLTR122 were used to condition DME medium containing no supplements. A 48-h conditioned medium (300 ml) was passed directly through a 1-ml column of heparin-Sepharose (Pharmacia) at room temperature. The column was washed extensively with 0.45 M NaCl–20 mM Tris (pH 7.5) and then washed in succession with Tris buffer containing NaCl at 0.6 M (3 ml), 0.8 M (2 ml), 1.0 M (2 ml), 1.5 M (2 ml), and 2.0 M (2 ml). Fractions (1 ml) were collected and were diluted 1:100 with defined medium containing insulin (40); they were then assayed for stimulation of BALB/c 3T3 cells.

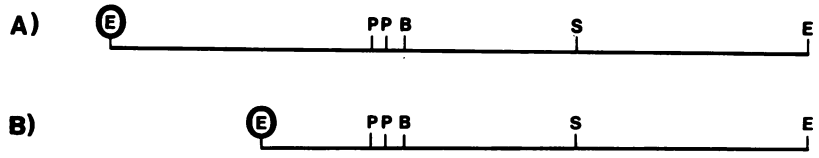
Expression of a FGF-5 fusion protein in *Escherichia coli*. The 1.1-kilobase-pair (kbp) *Eco*RI fragment containing the FGF-5 cDNA clone 1-2-2 was inserted into the *Eco*RI site of the expression plasmid pATH3 (31), generating the construct pTrpE-FGF5. pTrpE-FGF5 can direct synthesis in *E. coli* of a fusion protein containing, from N to C termini, approximately 35 kilodaltons of the TrpE protein, 5 kilodaltons specified by 5′ FGF-5 cDNA sequences, and the entire FGF-5 polypeptide. *E. coli* HB101 carrying either pTrpE-FGF5 or the parent vector pATH3 was cultured with indoleacrylic acid to maximize expression of the TrpE promoter (31). Harvested cells were lysed by sonication, and the supernatant fraction after centrifugation at 15,000 rpm was assayed for mitogenic activity towards BALB/c 3T3 cells, as described above.

RESULTS

RNA transcribed from the rearranged oncogene can encode a protein related to FGFs. Transformed NIH 3T3 cells bearing the rearranged human oncogene express oncogene-derived RNA species of approximately 1.6 and 4.0 kilobases (kb) in length (39). RNA from such transformed cells had been used to construct a cDNA library, which was screened to yield a clone homologous to the oncogene. This cDNA isolate, termed 1-2-2, had strong transforming activity when linked to murine retrovirus promoter elements (39).

The complete nucleotide sequence of the 1-2-2 cDNA clone was determined and is presented in Fig. 1. The 1,120-base-pair sequence lacked the 3′ poly(A) tract, because the cDNA cloning procedure involved *Eco*RI digestion, which cut the native cDNA at least once. The single strand shown corresponded to that of the RNA, as demonstrated by the ability of an oligonucleotide of complementary sequence to prime reverse transcription of the oncogene-encoded RNA (see below). The cDNA sequence contained two ATG-initiated open reading frames (ORFs); ORF-1 and ORF-2 can specify polypeptides of 38 and 267 amino acid residues, respectively. The two reading frames slightly overlapped, with the ORF-1 termination codon, TGA, situated one nucleotide downstream from the ORF-2 initiator ATG.

The protein specified by ORF-2 bore a leucine-rich hydrophobic amino terminus, which may serve as a signal sequence for cotranslational transport into the endoplasmic reticulum (4). The protein's lack of other extensive tracts of



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ORF-1.....METSerThrArgCysGlyGluAlaGlyGlnSerArgGlyThrGlnProHisArgGlyTyrArg      20
CCTCTCCCCTTCTCTTCCCCGAGGCTATGTCCACCCGGTGCGGCGAGGCGGGCCAGAGCAGAGGCACGCAGCCGCACAGGGGCTACAGA    89

AlaGlnAsnGlnProTyrLysMetHisLeuGlyProProArgLeuGluGluEND                        38
GCCCAGAATCAGCCCTACAAGATGCACTTAGGACCCCCGCGGCTGGAAGAATGAGCTTGTCCTTCCTCCTCCTCCTCTTCTTCAGCCAC  178
ORF-2.....METSerLeuSerPheLeuLeuLeuLeuPheSerHis                               13

CTGATCCTCAGCGCCTGGGCTCACGGGGAGAAGCGTCTCGCCCCCAAAGGGCAACCCGGACCCGCTGCCACTGATAGGAACCCTAGAGGC  268
LeuIleLeuSerAlaTrpAlaHisGlyGluLysArgLeuAlaProLysGlyGlnProGlyProAlaAlaThrAspArgAsnProArgGly   43

TCCAGCAGCAGACAGAGCAGCAGTAGCGCTATGTCTTCCTCTTCTGCCTCCTCCTCCCCCGCAGCTTCTCTGGGCAGCCAAGGAAGTGGC  358
SerSerSerArgGlnSerSerSerSerAlaMetSerSerSerSerAlaSerSerSerProAlaAlaSerLeuGlySerGlnGlySerGly   73

TTGGAGCAGAGCAGTTTCCAGTGGAGCCTCGGGGCGCGGACCGGCAGCCTCTACTGCAGAGTGGGCATCGGTTTCCATCTGCAGATCTAC  448
LeuGluGlnSerSerPheGlnTrpSerLeuGlyAlaArgThrGlySerLeuTyrCysArgValGlyIleGlyPheHisLeuGlnIleTyr  103

CCGGATGGCAAAGTCAATGGATCCCACGAAGCCAATATGTTAAGTGTTTTGGAAATATTTGCTGTGTCTCAGGGGATTGTAGGAATACGA  538
ProAspGlyLysValAsnGlySerHisGluAlaAsnMetLeuSerValLeuGluIlePheAlaValSerGlnGlyIleValGlyIleArg  133

GGAGTTTTCAGCAACAAATTTTTAGCGATGTCAAAAAAAGGAAAACTCCATGCAAGTGCCAAGTTCACAGATGACTGCAAGTTCAGGGAG  628
GlyValPheSerAsnLysPheLeuAlaMetSerLysLysGlyLysLeuHisAlaSerAlaLysPheThrAspAspCysLysPheArgGlu  163

CGTTTTCAAGAAAATAGCTATAATACCTATGCCTCAGCAATACATAGAACTGAAAAAACAGGGCGGGAGTGGTATGTTGCCCTGAATAAA  718
ArgPheGlnGluAsnSerTyrAsnThrTyrAlaSerAlaIleHisArgThrGluLysThrGlyArgGluTrpTyrValAlaLeuAsnLys  193

AGAGGAAAAGCCAAACGAGGGTGCAGCCCCCGGGTTAAACCCCAGCATATCTCTACCCATTTTCTTCCAAGATTCAAGCAGTCGGAGCAG  808
ArgGlyLysAlaLysArgGlyCysSerProArgValLysProGlnHisIleSerThrHisPheLeuProArgPheLysGlnSerGluGln  223

CCAGAACTTTCTTTCACGGTTACTGTTCCTGAAAAGAAAAATCCACCTAGCCCTATCAAGTCAAAGATTCCCCTTTCTGCACCTCGGAAA  898
ProGluLeuSerPheThrValThrValProGluLysLysAsnProProSerProIleLysSerLysIleProLeuSerAlaProArgLys  253

AATACCAACTCAGTGAAATACAGACTCAAGTTTCGCTTTGGATAATATTAATCTTGGCCTTGTGAGAAACCATTCTTTCCCCTCAGGAGT  988
AsnThrAsnSerValLysTyrArgLeuLysPheArgPheGlyEND                                267

TTCTATAGGTGTCTTCAGAGTTCTGAAGAAAAATTACTGGACACAGCTTCAGCTATACTTACACTGTATTGAAGTCACGTCATTTGTTTC 1078

AGTGTGACTGAAACAAAATGTTTTTTGATAGGAAGGAAACTG                                   1120
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FIG. 1. Structures and sequence of FGF-5 cDNA clones. (A) The structure of the 1-2-2 cDNA clone corresponding to a transcript of a rearranged, activated human oncogene. Several restriction endonuclease cleavage sites are indicated: E, *Eco*RI; B, *Bam*HI; S, *Sma*I, P, *Pst*I. (B) Structure of a homologous cDNA clone isolated from a library of human brain stem RNA (1-day old, autopsy). The library was kindly provided by R. Lazzarini. The circled *Eco*RI sites at the left ends of both cDNAs are part of the synthetic linker sequences added during cloning. (C) Sequence of the 1-2-2 cDNA clone depicted in panel A. The nucleotide sequence (1,120 bases) is shown along with the predicted amino acid sequences specified by the two ORFs, ORF-1 and ORF-2. The homologous brain cDNA sequence is identical to bases 246 to 1120, which are depicted here.

hydrophobic residues suggests that the ORF-2 product can be secreted. The predicted protein also bears a consensus sequence for N-linked glycosylation, Asn-Gly-Ser (residues 109 to 111). When the ORF-2 protein sequence was compared with sequences in the PIR-NBRF (Protein Identification Resource, National Biomedical Research Foundation) data base, substantial homology was detected between the ORF-2 protein and both acidic and basic fibroblast growth factors. The recently described *int-2* and *hst*/KS3 predicted protein sequences are also homologous to, but distinct from, the ORF-2 protein, which we here term FGF-5.

A comparison of the FGF-5 amino acid sequence with those for other FGF family proteins is shown in Fig. 2. Two blocks of FGF-5 amino acid residues (90 to 180 and 187 to 207) showed substantial homology to the other proteins, ranging from 40.2% (versus acidic FGF) to 50.4% (versus *hst*/KS3). Within these homology blocks, the five proteins were identical at 20% of the residues, and allowing for conservative amino acid substitutions, the five proteins shared 29% homology. Nucleotide sequence homology between the coding sequences of FGF-5 and those of related genes was minimal.

The sequences of the FGF-related proteins differed in several respects. First, the five sequences differed in the length and sequence of residues between the two homology blocks and distal to them. Second, the FGF-5 sequence was unique in bearing an insertion within the second homology block (Cys-Ser, residues 201 and 202). Lastly, the amino-terminal sequences of the FGF-5, *hst*/KS3, and *int-2* proteins were extensively hydrophobic, while those of acidic and basic FGF precursor proteins were not, suggesting differences in posttranslational trafficking amongst the FGF-like proteins.

Similarity in structures of the FGF-5 oncogene and other FGF-related genes. The structure of the rearranged, transforming FGF-5 oncogene is illustrated in Fig. 3A. The portion of the oncogene transcripts represented in the transforming cDNA clone 1-2-2 derived from three exons in the gene. The DNA sequences at the exon-intron boundaries and their positions with respect to the coding sequence are presented in Fig. 3B. Exon I spanned coding sequences from the initiation codon through the first nucleotide of serine codon 118, while exon II terminated after serine codon 152. Since the splice boundaries were at points within the FGF family homology blocks, we could compare these exon boundaries with those within the *hst*/KS3, *int-2*, and basic

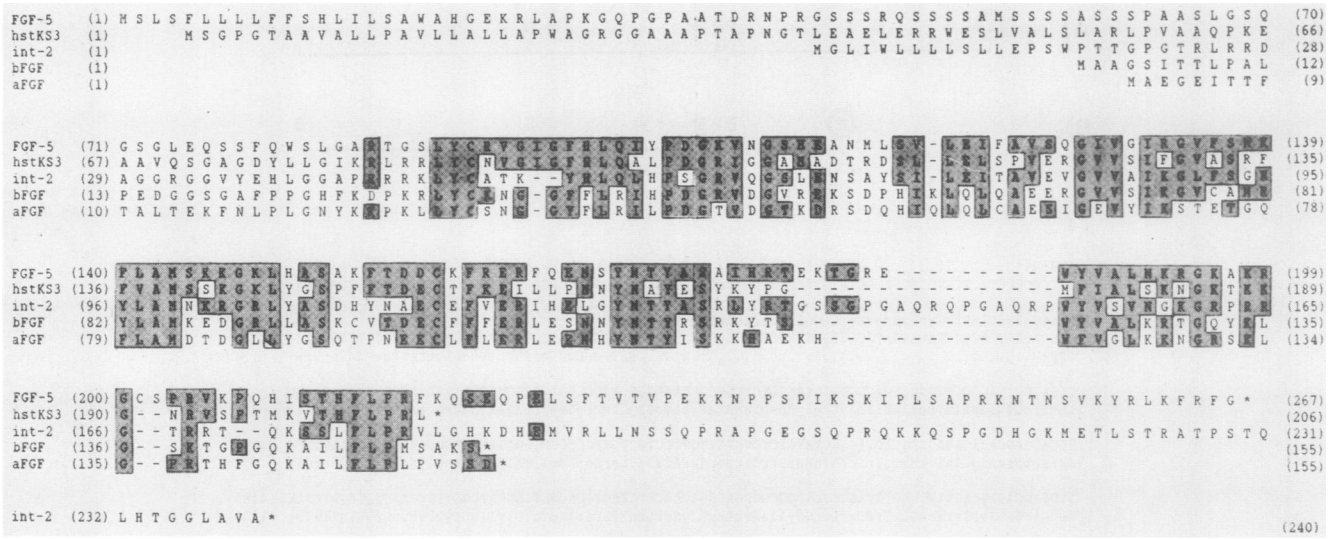

FIG. 2. Homology between the predicted FGF-5 protein and other FGF-related proteins. The predicted human FGF-5 amino acid sequence (from ORF-2) is aligned for maximum homology with sequences of the precursor proteins encoded by human *hst*/KS3 (30), murine *int-2* (21), human basic FGF (2), and human acidic FGF (15). Amino acid positions of identity or conservative substitution between FGF-5 and related proteins are boxed and shaded. Conservative substitutions are defined as D↔E, K↔R, S↔T, I↔L↔V, and F↔Y. Within the regions of nucleotides 90 to 180 and 187 to 217 of the FGF-5 sequence, there is 50.4% sequence identity with *hst*/KS3, 47.5% identity with *int-2*, 43.4% with basic FGF, and 40.2% with acidic FGF.

FGF genes (2, 21, 38). The exon boundaries of both the *hst*/KS3 and *int-2* oncogenes were positioned identically to those of the FGF-5 gene. The exon II-exon III boundary of the basic FGF gene also aligned perfectly, while the exon I-exon II boundary of this gene was shifted by three nucleotides. Hence, the FGF-related genes have evolved with virtually no deviation from the exon structure of their ancestral gene. Interestingly, the FGF-related genes bore introns of dramatically different lengths: *hst*/KS3 introns total 1.1 kbp, FGF-5 introns total 19 kbp, and basic FGF gene introns exceed 30 kbp.

Rearrangement accompanying gene transfer had positioned a retroviral enhancer next to the promoter of the FGF-5 gene. Transfection of NIH 3T3 cells with DNA from the VMCUB2 human bladder carcinoma cell line had fortuitously resulted in a rearrangement between the FGF-5 gene in the tumor DNA and a cotransfected plasmid, pLTRneo (39). This plasmid contains a Tn*5* neomycin resistance gene flanked by MLV LTR sequences. The rearranged gene was expressed at high levels in the transformed cells, presumably because of the influence of the MLV LTR (39). The 5′ end of the FGF-5 gene had recombined with the pLTRneo plasmid (diagrammed in Fig. 3A). In order to map the recombination event which had occurred, we sequenced a portion of the rearranged FGF-5 gene in the 5′ region. We also analyzed restriction endonuclease cleavage sites of the normal and rearranged FGF-5 genes by filter blot hybridization.

A *Hin*dIII recognition site just upstream from exon I was not disrupted by the rearrangement which activated the FGF-5 gene (shown schematically in Fig. 3C). The sequence of the rearranged FGF-5 gene more than 60 base pairs upstream from this *Hin*dIII site corresponded to MLV LTR sequences from the pLTRneo plasmid (Fig. 3D). The orientation of the juxtaposed LTR does not allow for transcription of the FGF-5 gene from the LTR promoter. However, despite a 41-nucleotide deletion suffered by the LTR during rearrangement, its 75-base-pair direct repeat elements constituting the transcriptional enhancer (18) were intact.

The MLV LTR should be driving transcription from the native promoter of the FGF-5 gene or a cryptic promoter. To localize the start site for transcription, we mapped the 5′ end of FGF-5 RNA by the method of primer extension. A synthetic oligonucleotide complementary to FGF-5 RNA and corresponding to nucleotides 105 to 121 of the cDNA clone was annealed with RNA from 3T3 cells transformed by the rearranged FGF-5 gene. The annealed primer was extended with reverse transcriptase, and the products were resolved on a urea-polyacrylamide gel. The longest primer extension product obtained was 143 bases in length (Fig. 4, lane c). Hence, the 5′ end of one or both of the FGF-5 RNA species extended only 22 bases beyond the sequences present in the cDNA clone. The transcription cap site defined by this experiment was within the native sequence of the FGF-5 gene and was positioned downstream from a TATAA box presumptive promoter element (positions −31 to −27; Fig. 3D). The smaller primer extension products may be the consequence of RNA degradation, secondary structures in the RNA template, or downstream alternative transcription initiation sites.

Expression of the native FGF-5 gene. We examined whether the native FGF-5 gene specifies the same RNA transcripts and protein as does the rearranged, LTR-activated gene. One approach we have used is to characterize cDNA clones corresponding to transcripts of the native FGF-5 gene. On the basis of preliminary data suggesting expression of FGF-5 in the fetal brain, we chose to screen a cDNA library constructed in lambda gt11 vector by using RNA obtained at autopsy from a 1-day-old human brain stem (library kindly provided by R. Lazzarini). Four clones hybridizing to the FGF-5 gene were detected upon library screening, one of which was subcloned and fully sequenced. This brain cDNA clone was 245 nucleotides shorter than the 1-2-2 FGF-5 cDNA clone (Fig. 1B), but the sequence of the brain cDNA was identical to that of bases 246 to 1120 in the 1-2-2 clone sequence (Fig. 1C).

We also looked for expression of FGF-5 within a panel of

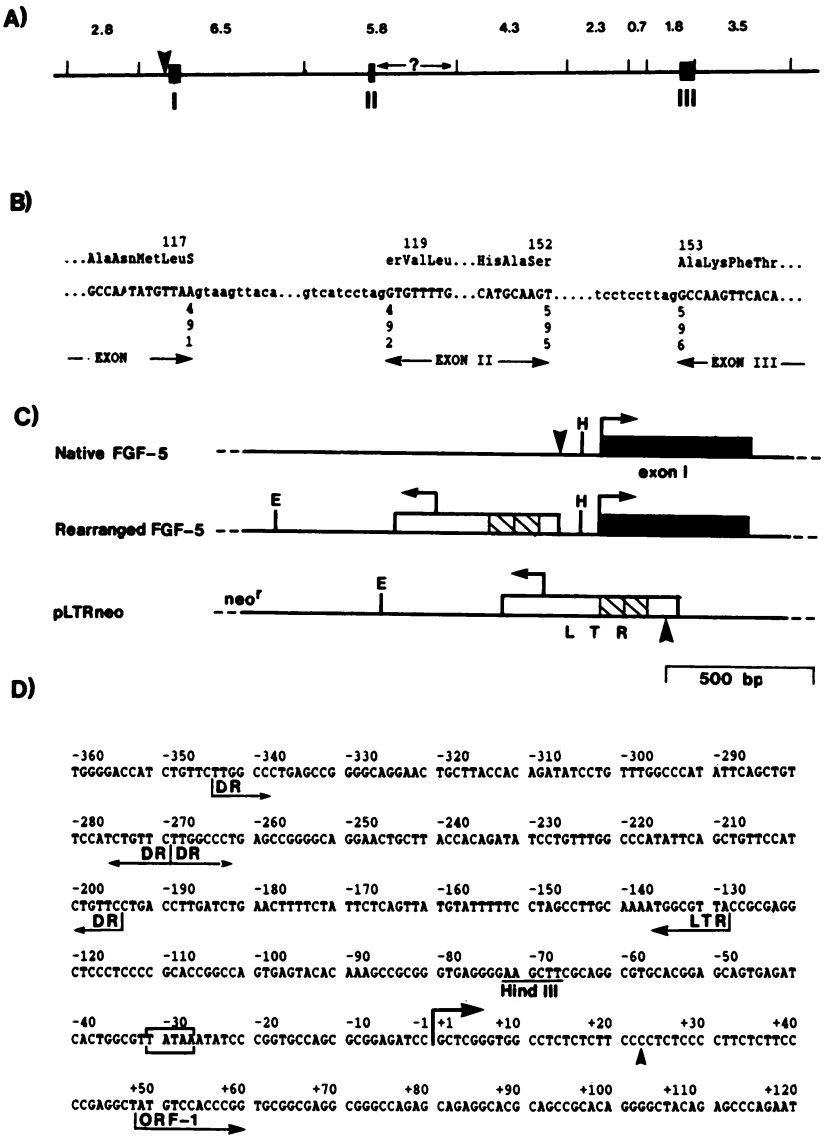

FIG. 3. Structure of the rearranged, activated FGF-5 oncogene. (A) Structural map of the rearranged FGF-5 gene. The positions of *Eco*RI cleavage sites (|) and the sizes of the *Eco*RI fragments (in kilobase pairs) are indicated. The three exons (■) are marked I, II, and III. The precise locations of exons I and III were obtained by mapping restriction sites in the genomic DNA which correspond to sites in the cDNA clone. Exon II lies within the 5.8-kbp *Eco*RI fragment, but its precise position was not determined (signified by the "?" symbol). The point of rearrangement between the native FGF-5 gene and plasmid pLTRneo is indicated (▼). (B) Sequences at the exon-intron boundaries. Exon sequences are shown in uppercase letters, and intron sequences are shown in lowercase letters. Only sequences near the boundaries are shown. Exon sequences are numbered according to the corresponding FGF-5 1-2-2 cDNA clone sequence (Fig. 1C). The corresponding amino acid sequence of FGF-5 is shown above the exon sequences. (C) Schematic diagram illustrating the rearrangement between the native FGF-5 gene and plasmid pLTRneo, which generated the activated FGF-5 gene. Symbols: ■, exon I of FGF-5 gene; →, site of FGF-5 transcription initiation; □, one of the two LTR sequences in pLTRneo; ←, site of initiation of LTR transcription by the promoter; ▨, 75-base-pair repeats which contain the enhancer (18); ▼, break points in pLTRneo and native FGF-5 gene accompanying rearrangement. E, *Eco*RI site; H, *Hin*dIII site; bp, base pairs. (D) Sequence of rearranged FGF-5 gene in the promoter region. The sequences are numbered corresponding to the site of transcription initiation (+1), which was mapped by primer extension analysis. The arrowhead at +23 corresponds to the first base in the 1-2-2 cDNA clone (Fig. 1C). A TATAA box presumptive promoter element (positions −31 to −27) is bracketed. LTR sequences in the rearranged gene lie upstream from −129 (29). The direct repeat (DR) elements of the LTR are marked.

human tumor cell lines of solid tumor origin. Cytoplasmic RNAs were prepared from 13 such cell lines, and the samples were assayed for FGF-5 transcripts by gel electrophoresis and filter blot hybridization. None of these cell lines had shown any evidence for FGF-5 gene rearrangement (data not shown). Two of the cell lines, hepatoma SKHEP1 and bladder carcinoma 639V, expressed two RNA species homologous to the FGF-5 cDNA probe (Fig. 5, lanes h and k). A third cell line, endometrial carcinoma HEC-1A, expressed FGF-5 RNA at lower levels (lane j), while the other tumor cell lines did not express FGF-5 detectably. (The cDNA probe also hybridized weakly to human 28S rRNA.)

The FGF-5 transcripts in the tumor cell lines were indistinguishable in size and relative abundance from the two

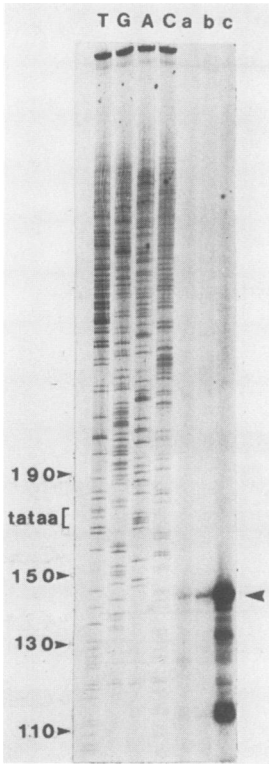

FIG. 4. Mapping the 5′ end of FGF-5 RNAs by primer extension analysis. Total cytoplasmic RNAs (10 μg) were annealed with the oligonucleotide 5′-[^{32}P]PO_4-CCTAAGTGCATCTTGTA-3′ complementary to FGF-5 RNA (bases 105 to 121 of the 1-2-2 cDNA clone), and the mixture was incubated in buffer containing deoxynucleoside triphosphates and avian myeloblastosis virus reverse transcriptase. Reaction products were resolved on an 8 M urea–12% polyacrylamide sequencing gel. RNAs from human tumor cell lines 639V (lane a) and SKHEP-1 (lane b) and from 3T3 transformant VMCUB2-1 (lane c) were used as templates. The same oligonucleotide (but unlabeled) was used to prime DNA sequencing reactions by using a subclone of the rearranged FGF-5 gene as the template. Sequencing reactions are designated A, C, G, and T, corresponding to the sense strand of the FGF-5 gene, and lengths (in bases) along the sequence ladder are indicated on the left. A TATAA box upstream of the transcribed sequences is indicated.

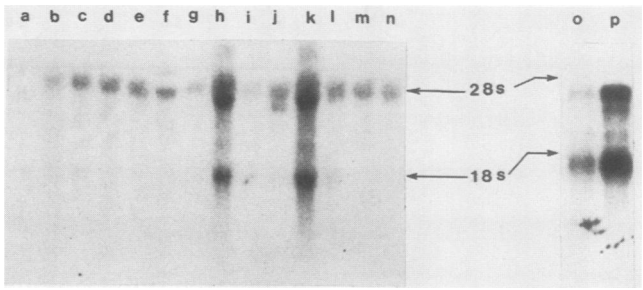

FIG. 5. Northern blot analysis of FGF-5 RNA in human tumor cell lines. Total cytoplasmic RNAs (10 μg; lanes a to n) or poly(A)-selected cytoplasmic RNAs (1 μg; lanes o and p) were subjected to electrophoresis through 1.5% agarose gels containing 2.2 M formaldehyde. Gel-embedded RNA was transferred to nitrocellulose, hybridized with nick-translated FGF-5 1-2-2 cDNA, and autoradiographed. Total RNAs were from NIH 3T3 cells (lane a) and human tumor cell lines VMCUB1 (lane b), VMCUB2 (lane c), Calu4 (lane d), KNS62 (lane e), BT20 (lane f), MDAMB469 (lane g), SKHEP-1 (lane h), MCF-7 (lane i), HEC-1A (lane j), 639V (lane k), 253J (lane l), HT29 (lane m), and SH1 (lane n). Poly(A) RNAs were from 639V (lane o) and from NIH 3T3 transformant VMCUB2-1 bearing the rearranged, LTR-activated FGF-5 gene (lane p). The positions of 18S (1.9-kb) and 28S (4.5-kb) rRNAs are indicated.

RNA species transcribed from the rearranged, LTR-activated FGF-5 gene in transformed 3T3 cells (Fig. 5, lanes o and p). By primer extension analysis, we showed that at least one of the native FGF-5 transcripts in SKHEP1 and 639V cells had precisely the same 5′ end as that which was characterized for a transcript of the rearranged gene (Fig. 4, lanes a and b). Taken together, these data confirm that RNA species encoded by native and rearranged FGF-5 genes are the same.

Secretion of a mitogen functionally related to FGFs by FGF-5-transformed 3T3 cells. Our sequence data suggested that the FGF-5 gene encodes a secreted growth factor. We assayed for mitogenic activity secreted from 3T3 cells transformed by the rearranged FGF-5 gene or by FGF-5 cDNA linked to MLV LTR sequences. Conditioned media from such transformed cell cultures (termed VMCUB2-1 and 3T3-LTR122, respectively) were serially diluted and assayed for the ability to stimulate DNA synthesis in quiescent BALB/c 3T3 fibroblast cultures. These transformed cells secreted a mitogenic activity that was detectable at 1:8 dilutions (Table 1). Secretion of mitogenic activity is not a property of transformed cells per se, as NIH 3T3 cells transformed by activated human H-*ras* or viral *src* oncogenes released little or no mitogenic activity (Table 1).

As a means of assessing whether the mitogen secreted by FGF-5-transformed cells is, indeed, FGF-5, we tested whether this mitogen has properties diagnostic for FGFs. One property of acidic and basic FGFs is their ability to strongly bind to the glycosaminoglycan heparin (6, 20, 28). Elution of FGFs from heparin affinity resins requires NaCl concentrations of 1.0 M or greater. By contrast, platelet-derived growth factor, a basic protein which binds heparin by weaker ionic interactions, elutes at approximately 0.5 M NaCl (36). Mitogenic conditioned medium from FGF-5 cDNA-transformed 3T3 cells was passed directly over a heparin-Sepharose column, which was washed extensively with buffered 0.45 M NaCl, and then eluted with a stepwise increase in salt concentrations. Dilutions of column fractions were assayed for the ability to stimulate quiescent BALB/c 3T3 cells. The peak of mitogenic activity eluted in the 1.0 and 1.5 M NaCl fractions (Fig. 6).

A second property of FGF is their broad spectrum of mitogenicity, including their activity towards vascular endothelial cells. Conditioned medium from FGF-5-transformed cells was tested for ability to stimulate the proliferation of fetal bovine heart endothelial cells. Fetal bovine heart endothelial cell cultures were fed with conditioned medium diluted into DME medium containing 1.5% calf serum and 5 μg of heparin per ml. Cell growth over 6 days was determined by counting trypsinized cells. Whereas cell cultures without conditioned medium underwent only 1.5 population doublings during the assay, 1:2 and 1:8 dilutions of conditioned medium stimulated growth to 3.3 and 3.4 population doublings, respectively. This stimulation is comparable with that induced by partially purified basic FGF (3.1 doublings). These data strongly suggest a functional similarity between FGF-5 and the well-characterized fibroblast growth factors.

Mitogenic activity of bacterial extract containing FGF-5 fusion protein. FGF-5 cDNA was cloned into the plasmid pATH3 (31) to generate the construct pTrpE-FGF5. In *E.*

TABLE 1. Stimulation of quiescent BALB/c 3T3 cells with conditioned media and bacterial cell extracts[a]

Conditioned medium or extract	Dilution	[^{3}H]thymidine incorporation (cpm/10,000 cells)
Conditioned medium from:		
3T3-*ras*	1:2	1.1
	1:4	1.5
	1:8	1.0
3T3-*src*	1:2	1.5
	1:4	1.1
	1:8	1.1
VMCUB2-1	1:2	23.0
	1:4	9.5
	1:8	3.2
3T3-LTR122	1:2	130.0
	1:4	99.1
	1:8	40.8
Extract from:		
HB101(pTrpE-FGF5)	1:300	140.0
	1:1,000	105.0
HB101(pATH3)	1:300	1.2
Without supplements		0.9
With 10% calf serum		146.0

[a] BALB/c 3T3 cells were plated in culture wells (20,000 cells per 2.0-cm^2 well) in serum-containing medium and maintained for 5 days without refeeding, allowing the cells to form quiescent, serum-exhausted monolayers. Cultures were refed with serum-free medium containing dilutions of conditioned medium from transformed cells or dilutions of extracts from bacterial cultures. [^{3}H]thymidine (4 μCi/ml) was added 15 h later, and after 3 h of incubation, the cultures were fixed in 15% trichloroacetic acid, their DNA was dissolved in 0.5 N NaOH, and incorporated label was assayed by liquid scintillation.

coli, pTrpE-FGF5 can direct the synthesis of a fusion protein containing the FGF-5 polypeptide linked to the bacterial TrpE gene product. Extracts were made from bacteria harboring either pTrpE-FGF5 or the parental vector pATH3. A 1:300 dilution of extract from cells containing pTrpE-FGF5 induced maximal mitogenic stimulation of quiescent BALB/c 3T3 cells, whereas extract from pATH3-containing cells lacked detectable activity (Table 1). Furthermore, the mitogenic activity in cells containing pTrpE-FGF5 displayed the same affinity for heparin-Sepharose resin as the growth factor secreted from FGF-5 transformed NIH 3T3 cells (data not shown). We conclude that FGF-5 is a mitogenic protein.

DISCUSSION

Potential functions of FGF-5. FGF-5 bears functional similarities to other fibroblast growth factors. This new protein is mitogenic towards fibroblasts and, most likely, stimulates the growth of vascular endothelial cells. We do not yet know whether these mitogenic activities reflect functions normally performed by FGF-5 in vivo. To determine the normal roles of the FGF-5 gene, it will be necessary to define the timing and location of FGF-5 expression and to characterize the range of biological responses which FGF-5 can elicit. One early clue to FGF-5 function is its expression in the neonatal brain. Since basic FGF can stimulate the survival of cultured cortical neurons and their elaboration of neurite processes (22), it is tempting to speculate that FGF-5 functions as a neurotrophic or neurotropic factor.

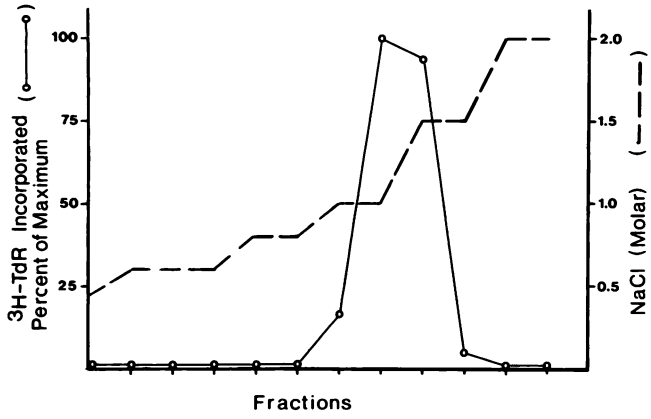

FIG. 6. Heparin-Sepharose chromatography of mitogenic activity secreted from transformed cells expressing FGF-5. NIH 3T3 cells transformed by plasmid pLTR122 (containing FGF-5 cDNA linked to LTR sequences) were used to condition 300 ml of serum-free medium. The conditioned medium was passed over a 1.0-ml heparin-Sepharose column at room temperature, and the column was washed with 20 ml of Tris buffer (pH 7.5) containing 0.45 M NaCl. Bound material was eluted stepwise with Tris-buffered NaCl solutions (0.6, 0.8, 1.0, 1.5, and 2.0 M). Ten microliters of the 1-ml fractions was assayed for the ability to stimulate DNA synthesis in quiescent BALB/c 3T3 cell cultures. Tritiated thymidine incorporation data are expressed as percentages of the maximum incorporation attainable in the assay by using 10% calf serum (1.5 × 10^6 cpm/100,000 cells).

We have found that 3 of 13 human tumor-derived cell lines expressed the FGF-5 gene. It will be important to determine whether FGF-5 is also expressed by any solid tumors. If so, this growth factor, which we expect to be efficiently secreted, may contribute to tumor growth by serving as either an autocrine factor or an angiogenic factor.

Structural features of the FGF-5 gene. The FGF-5 gene is transcribed to generate 1.6 and 4.0 kb of polyadenylated RNA species. We suspect that these RNAs differ exclusively in the length of the sequence 3′ to the FGF-5 ORF. Several pieces of evidence contribute to this interpretation. The same RNA species are expressed by the normal FGF-5 gene and by the rearranged gene, which has different upstream sequences. Second, both species are homologous to the cloned FGF-5 cDNA. Lastly, restriction enzyme analysis of several independent FGF-5 cDNA clones have failed to provide any evidence for differential splicing (X. Zhan, unpublished data). Whether the additional 3′ sequences in the 4.0-kb RNA contains an additional ORF, as exists 3′ to the *hst*/KS3 coding sequences (30), remains to be determined.

The significance of the upstream ORF in FGF-5 RNA is unclear. We could not find a sequence homology between the predicted ORF-1 protein and any data bank polypeptide sequences. It is possible that ORF-1 influences the translation efficiency of FGF-5. In at least one system, translation of a mRNA coding sequence was dramatically inhibited by the presence of an overlapping, upstream ORF (17). It will be of interest to determine whether ORF-1 is evolutionarily conserved and whether experimental deletion of ORF-1 alters the expression of FGF-5 protein.

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