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Cloning and expression of cDNA encoding human basic fibroblast growth factor

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A cDNA encoding human basic fibroblast growth factor (bFGF) was isolated from a human foreskin fibroblast cDNA library. The cDNA, 4 kilobases in size, had a coding sequence, 5′ and 3′ untranslated regions, and a poly(A) chain. Isolation of additional cDNA clones that had a short 3′ untranslated region suggested the presence of multiple mRNA forms. By Northern blot analysis, at least five bFGF mRNA species were detected in cultured fibroblast cells. Transfection of the cDNA to COS cells resulted in the detection of mitogenic activity in the culture medium of the transfected cells, suggesting that a part of the synthesized protein might be secreted from cells despite its unusual short signal sequence.

Fibroblast growth factor; cDNA; mRNA; Secretion; (COS cell)

1. INTRODUCTION

Basic fibroblast growth factor (bFGF) is a mitogen originally isolated from bovine brain and pituitary [1]. It is also present in kidney, placenta, corpus luteum, adrenal gland, retina, macrophages and chondrosarcoma [2]. It has been shown to be mitogenic and to support the differentiation of a wide variety of neuroectoderm- and mesoderm-derived cells [3], in particular that of vascular endothelial cells [4].

The complete amino acid sequence of the bovine bFGF [5] has been reported, and the cDNA [6] has been isolated recently. For human bFGF, the partial amino terminal sequence of 30 amino acids has been reported [7], but the full sequence is not yet clear.

We have cloned a full-length cDNA molecule encoding human bFGF, and report here the nucleotide sequence of cDNA and the deduced primary structure of the precursor protein molecule. We also present blot analysis of bFGF mRNA synthesized in fibroblast cells and evidence for the expression of the cDNA transfected to COS cells.

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2. MATERIALS AND METHODS

2.1. *Oligonucleotide probes*

Two oligonucleotide probes (probes 1,2) of 23 bases (b) in length, each containing the possible coding sequences for the bovine bFGF, were synthesized. Probe 1 was a 16 mixture with the sequence 5′-GGRTCYTTRAARTGGCCAGGAGG corresponding to the amino acid residues of 13–20 (Pro-Pro-Gly-His-Phe-Lys-Asp-Pro), probe 2 being a 32 mixture with the sequence 5′-TCRAARAARAARCAYTCGTCGGT corresponding to the amino acid residues of 89–96 (Thr-Asp-Glu-Cys-Phe-Phe-Phe-Gln), where R is A/G, and Y is T/C. To lower the population of the probes, a unique nucleotide was chosen at certain third positions at degenerated codons (indicated by

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underline). The probes were labelled with [γ-^{32}P]ATP (5000 Ci/mmol) by T_4 polynucleotide kinase.

2.2. *cDNA screening*

A human foreskin fibroblast cDNA library, constructed with pcDV1 vectors [8], and consisting of about 2×10^6 independent colonies of *E. coli* χ 1776, was kindly provided by Dr H. Okayama. The total plasmid prepared from this library was transfected to *E. coli* DH 1 to prepare a second library. The *E. coli* colonies from the second library were screened by the high-density colony hybridization method [9]. Two replica nitrocellulose filters were prepared from the master filters, and each was probed with ^{32}P-labelled oligonucleotides. Hybridization was carried out in 5 × SSPE (0.18 M NaCl/10 mM NaH_2PO_4, pH 7.4/1 mM EDTA pH 7.4)/5 × Denhardt's/sonicated heat-denatured salmon sperm DNA (100 μg/ml)/0.1% SDS for 16 h at 35°C [10]. The filters were washed by immersing them twice with a sufficient volume of 5 × SSC (0.15 M NaCl/0.015 M sodium citrate) containing 0.1% SDS at room temperature for 60 min, and twice more with the same solution at 48°C (probe 1) or 45°C (probe 2) for 60 min. The filters were then dried and autoradiographed to Kodak X-AR film at −70°C with intensifying screens.

2.3. *DNA sequence analysis*

Nucleotide sequence analysis was carried out by the chain-termination method [11] using restriction fragments of cDNA subcloned in the phage M13 vectors, mp10 and mp11.

2.4. *Analysis of RNA*

Human foreskin fibroblast cells (Flow 7000) were obtained from Flow Laboratories, and human embryonic lung cells (HEL) were provided by Dr I. Yamane (Tohoku University). Human colon adenocarcinoma cell lines WiDr and G361 were obtained from the Institute for Fermentation, Osaka.

Poly(A) RNA was prepared by guanidinium isothiocyanate disruption of cells and by centrifugation through 5.7 M cesium chloride [12], with further purification by oligo(dT)-cellulose chromatography. Poly(A) RNA was denatured, size-fractionated on 1.4% formaldehyde/agarose gels, and transferred to a nitrocellulose membrane [13]. Hybridizations were performed in 50% (v/v) formamide/10% (w/v) dextran sulfate/5 × SSPE/5 × Denhardt's solution/0.1% SDS/sonicated-denatured salmon sperm DNA (100 μg/ml) at 42°C for 16 h. The blots were washed three times in 0.1% SDS/2 × SSC for 20 min at room temperature, and then in 0.1% SDS/0.1 × SSC for 30 min at 55°C. The dried filters were exposed to Kodak X-AR film at −70°C with an intensifying screen.

2.5. *Transfection of DNA to COS cells*

Monkey COS 7 cells [14] were obtained from Dr P. Berg and cultured in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal bovine serum. Monolayers of COS 7 cells in a 6 cm diameter dish were transfected with 10 μg DNA according to the calcium phosphate coprecipitation method [15]. After 4 h at 37°C, the medium was replaced with 5 ml fresh DME medium containing 0.5% fetal bovine serum and cultured for 72 h. The medium was then collected and assayed for mitogenic activity.

2.6. *Biological assays*

The mitogenic activity of the COS cell medium was assayed using BALB/c3T3 cells. FGF isolated from bovine brain (Takara Shuzo, Kyoto) was used as a standard of the mitogen. BALB/c3T3 cells were plated at a density of 2×10^4 cells per 35 mm dish in 2 ml DME medium containing 5% calf serum. The next day, the medium was replaced with DME medium supplemented with 0.5% calf serum and 100 μl aliquots of sample were added. After 2 days in culture, duplicated plates were trypsinized and the cell number determined.

[^{3}H]Thymidine incorporation assay of BALB/c3T3 cells was carried out as described by Maciag et al. [16]. Cells were plated at a density of 2×10^3 cells per well of a microtiter plate (96 wells, Nunc), and the next day, the medium was replaced with 0.2 ml DME medium supplemented with 0.5% calf serum. 20 h after the addition of a 10 μl sample, [^{3}H]thymidine (1 μCi, 5000 Ci/mmol) was added to each well, and after 6 h, the cells were collected by trypsinization and the incorporated [^{3}H]thymidine counted.

3. RESULTS

3.1. *cDNA cloning and nucleotide sequence analysis*

A cDNA library, constructed from human foreskin fibroblast RNA in the Okayama-Berg vector, pcDV1, was screened. Two mixtures of probes both 23 nucleotides long were designed from the amino acid sequence of bovine bFGF. Out of 5 × 10 colonies, one cDNA clone was isolated which hybridized to both probes. The isolated plasmid, designated pTB627, has a long inserted cDNA of about 4 kb, and the restriction endonuclease cleavage map of the cDNA is shown in fig.1. The nucleotide sequence of the 4 kb insert cDNA in pTB627 and the deduced amino acid sequence of human bFGF are shown in fig.2. The amino-terminal amino acid of the mature protein was deduced from the published partial amino-terminal amino acid sequence of human bFGF [7]. The protein consists of 146 amino acids and its sequence coincides well with that reported for bovine bFGF. The two sequences differ in two amino acids: the 112th and 128th amino acid residues of human bFGF are threonine and serine and those of

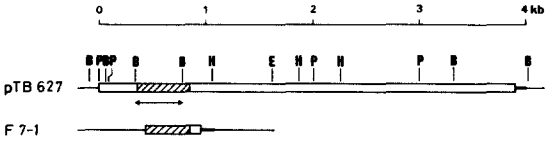

Fig.1. Restriction maps of basic FGF cDNA. pTB627 and F7-1 cDNA are shown. Thin lines and bold lines indicate vector and poly(A) tails, respectively. Shaded and open boxes indicate coding and untranslated regions, respectively. The *Bam*HI 430 b DNA fragment of pTB627 cDNA is shown by a bidirectional arrow. Restriction sites: B, *Bam*HI; P, *Pst*I; H, *Hind*III; E, *Eco*RI.

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GCCAGATTAGCGGACGCGTGCCCGCGGTTGCAACGGGATCCCGGGCGCTGCAGCTTGGGAGGCGGCTCTCCCCAGGCGGCGTCCGCGGAGACAACCATCCGTGAACCCCAGGTCCCGGCG   120
CGCCGGCTCGCCGCGCACCAGGGGCCGGCGGACAGAAGAGCGGCCGAGCGGCTCGAGGCTGGGGGACCCGGCGCGGCCGCGCGCTGCCGGGCGGGAGGCTGGGGGGCCGGGGCGGGGCCG   240

TGCCCCGGAGCGGGTCGGAGGCCGGGGCCGGGGCCGGGGGACGGCGGCTCCCCGCGCGGCTCCAGCGGCTCGGGGATCCCGGCCGGGCCCCGCAGGACCATGGCAGCCGGGAGCATCACC   360
                                                                                                                  METAlaAlaGlySerIleThr
                                                                                                                    -9
ACGCTGCCCGCCTTGCCCGAGGATGGCGGCAGCGGCGCCTTCCCGCCCGGCCACTTCAAGGACCCCAAGCGGCTGTACTGCAAAAACGGGGGCTTCTTCCTGCGCATCCACCCCGACGGC   480
ThrLeuProAlaLeuProGluAspGlyGlySerGlyAlaPheProProGlyHisPheLysAspProLysArgLeuTyrCysLysAsnGlyGlyPhePheLeuArgIleHisProAspGly
   -1  1                    10                  20                  30
CGAGTTGACGGGGTCCGGGAGAAGAGCGACCCTCACATCAAGCTACAACTTCAAGCAGAAGAGAGAGGAGTTGTGTCTATCAAAGGAGTGTGTGCTAACCGTTACCTGGCTATGAAGGAA   600
ArgValAspGlyValArgGluLysSerAspProHisIleLysLeuGlnLeuGlnAlaGluGluArgGlyValValSerIleLysGlyValCysAlaAsnArgTyrLeuAlaMetLysGlu
   40                  50                  60                  70
GATGGAAGATTACTGGCTTCTAAATGTGTTACGGATGAGTGTTTCTTTTTTGAACGATTGGAATCTAATAACTACAATACTTACCGGTCAAGGAAATACACCAGTTGGTATGTGGCACTG   720
AspGlyArgLeuLeuAlaSerLysCysValThrAspGluCysPhePhePheGluArgLeuGluSerAsnAsnTyrAsnThrTyrArgSerArgLysTyrThrSerTrpTyrValAlaLeu
   80                  90                  100                 110
AAACGAACTGGGCAGTATAAACTTGGATCCAAAACAGGACCTGGGCAGAAAGCTATACTTTTTCTTCCAATGTCTGCTAAGAGCTGATTTTAATGGCCACATCTAATCTCATTTCACATG   840
LysArgThrGlyGlnTyrLysLeuGlySerLysThrGlyProGlyGlnLysAlaIleLeuPheLeuProMetSerAlaLysSer***
  120                 130                 ↓140                146
AAAGAAGAAGTATATTTTAGAAATTTGTTAATGAGAGTAAAAGAAAATAAATGTGTAAAGCTCAGTTTGGATAATTGGTCAAACAATTTTTTATCCAGTAGTAAAATATGTAACCATTGT   960
CCCAGTAAAGAAAAATAACAAAAGTTGTAAAATGTATATTCTCCCTTTTATATTGCATCTGCTGTTACCCAGTGAAGCTTACCTAGAGCAATGATCTTTTTCACGCATTTGCTTTATTCG  1080
AAAAGAGGCTTTTAAAATGTGCATGTTTAGAAACAAAATTTCTTCATGGAAATCATCATATACATTAGAAAATCACAGTCAGATGTTTAATCAATCCAAAATGTCCACTATTTCTTATGT  1200
CATTCGTTAGTCTACATGTTTCTAAACATATAAATGTGAATTTAATCAATTCCTTTCATAGTTTTATAATTCTCTGGCAGTTCCTTATGATAGAGTTTATAAAACAGTCCTGTGTAAACT  1320
GCTGGAAGTTCTTCCACAGTCAGGTCAATTTTGTCAAACCCTTCTCTGTACCCATACAGCAGCAGCCTAGCAACTCTGCTGGTGATGGGAGTTGTATTTTCAGTCTTCGCCAGGTCATTG  1440
AGATCCATCCACTCACATCTTAAGCATTCTTCCTGGCAAAAATTTATGGTGAATGAATATGGCTTTAGGCGGCAGATGATATACATATCTGACTTCCCAAAAGCTCCAGGATTTGTGTGC  1560
TGTTGCCGAATACTCAGGACGGACCTGAATTCTGATTTTATACCAGTCTCTTCAAAACCTTCTCGAACCGCTGTGTCTCCTACGTAAAAAAAGAGATGTACAAATCAATAATAATTACAC  1680
TTTTAGAAACTGTATCATCAAAGATTTTCAGTTAAAGTAGCATTATGTAAAGGCTCAAAACATTACCCTAACAAAGTAAAGTTTTCAATACAAATTCTTTGCCTTGTGGATATCAAGAAA  1800
TCCCAAAATATTTTCTTACCACTGTAAATTCAAGAAGCTTTTGAAATGCTGAATATTTCTTTGGCTGCTACTTGGAGGCTTATCTACCTGTACATTTTTGGGGTCAGCTCTTTTTAACTT  1920
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CACCGAAATGCTGGAGGTGTTTGATCAGTTTTCAAGAAACTTGGAATATAAATAATTTTATAATTCAACAAAGGTTTTCACATTTTATAAGGTTGATTTTTCAATTAAATGCAAATTTAT  2160
GTGGCAGGATTTTTATTGCCATTAACATATTTTTGTGGCTGCTTTTTCTACACATCCAGATGGTCCCTCTAACTGGGCTTTCTCTAATTTTGTGATGTTCTGTCATTGTCTCCCAAAGTA  2280
TTTAGGAGAAGCCCTTTAAAAAGCTGCCTTCCTCTACCACTTTGCTGAAAGCTTCACAATTGTCACAGACAAAGATTTTTGTTCCAATACTCGTTTTGCCTCTATTTTACTTGTTTGTCA  2400
AATAGTAAATGATATTTGCCCTTGCAGTAATTCTACTGGTGAAAAACATGCAAAGAAGAGGAAGTCACAGAAACATGTCTCAATTCCCATGTGCTGTGACTGTAGACTGTCTTACCATAG  2520
ACTGTCTTACCCATCCCCTGGATATGCTCTTGTTTTTTCCCTCTAATAGCTATGGAAAGATGCATAGAAAGAGTATAATGTTTTAAAACATAAGGCATTCGTCTGCCATTTTCAATTAC  2640
ATGCTGACTTCCCTTACAATTGAGATTTGCCCATAGGTTAAACATGGTTAGAAACAACTGAAAGCATAAAAGAAAAATCTAGGCCGGGTGCAGTGGCTCATGCCCATATTCCCTGCACTT  2760
TGGGAGGCCAAAGCAGGAGGATCGCTTGAGCCCAGGAGTTCAAGACCAACCTGGTGAAACCCCGTCTCTACAAAAAAACACAAAAAATAGCCAGGCATGGTGGCGTGTACATGTGGTCTC  2880
AGATACTTGGGAGGCTGAGGTGGGAGGGTTGATCACTTGAGGCTGAGAGGTCAAGGTTACAGTGAGCCATAATCGTGCCACTGCAGTCCAGCCTAGGCAACAGAGTGAGACTTTGTCTCA  3000
AAAAAAGAGAAATTTTCCTTAATAAGAAAAGTAATTTTTACTCTGATGTGCAATACATTTGTTATTAAATTTATTATTTAAGATGGTAGCACTAGTCTTAAATTGTATAAAATATCCCCT  3120
AACATGTTTAAATGTCCATTTTTATTCATTATGCTTTGAAAAATAATTATGGGGAAATACATGTTTGTTATTAAATTTATTATTAAAGATAGTAGCACTAGTCTTAAATTTGATATAACA  3240
TCTCCTAACTTGTTTAAATGTCCATTTTTATTCTTTATGTTTGAAATAAATTATGGGGATCCTATTTAGCTCTTAGTACCACTAATCAAAAGTTCGGCATGTAGCTCATGATCTATGCT  3360
GTTTCTATGTCGTGGAAGCACCGGATGGGGGTAGTGAGCAAATCTGCCCTGCTCAGCAGTCACCATAGCAGCTGACTGAAAATCAGCACTGCCTGAGTAGTTTTGATCAGTTTAACTTGA  3480
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TTTTAATCAAGATAGTGTGCTTTATTCTGTTGTATTTTTTATTATTTTAATATACTGTAAGCCAAACTGAAATAACATTTGCTGTTTTATAGGTTTGAAGACATAGGAAAAACTAAGAGG  3720
TTTTATTTTTGTTTTTGCTGATGAAGAGATATGTTTAAATACTGTTGTATTGTTTTGTTTAGTTACAGGACAATAATGAAATGGAGTTTATATTTGTTATTTCTATTTTGTTATATTTAA  3840
TAATAGAATTAGATTGAAATAAAATATAATGGGAAAT(A)n
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Fig.2. Nucleotide sequence and predicted amino acid sequence of human basic FGF cDNA. The poly(A) additional signals and its analogues are underlined. The position of the start of poly(A) stretch in F7-1 cDNA is indicated by the arrow.

bovine bFGF are serine and proline. The nucleotide sequence has an open reading frame extending upstream about 300 bases from the codon for the amino-terminal proline of the mature protein to the end of the cDNA. However, the extremely GC-rich feature of this upstream region (82% of the 340 nucleotides are GC) suggests that it may be partly a noncoding sequence. In this region, only one ATG codon is found and it is in the same translational reading frame as the mature protein at the nine amino acids upstream from the amino-terminal proline. This ATG is flanked by sequences (GGACCATGG) that fulfill the Kozak [17] criteria for initiation codons (CCA/GCCATGG). So it is suggested that the translation of the precursor molecule may start at this initiator ATG codon as supposed in bovine bFGF cDNA [6]. The 3′ untranslated region of the cDNA consists of 3070 nucleotides of an AT-rich sequence. The poly(A) additional signal AATAAA [18] is found 14 nucleotides upstream of the poly(A) homopolymer stretch.

3.2. *Additional cDNA clones having different polyadenylation sites*

By re-screening the cDNA library using a nick-translated pTB627 cDNA as probe, four additional cDNA clones were isolated. The size of the cDNA inserts of these clones varied from 500 to 700 b and their restriction endonuclease maps were the same. The plasmid containing the longest cDNA insert was designated F7-1. The nucleotide sequence of this insert was found to be identical to pTB627 cDNA, although F7-1 cDNA has an extremely short 3′ untranslated region (fig.1). The poly(A) chain is initiated 97 nucleotides downstream from the termination codon and is preceded by the poly(A) additional signal 13 nucleotides upstream (denoted in fig.2). The existence of different polyadenylation sites in the cDNAs suggested the presence of multiple forms of human bFGF mRNA. Moreover, an additional AATAAA sequence (at position 3286) or analogous sequences of it could be found in the 3′ untranslated region of pTB627 cDNA.

3.3. *RNA analysis by blot hybridization*

Poly(A) RNA was extracted from human embryonic lung cells (HEL), foreskin fibroblasts (Flow 7000) and colon adenocarcinoma cell lines WiDr and G361. Northern blot analyses were made with these RNAs, using a nick-translated pTB627 cDNA as probe. Multiple forms of bFGF mRNA were detected in HEL and Flow 7000 poly(A) RNA (fig.3). At least five mRNA species, 6.4, 4.3, 2.6, 2.0 and 1.4 kb in length, could be identified on the radioautogram. The 4.3 and 1.4 kb mRNA species might correspond to the cDNA clone pTB627 and F7-1, respectively. No basic FGF mRNA could be detected in WiDr and G361 which are reported to have high angiogenic activity [26].

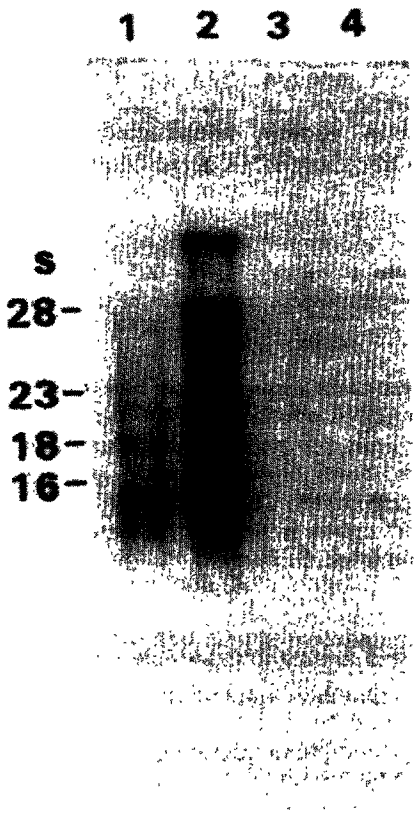

Fig.3. Blot hybridization analysis of the basic FGF mRNA. Poly(A) RNA, 15 μg per lane, was fractionated on a 1.4% formaldehyde/agarose gel, transferred to nitrocellulose and hybridized to a nick-translated *Bam*HI 430 b fragment of pTB627 cDNA. The migrating positions of the 28, 23, 18 and 16 S ribosomal subunits are indicated. Lanes: a, Flow 7000; b, HEL; c, WiDr; d, G361.

Table 1

Mitogenic activity of the conditioned medium of COS 7 cells transfected with pTB627

Culture medium	Proliferation (cells/dish)	[^{3}H]Thymidine incorporation (cpm)
COS 7 cells	1.2×10^5	1782
COS 7 cells transfected with pTB627	2.1×10^5	17186
Bovine FGF	1.9×10^5	14607

Monolayer of COS 7 cells were transfected with pTB627 by the calcium-phosphate coprecipitation method. The culture medium of control and DNA transfected cell was added to the culture of BALB/c3T3 cells and mitogenic activity was determined by cell proliferation or by incorporation of [^{3}H]thymidine. For the standard, FGF isolated from bovine brain was added to the culture at a final concentration of 0.2 ng/ml

3.4. *Expression of cDNA in COS cells*

pTB627 has a full-length cDNA inserted in the pcD vector which is designed to express inserted cDNA in mammalian cells under the control of the SV 40 promoter. Monkey COS 7 cells were transfected with pTB627, and the mitogenic activity of the culture medium was examined. As shown in table 1, the conditioned medium of COS cells transfected with pTB627 stimulated the growth of BALB/c3T3 cells. Its activity was higher than that of the medium containing 4 ng/ml of FGF isolated from bovine brain. The result confirmed that pTB627 cDNA was encoding the bFGF, and indicated that at least a part of the gene product was secreted into the medium after the cDNA was transfected to COS cells.

4. DISCUSSION

The amino acid sequence of human bFGF predicted from the nucleotide sequence of pTB627 cDNA coincided well with that of bovine bFGF. The two sequences differ in only two amino acids. The nucleotide sequence is also highly conserved, and the homology between the sequence in the coding region of human and bovine bFGF is about 95%. In the 5′ and 3′ untranslated regions, highly homologous sequences were observed with adequate alignment of both sequences. Human bFGF cDNA harbored in pTB627 has long 5′ and 3′ untranslated regions. In particular, the 3′ untranslated region extends over 3 kb to the poly(A) tail. At least three poly(A) additional signals and a few of their analogues are present in this region. Isolation of the additional cDNA clone F7-1, which has a short 3′ untranslated region, and the detection of multiple forms of bFGF mRNA in human fibroblast cells can be partly explained by multiple transcriptional termination after the poly(A) additional signals in the cDNA.

Since bFGF is a growth factor that functions extracellularly, it would be expected to have a signal peptide to direct its secretion. The region of nine amino acids preceding the mature peptide has the appearance of a hydrophobic signal peptide core sequence [20], but differs from other usual leader sequences of the proteins to be secreted by being extremely short. This feature resembles that of interleukin-1 which also has no clear signal peptide sequence [21]. By transfection of the plasmid to COS cells, the growth-stimulating activity for BALB/c3T3 cells was detected in the culture medium, suggesting that the nine amino acid region may function as leader peptide. However, we have detected about 10-times higher mitogenic activity in the lysate of DNA-transfected COS cells than in the culture medium (unpublished). So, the nine amino acids may not act sufficiently as a leader peptide and some regulatory mechanisms may exist for the secretion of bFGF.

bFGF has been isolated from several tissues, but no one has reported detecting it in fibroblast cells. We have isolated bFGF cDNA from a fibroblast cDNA library and have also detected bFGF mRNA in cultured fibroblast cells. In these cells bFGF may function by an autocrine mechanism to stimulate themselves and also by a paracrine mechanism to the endothelial cells surrounded by them.

bFGF has been isolated from a hepatoma cell line (SK-HEP 1) [22], and the reason why we failed to detect bFGF mRNA in the colon adenocarcinoma cells WiDr and G361, reported to have high angiogenic activity, is not known. Other cell lines must be examined to clarify the profile of the biosynthesis of bFGF.

ADDENDUM

In the course of the preparation of this manuscript, J.A. Abraham et al. [23] reported the cloning of human bFGF cDNA.

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