Vol. 157, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1988 Pages 256-263

Complementary DNA cloning and sequencing of rat ovarian basic fibroblast growth factor and tissue distribution study of its mRNA

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Received October 17, 1988

Three cDNA clones encoding rat basic fibroblast growth factor (FGF) were isolated from 10^6 independent clones prepared from a pregnant mare serum gonadotropin (PMSG)-stimulated rat ovarian cDNA library. One of the cDNA clones contained the entire coding sequence for basic FGF. The other two possessed the sequence coding the carboxy terminal 61 amino acids of rat basic FGF, the putative upstream intron sequence, and a 3'-noncoding region. The cDNAs encoding rat basic FGF predict a molecule consisting of 154 amino acid residues, which is one amino acid shorter than the human and bovine basic FGF. Otherwise, there are only 5 conservative amino acid substitutions between the rat and the human/bovine sequences.

Poly A^+ RNA from brain cortex and hypothalamus show a single 6.0 kb band that hybridizes to the cloned cDNA probe by Northern analyses. The observation that basic FGF mRNA is below the limits of detection in adrenal, spleen, heart, lung, kidney, liver, stomach, small intestine, large intestine, testis, and ovary support the notion that the high levels of the protein found in these tissues is due to storage of the mitogen in the extracellular matrix and not continuous gene expression. The significance of the abundance of mRNA in tissues which are not undergoing either active angiogenesis or cell proliferation (hypothalamus and brain cortex) is unclear but emphasizes the potential neuronotrophic function of basic FGF.

Basic FGF is a potent mitogen for vascular and capillary endothelial cells as well as many other cells of mesodermal or neuroectodermal origin (1,2). It has been purified from various tissues including pituitary, hypothalamus, brain, retina, kidney, adrenal gland, thymus, corpus luteum, and placenta (1,2). The structures of bovine and human basic FGF have been determined by both protein (3-5) and DNA sequencing (5-8). In both species, basic FGF exists in several microheterogeneous forms of 130-157 amino acids (1,2). While some molecular forms

Abbreviations: FGF = fibroblast growth factor; PMSG = pregnant mare serum gonadotropin

0006-291X/88 $1.50

of basic FGF are thought to be due to the conditions of extraction (9,10), *in vitro* transcription and translation analyses with human basic FGF gene have revealed multiple forms of basic FGF derived from alternative initiation codons (11). Regardless, all of the microheterogeneous forms of basic FGF appear to have identical biological activity.

In an effort to examine the regulation of basic FGF synthesis and secretion, we have been studying the effects of this growth factor in the rat. In this model, human basic FGF is a potent angiogenic factor that can stimulate vascularization of the vasa vasorum, angiogenesis in the brain, and promote peripheral and optic nerve regeneration (12-15). Because the laboratory rat is a highly developed experimental model to study the potential physiological role of endogenous basic FGF, we sought to characterize rat basic FGF. In this study, we establish the nucleotide sequence of cDNAs encoding rat basic FGF and the predicted primary structure of this mitogen. Its homology to human and bovine basic FGF is presented and its potential role in ovarian physiology is discussed.

MATERIALS AND METHODS

Tissue and RNA preparation. Sprague-Dawley rats were kept under an environment recommended by the NIH guidelines for housing laboratory animals. All tissues were collected from 6-week-old animals. Prior to the preparation of the rat ovarian cDNA library, female rats were injected with 50 IU PMSG and then, 48 hours later, their ovaries were excised. All of the tissues were frozen in liquid nitrogen immediately after excision, and stored at -80°C. Total RNA was prepared by the conventional guanidine isothiocyanate method (16) and poly A^+ RNA was selected by oligo(dT) cellulose column chromatography.

cDNA library construction and screening. Preparation of the rat ovarian cDNA library has been reported elsewhere (17). Briefly, oligo(dT)-primed double-stranded cDNAs were prepared, ligated to a synthetic adaptor containing EcoRI, XbaI, and XhoI restriction sites and then were inserted into the EcoRI site of the lambda-gt10 vector. The constructed cDNAs were packaged *in vitro* with lambda packaging extract (Gigapack Gold, Stratagene, San Diego, CA).

One million independent plaques (5×10^4 plaques per plate) were lifted onto nylon membrane filters and were hybridized with a ^{32}P-labeled synthetic human basic FGF probe in a solution of 40% formamide, 1 M NaCl, 1% SDS, 10% dextran sulfate, and 0.2 mg/ml denatured salmon sperm DNA at 37°C for 15 hr. The filters were washed with vigorous agitation in 2X SSC, 0.1% SDS for 15 min at room temperature, and then incubated in 0.2X SSC, 0.1% SDS for 10 min at 50°C. The washed filters were exposed to X-Ray films with intensifying screens at -80°C overnight. Positive plaques were screened again to isolate the pure plaques. The synthetic probe encoding the entire human basic FGF sequence was a generous gift of Drs. Paulo Sarmientos and Marco Soria of Farmitalia Carlo Erba, Milano, Italy.

Subcloning and DNA sequencing. DNAs obtained after individual amplification of the isolated phage clones were digested with Xho I (none of the insert fragments had Xho I sites) and subcloned into the Xho I site of pBluescript SK^+ (Stratagene, San Diego, CA). The DNA subcloned into pBluescript were sequenced for both strands by the double strand dideoxy chain termination method (18) using Sequenase (United States Biochem. Co., Cleveland, OH) and synthetic oligonucleotide primers (17-mers) derived from the common DNA sequence regions for both the human and bovine basic FGF cDNAs. Synthetic oligonucleotide primers were synthesized with an Applied Biosystems Model 380A DNA synthesizer and purified by gel filtration.

Northern blotting analyses. Poly A^+ RNAs were electrophoresed on a 0.66 M- formaldehyde-agarose gel and then transferred onto nylon membrane filters. Hybridization was performed with a ^{32}P-labelled probe that contains only the coding region of the cloned rat basic FGF cDNA in a solution of 50% formamide, 1 M NaCl, 1%SDS, 10% dextran sulfate, and 0.2 mg/ml denatured salmon sperm DNA. After 15 hr at 37°C, the filters were washed with vigorous agitation in 2X SSC, 0.1% SDS for 15 min at room temperature and then were incubated in 0.1X SSC, 0.1% SDS for 1 hr at 65°C. Molecular weight estimation of the hybridizing band was obtained from the relative migration rates of a commercially available RNA ladder (Bethesda Research Labs, Gaithersburg, MD) containing 9.5, 7.5, 4.4, 2.4, 1.4 and 0.3 kb polyadenylylated RNA standards.

RESULTS AND DISCUSSION

We used a PMSG-stimulated rat ovarian cDNA library to screen for cDNAs encoding rat basic FGF because a PMSG-stimulated ovary contains a large number of proliferating granulosa cells and these cells produce basic FGF *in vitro* (19). Because basic FGF may play a role in this process, we reasoned that the stimulated ovary should contain enough basic FGF mRNA to be detectable (20). In spite of this manipulation, only 3 positive plaques out of one million independent clones were detected in the screening. Fig 1 shows a schematic representation of each of these clones.

One clone (RObFGF103) was found to contain the entire coding region for rat basic FGF. Unlike the human and bovine basic FGF cDNAs which encode 155 amino acid residues, this rat cDNA encodes a protein of 154 amino acids (Fig. 2). The other two clones (RObFGF101 and 102) appeared indistinguishable from each other. They encode the carboxy terminal 61 amino acids of rat basic FGF preceded by an unrelated DNA sequence. This 5'-region in clones RObFGF101 and 102 meets the criteria of an intron sequence (data not shown) because: 1) there is no in-frame methionine codon in the region upstream of the 61 amino acids in the open reading frame; 2) the two nucleotides upstream of the first amino acid codon of this 61-amino acid tract are AG, a consensus 3'-terminal sequence of introns (21); and 3) the coding regions in the human basic FGF gene is also interrupted by an intron at the identical position.

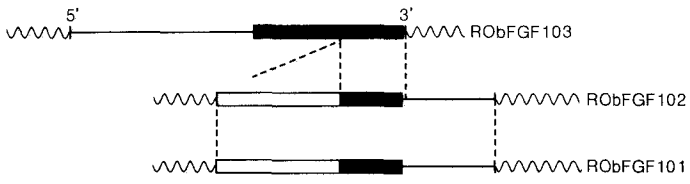

Fig. 1. Schematic representation of the rat basic FGF cDNA clones.

The coding region is represented by a filled box and putative intron region by an open box. The 5' and 3' noncoding regions are represented by solid line and the vector regions are shown by the wavy lines.

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   1 GGCACCCCATTCCTGGCCTCTGTCTCCCGCACCCTATCCCTTCACAGCCTGTGCTCTAGGGGACTGGAGA   70
  71 TTTCCAAAACCTGACCCGATCCCTCCCCAGTTCAGTTCCTTCTACTGCTTTGGGTGGAAGGCTGGTCGTT  140
 141 GTGTTAAAAGGCAGGAAGGGAGAAAGTTGCATTTAAACTTTAGGAGCTGCGTCACGGCAGTCTCCTGGAG  210
 211 AAAGCTCCGCCGAACGGGACAGATTCTTTTTGCAACTTGGAGGCGCCGGGCGTGGGGAGGAGGCGGCGCG  280
 281 CGGGGCGGGGGCGCGCGGGGCCGGGGTGCAGGCGGGGACGCGGGGTGACGCGGGCCCGGGCCGCTGTAGC  350
 351 ACACAGGGGCTCGGTCTCTCGGCTTCAGGCGGAGTCCGGCTGCACTAGGCTGGGAGCGCGGCGGGACGCG  420
 421 AACCGGGAGGCTGGCAGCCCGCGGGCGAGCCGCGCTGGGGGGCCGAGGCCGGGGTCGGGGCCGGGGAGCC  490
                                                    -9                  -1
 491 CCGAGAGCTGCCGCAGCGGGGTCCCGGGGCCGCGGAGGGGCCATGGCTGCCGGCAGCATCACTTCGCTTC  560
                                                      M  A  A  G  S  I  T  S  L
     +1                          +10                         +20
 561 CCGCACTGCCGGAGGACGGCGGCGGCGCCTTCCCACCCGGCCACTTCAAGGATCCCAAGCGGCTCTACTG  630
      P  A  L  P  E  D  G  G  G  A  F  P  P  G  H  F  K  D  P  K  R  L  Y  C
               +30                         +40
 631 CAAGAACGGCGGCTTCTTCCTGCGCATCCATCCAGACGGCCGCGTGGACGGCGTCCGGGAGAAGAGCGAC  700
        K  N  G  G  F  F  L  R  I  H  P  D  G  R  V  D  G  V  R  E  K  S  D
           +50                         +60                         +70
 701 CCACACGTCAAACTACAGCTCCAAGCAGAAGAGAGGAGTTGTGTCCATCAAGGGAGTGTGTGCGAACC  770
       P  H  V  K  L  Q  L  Q  A  E  E  R  G  V  V  S  I  K  G  V  C  A  N
                        +80                         +90
 771 GGTACCTGGCTATGAAGGAAGATGGACGGCTGCTGGCTTCTAAGTGTGTTACAGAAGAGTGTTTCTTCTT  840
      R  Y  L  A  M  K  E  D  G  R  L  L  A  S  K  C  V  T  E  E  C  F  F  F
                  +100                        +110
 841 TGAACGCCTGGAGTCCAATAACTACAACACTTACCGGTCACGGAAATACTCCAGTTGGTATGTGGCACTG  910
        E  R  L  E  S  N  N  Y  N  T  Y  R  S  R  K  Y  S  S  W  Y  V  A  L
           +120                        +130                        +140
 911 AAACGAACTGGGCAGTATAAACTCGGATCCAAAACGGGGCCTGGACAGAAGGCCATACTGTTTCTTCCAA  980
       K  R  T  G  Q  Y  K  L  G  S  K  T  G  P  G  Q  K  A  I  L  F  L  P

 981 TGTCTGCTAAGAGCTGACTCTCTTTAGACACTGTCACTCTCAGGCAGTCCCCTGTGGTAGAGCTTGTAGA 1050
      M  S  A  K  S end
1051 ACAGGCCTGTGTATACTGCAGGGAGTTCCTCCATGCTGAGGTCAATCTTGTCAAACCCTTCCCTGTGTCC 1120
1121 GTATAGTAGTAGCCTAGCCACCCTGCTGGTGATGGGGGTTGTGTGTTTAGTCCTGGCCAGGCTTTCTAGA 1190
1191 TCCATCCATTCACACTTCAAGCATTCCTGCTGGCAGAAGTTGATGGTGAAGGAACGCGGCTGCAGGCGGC 1260
1261 AGATCAGGTCTC                                                          1272
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Fig. 2. Nucleotide and deduced protein sequence of the rat basic FGF cDNA.

The nucleotides are numbered at both ends, and amino acids in one-letter code, are numbered throughout. The nearest upstream in-frame stop codon to the putative initiation methionine codon is boxed. The location of the putative intron is shown by arrow head. The DNA sequences upstream to nucleotide #282 and downstream to nucleotide #1018 are different from the corresponding regions of the sequence published by Kurokawa et al. (24), as well as some other deficiencies described in the text.

The first methionine codon at nucleotides #533-535 in the open reading frame of the composite cDNA sequences of rat basic FGF shown in Fig. 2, is likely to be the initiation site of the translation. This is supported by the fact that the sequence surrounding the ATG (GCCATGG) in the rat basic FGF cDNA sequence is in good agreement with the consensus sequence ($\begin{smallmatrix}A\\G\end{smallmatrix}$XXATGG) for the start of translation in eukaryotes proposed by Kozak (22). Thus, the cDNA encoding rat basic FGF predicts a molecule of 154 amino acids.

Comparison of the rat basic FGF amino acid sequence with human and bovine structures shows that there are five conservative amino acid substitutions and a deletion of one amino acid (Fig. 3). The high degree of the structural homology between species enabled us to sequence the whole coding region of the rat basic FGF using synthetic oligonucleotide primers (17-mers) based on the common DNA sequence regions in both human and bovine basic FGF

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          -9        +1        10        20        30        40        50        60
human     .......t.........S.....................................I.................
rat       maagsitslPALPEDGG-GAFPPGHFKDPKRLYCKNGGFFLRIHPDGRVDGVREKSDPHVKLQLQAEERGVVSIKG
bovine    .......t.........S.....................................I.................

          70        80        90        100       110       120       130       140
human     ...................D.....................T.................................
rat       ANRYLAMKEDGRLLASKCVTEECFFFERLESNNYNTYRSRKYSSWYVALKRTGQYKLGSKTGPGQKAILFLPMSAK
bovine    ...................D.....................................P.................
```

Fig. 3. Amino acid sequence comparison of rat, human, and bovine basic FGF.

Sequences are numbered according to the original nomenclature established by Esch *et al.* (4) for bovine basic FGF with the putative amino terminal extensions in lower case. Dots indicate identity with the rat sequence at that position. A gap is inserted to allow maximal homology and is represented as a dash.

cDNAs. The major difference between these basic FGFs is the fact that, in the rat there is a deletion of serine at position 9 of original bovine basic FGF (4). In view of the fact that this change is outside of the heparin and receptor binding domain of basic FGF (23), it seems unlikely that there would be any effect of this change in the biological activity of rat basic FGF.

While this work was in progress, Kurokawa *et al.* (24) presented a brief note on the nucleotide sequence of rat basic FGF using cDNA isolated from a rat brain cDNA library. The coding region of their cDNA is identical to that of ours but some sequence differences can be noted upstream from and adjacent to the potential initiation methionine as well as in the 3' noncoding region. In particular, the cytosine at nucleotide #419 and the guanines at nucleotides #465 and 497 and the stretch of nucleotides GTGACGCGGG between nucleotides #324 and #335 (see Fig. 2) are absent in their clone. Although the significance of these differences is not clear, because Florkiewicz *et al.* (11) have proposed that the sequences encoding leucine in this open reading frame can act as initiators for the translation, it will be important to reconcile these structural differences.

Basic FGF has been purified from various tissues including pituitary, hypothalamus, brain, retina, kidney, adrenal gland, thymus, corpus luteum, placenta, and several tumors (reviewed in 1,2). However, the mRNA for basic FGF has been detected only in the hypothalamus (25) so far. Although these experiments were done with bovine and/or human tissues, this finding is in agreement with our Northern analyses which show a single 6.0 kb band only in the hypothalamus and brain cortex (Fig 4). Because blots hybridized with the human β-actin probe showed equivalent amounts of the mRNA in all lanes (data not shown), it seems likely that the paucity of the basic FGF mRNA might be caused by its short half-life. This possibility is supported by our observation that two of the three rat basic FGF cDNA clones are not the product of the fully processed mRNA. In a similar vein, the results support the notion that basic FGF may be stored in a bio-unavailable form and that its regulation is at the level of bioavailability rather than synthesis (26). With the availability of rat probes for rat tissues, it should be possible to use a more sensitive method like solution hybridization with S1 nuclease protection instead of the usual Northern analyses to detect basic FGF mRNA.

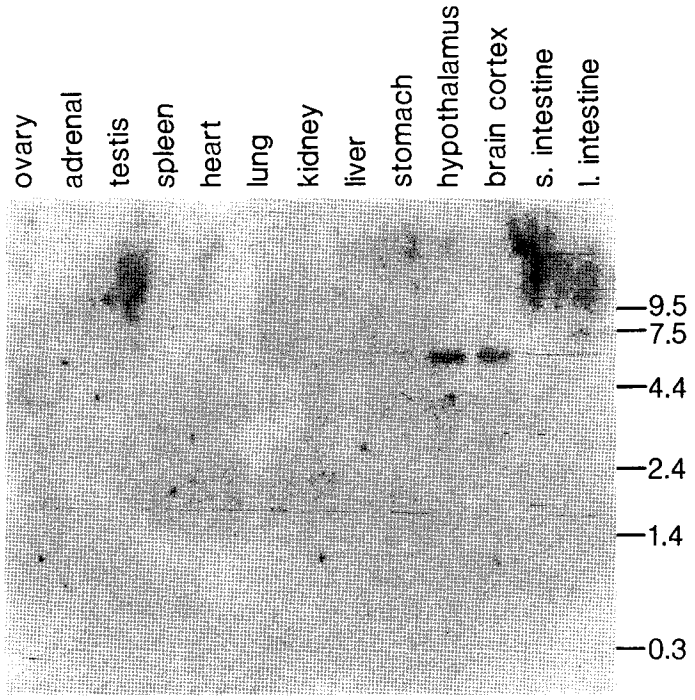

Fig. 4. Northern blot analyses of various rat tissues.

Five micrograms of poly A^+ RNA was prepared from each of the tissues listed and used in Northern analysis as described in the text. The numbers in kb correspond to the markers of a RNA ladder standard.

The availability of rat basic FGF probes should facilitate studies on the regulation of basic FGF in this experimental animal. Thus, it should be possible to determine what mechanisms regulate the expression of ovarian basic FGF. It is in this tissue in particular, that the angiogenic effects of basic FGF may play a critical role in modulating cell growth and function. The success of the onset of vascularization in the ovary and the development of a functional corpus luteum is a prerequisite for normal reproductive function, fetal growth and development. The identification of cDNAs encoding rat ovarian basic FGF is thus a natural extension of previous studies that established its presence in extracts of corpus luteum and its proposed identity with ovarian angiogenic factor (27). The results also support the observation of Sterling *et al.* (28) who failed to show the expression of basic FGF mRNA in adult bovine follicles of any size. The low abundance of cDNAs encoding rat basic FGF (3 clones) in a large library (10^6 independent clones) prepared from PMSG-treated rats supports the hypothesis that the synthesis of basic FGF in follicles is low. Thus it seems likely that the basic FGF gene is expressed only during development of the corpus luteum. Its dependence on luteinizing hormone, estrogen, and progesterone is currently under investigation. It is also interesting to note that the basic FGF mRNA is the most abundant in tissues like hypothalamus and brain cortex which are not undergoing either active angiogenesis or cell proliferation. This

result could provide an important clue to the function of basic FGF as a neuronotrophic factor and its differential synthesis and turnover in the central nervous system.

ACKNOWLEDGMENTS

We thank Dr. Roger Guillemin for his advice and encouragement, Drs. Paolo Sarmiento and Marco Soria for their generous gift of a synthetic probe encoding the entire sequence of human basic FGF, and Ms. Denise Higgins for secretarial assistance. This research was supported by Program Project Grants HD-09690 and DK-18811 from the NIH, and a grant from the Robert and Helen C. Kleberg Foundation.

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