A cDNA Encoding Fish Fibroblast Growth Factor-2, Which Lacks Alternative Translation Initiation*

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Here, we describe the isolation of a rainbow trout cDNA clone that contains the entire fibroblast growth factor-2 (FGF-2; basic FGF) coding region. Interestingly, the rainbow trout cDNA contains a translation stop codon just upstream of the primary initiating methionine codon and so cannot give rise to the longer forms of FGF-2 that are produced in mammals by alternative translation initiation at leucines farther upstream. Transfection of human FGF-2 cDNA into fish cells shows that fish cells can initiate protein synthesis at an upstream leucine CUG codon; surprisingly, however, synthesis is initiated only at the most 5’ CUG and not at the two subsequent CUG codons or the methionine AUG codon also used in mammalian cells. Like other FGF-2 proteins, bacterially produced rainbow trout FGF-2 protein binds tightly to heparin-Sepharose and also promotes the proliferation of fibroblast cells. However, the protein differs from all others previously identified at amino acids 121–123, which are part of the proposed highly conserved receptor-binding domain. Comparisons of the efficacies of recombinant wild-type and mutant rainbow trout FGF-2 proteins demonstrate that these three amino acids are critical to the activity of FGF-2.

Fibroblast growth factor-2 (FGF-2; also known as basic fibroblast growth factor) is a member of a family that now comprises nine related proteins sharing 30–55% sequence homology (1) and heparin binding affinity. Acidic FGF (FGF-1) and basic FGF (FGF-2) are the prototypes. Other members of the FGF family include the oncogene products FGF-3 (Int-2), FGF-4 (Hst/K-FGF), FGF-5, FGF-6, and FGF-7 (keratinocyte growth factor) and the more recently identified FGF-8 (androgen-induced growth factor) (2) and FGF-9 (glial activating factor) (3). FGF-2 has been cloned from human, bovine, mouse, rat, and Xenopus (4–9). The growth factor promotes the proliferation and differentiation of a wide range of mesoderm- and neuroectoderm-derived cells and, in Xenopus, is a potent inducer of mesoderm formation in developing embryos (9, 10). The strong mitogenic effect of FGF-2 on many cell types has suggested its use as a therapeutic drug in several clinical situations associated with tissue regeneration or repair (11, 12).

Two classes of FGF-2 cell-surface receptors have been described. The “low affinity” class has been identified as a heparan sulfate proteoglycan (13–15), whereas the “high affinity” class consists of at least four members, designated FGF receptors 1–4 (16, 17). Each member of this latter class has an immunoglobulin-like structure and an intracellular tyrosine kinase domain. Identifying the structure of the FGF-2 proteins that determines their interaction with these receptors has been of great interest. Two regions, corresponding to amino acids 33–77 and 115–124 in human FGF-2, have been proposed to be involved in the receptor binding and mitogenic activity of this growth factor (18). Three-dimensional structural studies, as well as other data, have indicated that the region corresponding to amino acids 115–124 in human FGF-2 is the core sequence required for the binding of FGF-2 to its receptors (19–21). In support of this assignment, this region is highly conserved among all the FGF-2 sequences obtained to date (4–9).

It has been known that several forms of FGF-2 are synthesized as a result of alternative translation initiation at an AUG start codon or two or more CUG codons located farther upstream (7, 22, 23). Subcellular localization studies have demonstrated that the AUG-initiated form is cytoplasmic, whereas the CUG-initiated forms contain a nuclear localization sequence and are mostly recovered in the nucleus (22, 24). Both forms are biologically active (25, 26), but differences in their functions have not yet been discerned.

We have undertaken the isolation of the rainbow trout homologue of FGF-2 to study its potential role in fish embryonic development, to further explore structure-function relationships through sequence comparisons, and to exploit its activities for fish cultivation. The cDNA we isolated encodes a 17.3-kDa protein that is highly homologous to mammalian FGF-2, but lacks the alternative translation start sites that give rise to the nuclear targeted forms of mammalian FGF-2 and also differs at amino acids 121–123 in the putative receptor-binding domain. To test the significance of this latter difference, we have used site-directed mutagenesis to change these amino acids in rainbow trout FGF-2 to those of mammals or to a completely different sequence and have tested their biological activities with fish and mammalian cell lines.

EXPERIMENTAL PROCEDURES

Isolation and Characterization of Rainbow Trout FGF-2 cDNA—To isolate the rainbow trout FGF-2 cDNA, a probe was first generated by PCR. Oligonucleotide primers were designed based on the amino acid sequence of the highly conserved region of FGF-2 and were then used to amplify rainbow trout testis cDNA as described previously (27). Products of the expected size were subcloned into the pSK vector (Stratagene) and sequenced. The primers that yielded a 357-bp fragment highly homologous to human (68.4%) and Xenopus (68.3%) FGF-2 proteins were as follows: primer 1, TA(T/C)TG(T/C)AA(A/G)AA(T/C)GG(A-C-T/G-G/G/A/G/C/T/G/T/C/T/C/T, and primer 2, CAT/A/G/C/T/G-G/G/G/A/G/C/G/T/G/T/C/T/T/G/T/G/G/A/G/C-T/A/A/G/A/G/G/C/T/A/G/A/G/T/A/T/A/G/C/G/T/G/T/C/T/T/T/C/T/C/T/G. A rainbow trout testis cDNA library in Agt10 (random hexanucleotide-primed) was screened by hybridization with a probe made from the cloned PCR fragment.
**Molecular Cloning of Rainbow Trout FGF-2 cDNA**

Reverse Transcription-PCR—Reverse transcription-PCR was conducted as described previously (28) with some modifications. Briefly, poly(A)-selected RNA was prepared from various rainbow trout tissues and from a cell line (RTG-2) derived from rainbow trout ovary (29). The cDNAs were synthesized using a first strand synthesis kit (Stratagene). An oligonucleotide of the first strand reaction (from brain, ovary, and RTG-2) was subjected to PCR using primers F3 and F4, which generated a 633-bp product spanning the first methionine codon: primer F3, TTCGTGATAACATTTCAGCG (positions 43–62); and primer F4, CGTGTTCAAGCTTCTCC (positions 705 to 684). The resulting PCR fragments were subcloned into the pSR vector and then sequenced.

Western Blotting for Determination of Rainbow Trout FGF-2—Tissues were homogenized in lysis buffer (10 mM Tris-HCl, pH 7.5, 0.1% Triton X-100) in the presence of protease inhibitors as instructed by the supplier (Boehringer Mannheim). After clarification by centrifugation, the extracts were applied to heparin-Sepharose CL-6B (Pharmacia Biotech Inc.). The column was washed with lysis buffer containing 0.5 mM NaCl until the buffer containing 2 mM NaCl. The column was then dialyzed against distilled water at 4 °C and lyophilized. Dissolved samples or cultured cells solubilized in the same buffer were electrophoresed on a 16% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore Corp.). After blocking, the membranes were immersed in a solution containing antiserum from a rabbit immunized with recombinant rainbow trout FGF-2 that had been purified from *Escherichia coli* as described below. The membranes were then reacted with alkaline phosphatase-conjugated anti-rabbit IgG (Sigma), and the reacted bands were visualized by nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

**Bacterial Expression and Purification of Rainbow Trout FGF-2**—PCR fragment containing the entire open reading frame, which was synthesized from M13mp19 containing the cDNA encoding rainbow trout FGF-2, was subcloned into the pET vector. A human FGF-2 expression plasmid (pCMV-hFGF-2) carrying the 475-bp EcoRI fragment described below into the EcoRI site of pcDNA3 (Invitrogen). A human FGF-2 expression plasmid (pCMV-hmFGF-2) was constructed by inserting the DNA fragment extending from the first methionine codon to the stop codon of human FGF-2 (positions 938 to 919).

Western Blotting for Determination of Rainbow Trout FGF-2—Tissues were homogenized in lysis buffer (10 mM Tris-HCl, pH 7.5, 0.1% Triton X-100) in the presence of protease inhibitors as instructed by the supplier (Boehringer Mannheim). After clarification by centrifugation, the extracts were applied to heparin-Sepharose CL-6B (Pharmacia Biotech Inc.). The column was washed with lysis buffer containing 0.5 mM NaCl until the buffer containing 2 mM NaCl. The column was then dialyzed against distilled water at 4 °C and lyophilized. Dissolved samples or cultured cells solubilized in the same buffer were electrophoresed on a 16% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore Corp.). After blocking, the membranes were immersed in a solution containing antiserum from a rabbit immunized with recombinant rainbow trout FGF-2 that had been purified from *Escherichia coli* as described below. The membranes were then reacted with alkaline phosphatase-conjugated anti-rabbit IgG (Sigma), and the reacted bands were visualized by nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

**RESULTS**

Isolation of a Rainbow Trout cDNA Encoding the FGF-2 Homologue—As a first step in isolating the rainbow trout FGF-2 cDNA clone, we designed a set of degenerate oligonucleotide primers homologous to an amino acid sequence that is highly conserved among various species. These primers were then used to generate PCR fragments from rainbow trout testis cDNA to isolate a fragment that would serve as a screening probe. A PCR product of the length predicted from the human FGF-2 gene was isolated, cloned, and sequenced. The sequence encoded 115 amino acids that had 68.4% identity to the corresponding region of human FGF-2.

Using a probe generated from this fragment, ~5 × 10^5 plaques from a rainbow trout testis cDNA library were screened, and one strongly hybridizing clone was obtained. cDNA thus obtained was sequenced.

**Structure of Rainbow Trout FGF-2**—The rainbow trout FGF-2 contains an ATG initiation codon followed by an open reading frame that encodes a 155-amino acid protein with a molecular mass of 17,317 Da. The amino acid sequence has a >70% homology to all previously isolated FGF-2 proteins and shows complete continuity with each, except for the single amino acid missing from the mouse sequence (Fig. 1). As with other FGF-2 proteins, homology is higher in the C-terminal region, which contains the putative receptor-binding domains (18). Homology is also high between the rainbow trout and human FGF-2 N-terminal domains, including a dearth of hydrophobic residues, which indicates the absence of a classical signal peptide.

**Rainbow Trout FGF-2 Lacks an Alternative N-terminal Variant**—A novel feature of the rainbow trout FGF-2 cDNA is the absence of an upstream CUG codon, which could serve to initiate an alternative N-terminal form of the protein like the nuclear targeted versions found in mammalian cells. Instead, a TAG stop codon is present 15 bases upstream of the initiating ATG codon. To test whether this stop codon might be an artifact of cloning or particular to the tissue (testis) used for the cDNA library, PCR fragments spanning this codon were amplified and sequenced from rainbow trout brain, ovary, and RTG-2 cDNAs. The presence of the stop codon was confirmed in each case (data not shown). To check further for the presence of an N-terminal variant, Western blot analysis was performed using anti-rainbow trout FGF-2 antibody. Western blot analysis of rainbow trout FGF-2 lacked the alternative N-terminal initiation variants found for mammalian FGF-2.

**Tissue Distribution of Rainbow Trout FGF-2 mRNA**—We used the highly sensitive reverse transcription-PCR technique to survey the tissue distribution of FGF-2 mRNA in rainbow trout. Expression could be detected in all tissues tested as well as...
as the RTG-2 cell line (Fig. 3), with the highest levels observed in brain, testis, and the RTG-2 cell line. No amplification was seen when the avian myeloblastosis reverse transcriptase was omitted from the cDNA synthesis reaction (data not shown).

Alternative Translation Initiation in Fish Cells—It is possible that rainbow trout FGF-2 lacks upstream initiating CUG codons because fish cells are unable to recognize them. To test this, a plasmid carrying the full-length human FGF-2 cDNA under control of the cytomegalovirus promoter (pCMV-hFGF-2) was transfected into the RBCF-1 cell line derived from goldfish and, as a positive control, into COS-7 cells. As shown in Fig. 4B (lane 9), Western blot analysis of transfected cell lysate from COS-7 cells detected three bands with the expected apparent molecular masses of 18, 22.5, and/or 23, and 24 kDa (7, 22, 23). Surprisingly, transfection of the RBCF-1 cell line yielded only the largest species (24 kDa) (Fig. 4B, lane 6). As a further control, both the RBCF-1 and COS-7 cells were transfected with a 5′-truncated form of the human cDNA (pCMV-hmFGF-2) that lacks the initiating leucine codons, but retains the initiating methionine codon. Both cell lines produced the expected 18-kDa protein when transfected with this plasmid (Fig. 4B, lanes 5 and 8).

Biological Activities of Recombinant Rainbow Trout FGF-2 and Its Mutants—Although rainbow trout FGF-2 is highly homologous to mammalian FGF-2 in the C-terminal region, it does contain nonconservative substitutions at amino acids 121–123 within the putative receptor-binding domain, which is highly conserved among all other FGF-2 sequences known (Fig. 1). To evaluate the significance of this difference, we compared the activities of the wild-type human and rainbow trout FGF-2 proteins and also mutant rainbow trout FGF-2 proteins in which the human amino acids were inserted into positions 121–123 (FGF-2/TSW) or in which these three amino acids were replaced with alanines (FGF-2/AAA). Each of the recombinant proteins was produced in *E. coli* and purified by heparin-Sepharose CL-6B affinity chromatography. As shown in Fig. 5, the elution profiles of each of the FGF-2 proteins were similar, suggesting that the mutations do not affect the heparin binding affinity. The mutations also do not appear to affect the stability of the proteins, as both the wild-type and FGF-2/AAA mutant rainbow trout proteins showed complete stability when incubated for up to 48 h at 37 °C in the medium (data not shown). In addition, the rates of degradation of the two proteins by trypsin were indistinguishable, and heparin protected both from trypsin digestion (data not shown). The biological activity of the mutant and wild-type proteins was evaluated by testing their ability to induce proliferation of cultured fibroblast cell lines from goldfish (RBCF-1) and mice (BALB/3T3). The
human FGF-2 protein was 4- and 6-fold more potent than the wild-type rainbow trout FGF-2 protein when tested with BALB/3T3 cells and RBCF-1 cells, respectively (Fig. 6). Interestingly, when the human amino acids were substituted for those of rainbow trout in positions 121–123 (mutant FGF-2/AAA), activity was increased 2-fold when tested with either cell type (Fig. 6). These results suggest that amino acids 121–123 contribute to the activity of FGF-2. The importance of these three amino acids is further underscored by the nearly complete absence of activity of the FGF-2/AAA mutant with either cell line (Fig. 6).

DISCUSSION

By using PCR to generate a homologous probe, we have isolated a rainbow trout FGF-2 cDNA clone that contains the entire coding sequence for the protein. An unexpected finding was that the cDNA contains an in-frame TGA stop codon just upstream of the initiating ATG codon, so rainbow trout cannot produce the alternative forms of FGF-2 found in mammals. The absence of alternative forms was confirmed by Western blotting of rainbow trout tissue extracts. A search of the EMBL database revealed that an in-frame TGA stop codon is also present 15 bases upstream of the initiating ATG codon of *Xenopus* FGF-2, although its significance has not previously been remarked upon. Why rainbow trout lacks alternative translation initiation forms of FGF-2 awaits understanding of the roles of the different forms in mammals. Purified FGF-2 variants have the same mitogenic activity and seem to interact with the same cell-surface receptors. However, the alternative forms have been found to localize to different subcellular compartments, suggesting that they carry out distinct functions (22, 24, 37, 38): the human (22, 24) and rat (39) AUG-initiated forms have been found primarily in the nucleus. Similar observations have been made for FGF-3 (40), suggesting that the alternative initiation mechanism might be a common feature among mammalian members of the FGF family.

Although the presence of an in-frame stop codon just 15 bp upstream of the rainbow trout initiating methionine codon precludes alternative initiation of FGF-2 from farther 5′ CUG codons, we have shown that fish cells are capable of initiating from such codons when present in the proper context. Surprisingly, initiation occurred only from the most 5′ CUG codon in the fish cells, whereas mammalian cells use, in addition, two other CUG codons and the methionine codon. One possible explanation of this result, based on the scanning model of Kozak (41), is that initiation at the 5′ CUG is actually more efficient in fish cells than in mammalian cells, leaving too few scanning ribosomes to initiate at the subsequent sites.

The encoded protein sequence of the rainbow trout cDNA is highly homologous to that of other species, especially in its C-terminal half. In this conserved region, human FGF-2 residues 115–124 (YRSRKYSSWY) have been shown to make a somewhat open loop on the surface of the molecule, which is presumed to bind to the FGF receptors (19, 20). It is therefore of particular interest that three nonconservative amino acid substitutions are present in rainbow trout FGF-2 residues 121–123 (PEM) as compared with human (TSW) and mouse, bovine, and *Xenopus* (SSW) FGF-2 proteins (Fig. 1).

To determine whether the substitutions of these residues could affect the FGF-2 biological activity or its specificity, two mutant FGF-2 proteins have been characterized in which FGF-2 residues 121–123 (PEM) have been substituted with TSW and AAA by site-directed mutagenesis. The FGF-2/AAA
protein has similar heparin binding affinity compared with wild-type or FGF-2/TSW mutant protein (Fig. 5). Surprisingly, the mutant human (FGF-2/TSW) protein showed an increased activity relative to wild-type rainbow trout FGF-2 on the fish fibroblast cells as well as the mouse cells (Fig. 6). These results are consistent with previous studies that have suggested that residues 121–123 have an important role for both mitogenic function and heparin binding, but have presented data showing to claim little effect on heparin binding. We are beginning a more extensive mutagenesis program in an effort to further probe the functionality of this region of the protein.

In summary, we have isolated a cDNA that encodes the complete rainbow trout FGF-2. The cDNA reveals two striking differences from mammalian FGF-2: the absence of alternative N-terminal forms and the nonconservative substitution of amino acids in the region believed to be critical for receptor binding and mitogenic activity. The availability of recombinant rainbow trout FGF-2 should facilitate functional analysis of FGF-2 through comparative studies with the mammalian proteins and should also have practical applications to fish aquaculture.

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REFERENCES
1. Burgess, W. H., and Maciag, T. (1989) Annu. Rev. Biochem. 58, 575–606
2. Tanaka, A. K., Miyamoto, N., Minamino, M., Takeda, M., Sato, M., Matsuo, H., and Metzumoto, K. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8928–8932
3. Miyamoto, M., Narno, K. I., Seko, C., Matsumoto, S., Kondo, T., and Kurokawa, T. (1993) Mol. Cell. Biol. 13, 4251–4259
4. Abraham, J. A., Mergia, A., Whang, J. L., Tumolo, J., Friedman, J., Gospodarowicz, D., and Fiddes, J. C. (1988) EMBO J. 7, 2533–2538
5. Abraham, J. A., Mergia, A., Whang, J. L., Tumolo, Friedman, J., Merril, K. A., Gospodarowicz, D., and Fiddes, J. C. (1986) Science 233, 545–548
6. Shimakawa, S., Emoto, N., Koba, A., Mercado, M., Shibata, F., Cocks, K., Baird, A., and Ling, N. (1988) Biochem. Biophys. Res. Commun. 157, 256–263
7. Prats, H., Kaghad, H., Prats, A. C., Klagsbrun, M., Lelias, J. M., Liazun, P., Chalen, P., Toubier, P., Amalric, F., Smith, J. A., and Caput, D. (1989) Proc. Natl. Acad. Sci. U. S. A. 85, 1836–1840
8. Hebert, J. M., Basilio, C., Goldfarb, M., Haub, O., and Martin, G. R. (1990) Dev. Biol. 138, 454–463
9. Kimelman, D., Abraham, J. A., Haaparanta, T., Palisi, T. M., and Kirschner, M. W. (1988) Science 242, 1053–1056
10. Kimelman, D., and Kirschner, M. (1987) Cell 51, 869–877
11. ten Dijke, P., and Iwata, K. K. (1989) Bio/Technology 7, 793–798
12. Tsuki, R., and Rikfin, D. B. (1990) J. Exp. Med. 172, 245–251
13. Vlodavsky, I., Feldman, J., Sullivan, R., Fridman, R., Ishai-Michaeli, R., Sasse, J., and Klagesbrun, M. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2292–2296
14. Moscatelli, D. (1988) J. Cell Biol. 107, 753–759
15. Bashkin, P., Doctrow, S., Klagesbrun, M., Svahn, C. M., Folkman, J., and Vlodavsky, I. (1989) Biochemistry 28, 1737–1743
16. Moscatelli, D. (1988) J. Cell. Physiol. 131, 123–130
17. Neufeld, G., and Gospodarowicz, D. (1985) J. Biol. Chem. 260, 13860–13868
18. Baird, A., Schubert, D., Ling, N., and Guillemin, R. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2322–2328
19. Zhu, X., Komiyama, H., Chirina, A., Faham, S., Fox, G. M., Arakawa, T., Hsu, B. T., and Rees, D. C. (1991) Science 251, 90–93
20. Eriksson, A. E., Cousens, L. S., Weaver, L. H., and Matthews, B. W. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3441–3445
21. Thompson, L. D., Panteliano, M. W., and Springer, B. A. (1994) Biochemistry 33, 3831–3840
22. Bugler, B., Amalric, F., and Prats, H. (1991) Mol. Cell. Biol. 11, 573–577
23. Florkiewicz, R. Z., and Sommer, A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3978–3981
24. Renko, M., Quarto, N., Morimoto, T., and Rikfin, D. B. (1989) J. Cell. Physiol. 144, 108–114
25. Coultet, B., Prats, H., Bayad, F., and Amalric, F. (1991) Cell Regul. 2, 709–718
26. Quarto, N., Talarico, D., Florkiewicz, R., and Rikfin, D. B. (1991) Cell Regul. 2, 699–708
27. Yamada, S., Hata, J., and Yamashita, S. (1993) J. Biol. Chem. 268, 24361–24366
28. Inoue, K., Yamada, S., and Yamashita, S. (1995) J. Mol. Biotechnol. 1, 131–134
29. Wolf, K., and Quimby, M. C. (1986) Science 125, 1055–1066
30. Shima, A., Nikaido, O., Shinozuka, S., and Egami, N. (1980) Exp. Gerontol. 15, 305–314
31. Gluzman, Y. (1981) Cell 23, 175–182
32. Yamashita, S., Wada, K., Horikoshi, M., Gong, D. W., Kukubo, T., Hisatake, K., Yokotani, N., Malik, S., Roeder, R. G., and Nakatani, Y. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2839–2843
33. Yamashita, S., Hisatake, K., Kukubo, T., Doh, K., Roeder, R. G., Horikoshi, M., and Nakatani, Y. (1993) Science 261, 463–466
34. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 367–382
35. Gospodarowicz, D. (1974) Nature 242, 175–182
36. Hata, J. I., Tamaru, T., Yokoshima, S., Yamashita, S., Kabeno, S., Matsumoto, K., and Onodera, K. (1987) Cell Regul. 2, 1059–1068
37. Amalric, F., Bouchu, G., Bonnet, H., Brethouen, P., Roman, A. M., Truchet, I., and Quarto, N. (1994) Biochem. Pharmacol. 47, 111–115
38. Powell, P. P., and Klagsbrun, M. (1991) J. Cell. Physiol. 148, 202–210
39. Arland, M., Dixon, M., Peters, G., and Dickson, C. (1990) Nature 343, 662–665
40. Kezak, M. (1991) J. Biol. Chem. 266, 19867–19870