BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **223,** 19–23 (1996)
ARTICLE NO. 0839

The Basic Fibroblast Growth Factor (FGF-2) Antisense RNA (GFG) Is Translated into a MutT-Related Protein *in Vivo*

Audrey W. Li,* Catherine K. L. Too,† and Paul R. Murphy*,‡,[1]

*Departments of *Physiology and Biophysics, †Biochemistry, and ‡Obstetrics and Gynaecology, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7*

Received April 25, 1996

The basic fibroblast growth factor (FGF-2) gene is transcribed bidirectionally to yield multiple sense (coding) transcripts and a unique 1.5 kb antisense transcript which may regulate sense RNA stability. The antisense RNA also contains a long open reading frame that predicts a hypothetical protein with homology to the prokaryotic MutT antimutator proteins. However, translation of this protein has not previously been demonstrated. We employed antibodies against the conserved MutT-domain of the deduced human FGF-2 antisense protein (GFG) to demonstrate expression of an immunoreactive 24 kDa protein in liver extracts from *Xenopus laevis*, and two proteins of 28 and 35 kDa in rat liver. In rats, GFG protein expression detected by western blot was tissue-specific and correlated with the level of FGF-2 antisense mRNA expression. These findings demonstrate that, in addition to its possible RNA regulatory function, the FGF-2 antisense transcript is translated into a conserved MutT-related protein.

Basic fibroblast growth factor (bFGF, FGF-2) is a member of the heparin-binding FGF family of mitogens which mediate a variety of physiological processes including embryogenesis, mesoderm induction, differentiation, angiogenesis and wound healing (1). The FGF-2 gene transcribes multiple polyadenylated RNAs from both the sense and antisense strands (reviewed in 2). We have recently cloned a fragment of the human FGF-2 antisense cDNA which we call GFG (2) and have identified FGF-2 antisense RNA expression in polyA$^+$RNA from a variety of human and rat tissues (3, 4). Sense and antisense FGF-2 RNAs are expressed in a tissue-specific and developmentally regulated manner in the rat, with highest antisense expression in the early postnatal liver (4).

Although a role for the antisense RNA in regulating FGF-2 mRNA stability has been suggested, the antisense RNA also contains an open reading frame (ORF) which, in *Xenopus laevis*, predicts a hypothetical 24 kDa protein of unknown function (5, 6). This open reading frame is conserved in the human FGF-2 antisense cDNA sequence (2). The deduced amino acid sequence of the *Xenopus* and human FGF-2 antisense ORFs contain a conserved motif (MutT domain) characteristic of the *Escherichia coli* MutT and *Streptococcus pneumoniae* MutX antimutator proteins (7, 8). The MutT motif forms a loop-helix-loop nucleotide binding domain found in a limited number of other prokaryotic and eukaryotic proteins (9–11), including the human and rat MutT homologues, hMTH (12) and rMTH (13). The *E. coli* MutT gene product and the human and rodent homologues are 8-oxo-dGTPases which remove oxidatively damaged guanosine triphosphates from the intracellular nucleotide pool (12–14). The conservation of this domain in the deduced sequence of the *Xenopus* and human FGF-2 antisense ORFs supports the possibility that they encode a functional protein. However, translation of the FGF-2 antisense RNA *in vivo* has never been demonstrated. In the present study we used antibodies against the MutT domain of the hypothetical human FGF-2 antisense protein to identify the translation product of the FGF-2 antisense RNA in rat tissues.

MATERIALS AND METHODS

Chemicals. Deoxynucleotide triphosphates (dNTPs) and protein A-Sepharose were purchased from Pharmacia Fine

[1] To whom correspondence should be addressed at the Department of Physiology and Biophysics. Fax: (902)-494-1685.

0006-291X/96 $18.00

Chemicals, Dorval, Quebec, Canada. ^{32}P-αUTP and ^{35}S-methionine were from Amersham, Oakville, Ontario, Canada. The *Xenopus laevis*, FGF-2 antisense cDNA clone (XF11) was generously provided by Dr. David Kimelman, University of Washington, Seattle, WA. Protein molecular weight markers and 4–20% SDS acrylamide Ready Gels were from BioRad, Mississauga, Ontario, Canada. All other chemicals were from Sigma Chemical Company, St. Louis, Mo., unless otherwise noted.

Preparation of antiserum. A synthetic multi-antigen peptide (MAP) containing eight copies of the sequence SEPGE-DIGDTAVREVFEET on a branched lysine core was synthesized by Research Genetics Inc., Huntsville, AL. This sequence corresponds to an internal region of the deduced amino acid sequence of the human FGF antisense protein which has strong homology to the predicted protein in *Xenopus laevis*, (Fig 1). For immunization the MAP antigen was emulsified by mixing with an equal volume of Freund's Adjuvant and injected into three to four dorsal subcutaneous sites on New Zealand white rabbits for a total 0.5 mg of peptide per immunization. Serum was collected from the auricular artery two weeks after the third boost and immunoglobulins were purified by affinity chromatography on Protein A-Sepharose.

In vitro translation and immunoprecipitation. A coupled transcription/translation system (TnT, Promega, Madison, WI) was used for eukaryotic *in vitro* translation of the *Xenopus laevis* FGF-2 antisense cDNA. A 1.46 kb insert of the *Xenopus laevis* FGF-2 antisense cDNA was subcloned into the EcoR1 site of the expression vector pcDNA3 (In Vitrogen, San Diego, CA) in both orientations for expression with the T7 promoter. Circular plasmid DNA (1 μg) was added to a 25 μl reaction containing 12.5 μl of rabbit reticulocyte lysate, 40 μCi ^{35}S-methionine (specific activity 1000 Ci/mmol), 20 μM amino acid mixture (without methionine), 40 U ribonuclease inhibitor, 1x TnT buffer and T7 polymerase as recommended by the supplier. After incubation at 30 C for 120 minutes, the reaction was snap frozen and stored at −80 C until further analysis. For immunoprecipitation, one half of the reaction (12.5 μl) was diluted in ice cold NET buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris pH 7.4 containing 0.1% Nonidet P-40 and 0.25% gelatin). Anti-GFG IgG (5 μl) was added and the mixture was incubated with gentle shaking on ice for 60 min. Protein A-Sepharose (50 μl bed volume) was added and incubated for a further 60 min, after which the antigen-antibody-Protein A-Sepharose complex was collected by centrifugation at 12,000 xg for 20 seconds. After washing (2 × 20 minutes in 1 ml NET buffer, 1 × 15 minutes in 1 ml 10 mM Tris pH 7.4 containing 0.1% Nonidet P-40), the immunoprecipitate was eluted from the Protein A-Sepharose by boiling for 10 minutes in 40 μl Laemmli gel loading buffer. Radioactive bands were detected by autoradiography after electrophoretic separation on linear 4–20% gradient gels.

Western blotting. Tissues samples were collected from immature (<2 month old) *Xenopus laevis* and from 14 day old Sprague Dawley rat pups and homogenized in ice-cold extraction buffer consisting of 50 mM Tris pH 7.4 containing 150 mM NaCl, 1 mM EGTA, 1% NP-40, 0.25% deoxycholate, 1 mM phenylmethylsulfonylfluoride, and 3 μg/ml each pepstatin A, leupeptin and antipain. The crude homogenates were centrifuged for 10 minutes at 1000 ×g at 4 C, and the resulting supernatants were removed for further processing or stored frozen at −80 C until use. For immunoblot analysis the extracts were subjected to electrophoresis in 4–20% linear gradient SDS-polyacrylamide gels and transferred to nitrocellulose membranes as previously described (15). After blocking overnight at 4 C in Tris buffered saline/0.5% Tween-20 (TBS-T) containing 10% milk powder (blocking solution) the membranes were incubated for 1 hour at room temperature with polyclonal anti-GFG serum or pre-immune serum (1:500 dilution) or purified IgG (10 μg/ml) in blocking solution. The blots were then washed in TBS-T (4 × 10 minutes) and incubated for 1 hour at room temperature with goat-anti rabbit IgG-horse radish peroxidase conjugate (1:3000 in blocking solution). Immunoreactive bands were visualized by chemiluminescence using a commercially available kit and protocols recommended by the manufacturer (ECL, Amersham, Oakville, ONT). Blots were exposed to X-ray film (DuPont NEF-496) for 0.5 to 10 minutes at room temperature without an intensifying screen.

Northern hybridization. Total RNA was isolated by sequential extraction with guanidium isothiocyanate, phenol and chloroform as previously described (4). After electrophoretic separation in 1.5% agarose formaldehyde gels, the RNA was transferred to Nylon membranes by downward alkaline capillary blotting and probed with a strand-specific ^{32}P-labeled cRNA probe complementary to exons 3 and 4 of the human FGF-2 antisense gene (4).

RESULTS AND DISCUSSION

Conservation of the MutT domain in the human FGF-2 antisense ORF. As shown in Fig. 1, the predicted amino acid sequence of the human FGF-2 antisense protein (GFG) contains a consensus MutT domain with 53% identity to the *E. coli* MutT sequence, and 89% identity with the predicted *Xenopus* antisense protein. The MutT sequence motif is defined (7) as Gx_5Ex_4[UZ]xRE[Z]xEE where x may be any amino acid, U is a bulky aliphatic residue (S/T/A/G/C) and Z is a bulky hyrophobic residue (L/I/V/M/A). The human GFG MutT domain has greater similarity to the *E. coli* and *X. laevis* MutT domains (53–89% identity) than to the human and rat MTH sequences (46% identity). Preliminary data indicate that the rat GFG sequence is identical to the human GFG sequence in the MutT domain (16). Outside the conserved MutT domain the deduced human and *Xenopus* proteins are >70% identical, but have little similarity to the prokaryotic or mammalian

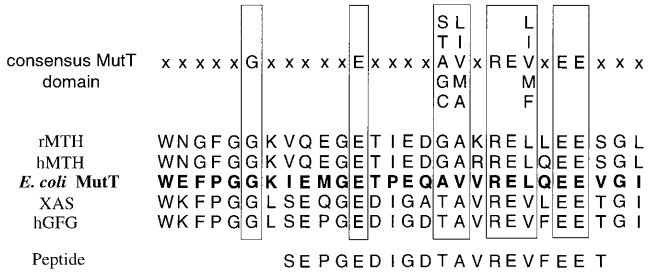

FIG. 1. Comparison of MutT domain sequences. The consensus MutT core motif and flanking amino acid sequence are shown at the top. The prototypic *E. coli* antimutator MutT domain (in bold) is flanked above by its human (hMTH) and rat (rMTH) homologues, and below by the hypothetical *Xenopus laevis* (XAS) and human (hGFG) FGF-2 antisense proteins. GenBank/EMBL accession numbers are L31408 (GFG), M27398 (XAS), D16581 (hMTH), D49977 (rMTH) and P08377 (MutT). The sequence of the immunizing peptide is shown at the bottom.

MutT proteins (not shown). A 19 amino acid sequence within the human GFG MutT domain was identified as a suitable immunogen (Fig. 1) and a synthetic multiple antigen peptide, $(SEPGEDIGDTAVREVFEET)_8$MAP was used to generate polyclonal antiserum for immunodetection of the GFG protein. As shown in Fig. 2A, the anti-GFG antiserum detected the immunizing multiple antigen peptide (predicted molecular size = 18.2 kDa) on western blots after SDS-PAGE.

Immunoprecipitation of recombinant Xenopus antisense FGF-2 protein. We next examined the ability of the antiserum to recognize full length recombinant GFG protein. Since the human GFG cDNA is not full length, we used the *Xenopus* FGF-2 antisense cDNA, generously provided by Dr. David Kimelman. The strong conservation of the predicted amino acid sequence in the MutT domain (100% between human and rat; 89% between human and *Xenopus*) supported the possibility that the polyclonal antiserum would detect the *Xenopus* protein. The 1.46 kb insert was subcloned into pcDNA3 in both orientations for *in vitro* transcription and translation. As shown in Fig. 2B, the anti-GFG antiserum specifically immunoprecipitated the predicted 24 kDa protein. We believe the less abundant larger band may be attributable to translation initiation upstream of the putative initiator AUG. The 183 nucleotides upstream of the presumptive initiator AUG in the Xenopus cDNA are in-frame and translation of a product with the additional 61 amino acids may explain the approximately 31 kDa band.

Western blot detection of native GFG protein. The pattern of antisense RNA expression in postnatal *Xenopus laevis* tissues has not been reported. However, we have previously demonstrated

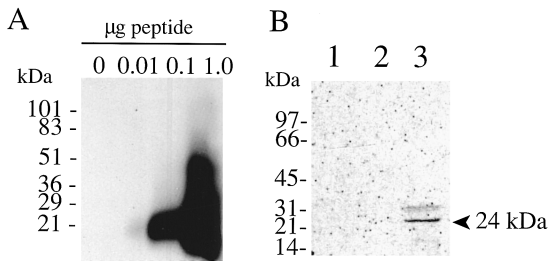

FIG. 2. (A) Immunoblot detection of the synthetic MutT peptide. Serial dilutions of the immunizing peptide (1.0–0.01 μg/lane) were subjected to SDS-PAGE and transferred to nitrocellulose for immunoblotting as described in Materials and Methods. Autoradiographic exposure time was 30 seconds. The predicted size of the multiple antigen peptide is 18.2 kDa. *(B)* Immunoprecipitation of *Xenopus* FGF-2 antisense protein. Following *in vitro* transcription/translation of the pcDNA3 vector alone (lane 1) and vector containing the *Xenopus* antisense cDNA in the noncoding or coding orientation (lanes 2 and 3 respectively), ^{35}S-methionine labeled samples were immunoprecipitated with anti-GFG IgG and protein A-Sepharose as described in *Materials and Methods.* Molecular masses (in kDa) were estimated from the migration of BioRad prestained (A) and nonstained (B) molecular weight markers.

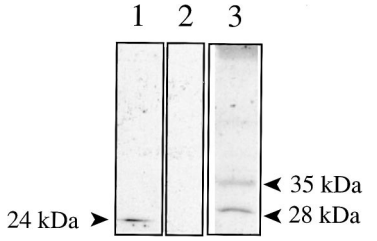

FIG. 3. Western blot detection of native FGF-2 antisense protein in liver extracts. Liver extracts from immature female *Xenopus laevis* (lanes 1 and 2) and 23 day old rat (lane 3) were subjected to SDS-PAGE on a linear 4–20% gradient gel and transferred to nitrocellulose for western blotting with anti-GFG antiserum (lanes 1, 3) or pre-immune serum (lane 2). Each lane contained 50 μg of protein.

that the antisense RNA is expressed in rat in a tissue- and age-specific pattern, with highest levels occurring in early post-natal liver (4). We therefore prepared crude extracts of *Xenopus* and rat liver for western blot analysis. As shown in Fig. 3, the anti-GFG antiserum specifically detected a prominent 24 kDa immunoreactive band in *Xenopus* liver extracts. In contrast, rat liver extracts consistently gave 2 immunoreactive products of 28 and 35 kDa (Fig 3, right panel). The larger size of the rat GFG protein is compatible with our recent observation (16) that the putative initiator methionine in the *Xenopus* sequence is replaced by a leucine in the rat sequence. The initiator methionine in the longer rat GFG ORF predicts the observed 35 kDa product. A potential CUG initiation codon with a flanking consensus Kozak sequence (17) may initiate translation of the 28 kDa immunoreactive band (16). Translation initiation of FGF-2 from CUG codons is well documented (18). Alternatively, the two bands may be derived by post-translational modification or proteolytic processing of the primary product. Although it is possible that the antiserum cross-reacts with other MutT-related proteins, it should be noted that all of the other MutT proteins reported to date are smaller than 20 kDa (8–13).

In order to establish that the expression of the immunoreactive 24 kDa band correlates with GFG mRNA expression, we next compared GFG mRNA expression with GFG-like immunoreactivity in rat liver and brain. GFG mRNA expression is highest in rat liver, and lowest in brain where FGF-2 sense mRNA is abundantly expressed (4). As shown in Fig. 4, the relative abundance of the

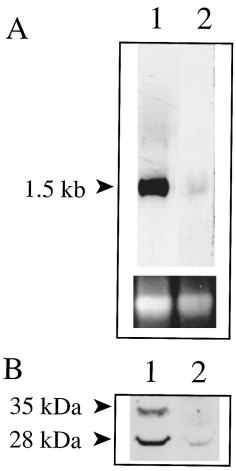

FIG. 4. Tissue-specific expression of GFG mRNA and protein. **(A)** Northern blot of total RNA from 20 day old rat liver (lane 1) and brain (lane 2) probed with the human GFG cRNA probe. Ethidium bromide stained 28S ribosomal RNA (lower panel) was used to confirm approximately equal loading. **(B)** Western blot of crude protein extracts from 20 day old rat liver (lane 1) and brain (lane 2). Each lane contained 100 μg of protein.

GFG-like immunoreactive bands in liver and brain correlated with the steady-state level of GFG mRNA in these tissues.

These findings demonstrate for the first time that the open reading frame of the FGF-2 antisense RNA is translated *in vivo*. Although its function remains unclear, the presence and conserved nature of the MutT domain signature in the deduced amino acid sequence identify it as a novel member of the MutT family of dNTPases (8). The *E. coli* MutT protein is an 8-oxo-dGTPase that hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP by the reaction $NTP^{4-}+H_2O \rightarrow NMP^{2-}+PP_i^{4-}+2H^+$ (19). Loss of activity of this gene in *E. coli* results in a 100–1000-fold increase in spontaneous mutation rates. The human and rodent homologues of the *E. coli* MutT protein are also 8-oxo-dGTPases, and have been shown to complement MutT function in MutT-deficient *E. coli.* (12). The FGF-2 antisense gene product appears to be another member of this family. In this regard it is interesting to note that the highest levels of GFG mRNA expression are seen in tissues with a high rate of oxidative metabolism (e.g. liver, kidney, adrenal) and in testis, which has a large population of rapidly dividing cells (3,4). This pattern of distribution is consistent with the postulated function of this protein in removing oxidatively damaged guanines from the nucleotide pool in cells with a high oxidative metabolism or high percentage of replicating cells. We are currently investigating the ability of the full length rat GFG protein to complement MutT 8-oxo-dGTPase activity in a MutT-deficient *E. coli* strain.

ACKNOWLEDGMENTS

We are grateful to Dr. David Kimelman (University of Washington) for the XF11 cDNA and to Dr. Richard Wassersug (Dalhousie University) for providing *Xenopus laevis* tissues for this study. Critical reading of the manuscript by Dr. W. H. Moger is greatly appreciated. This research was supported by grants to P.R.M. from the Medical Research Council of Canada and the Atlee Endowment.

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