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# MOLECULAR CLONING AND EXPRESSION OF THE MAMMALIAN BASIC FIBROBLAST GROWTH FACTOR ANTISENSE mRNA

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Submitted in Partial Fulfillment of the Requirements for the

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The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "Molecular Cloning and Expression of the Mammalian Basic Fibroblast Growth Factor Antisense mRNA"

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## ABSTRACT

The fibroblast growth factor (FGF-2) gene is bidirectionally transcribed wherein the sense DNA strand yields multiple mRNA species responsible for encoding FGF-2 protein, and the antisense strand generates a single 1.5 kb mRNA. Formation of double-stranded complexes between sense and antisense mRNAs has been suggested as a posttranscriptional mechanism in the regulation of FGF-2 gene expression. The majority of this thesis describes the cloning and characterization of the rat FGF-2 antisense (FGF-AS) mRNA.

Northern and RT-PCR analyses of various rat and human tissues reveal that the AS mRNA is expressed in a tissue-specific and developmentally regulated fashion. A neonatal rat liver library was constructed for the isolation of a full-length rat FGF-AS cDNA. Sequence analysis reveals that the rat FGF-AS transcript is a 1.1 kb polyadenylated RNA with a conserved ORF that specifies a novel 35 kDa protein. The rat AS and sense mRNAs contain 425 bp of complementary overlap at their 3' regions. Inverse levels of FGF-2 sense and AS mRNA observed in brain, liver, and certain tumor cell lines, are consistent with the possibility of AS regulation of FGF-2 mRNA.

The FGF-AS mRNA encodes a 35 kDa protein which is a novel member of the family of MutT/nudix hydrolyases. This diverse family of enzymes includes the antimutator 8-oxo-dGTPases which hydrolyze mutagenic 8-oxo-dGTP and thereby suppress the occurrence of spontaneous oxidative mutagenesis. Sequence-specific antibodies recognized the *in vitro* translated antisense protein and detected a 35 kDa protein in Western blots of rat and mouse tissues. FGF-AS mRNA expression in rat glioma cells was shown to increase in response to hydrogen peroxide-induced oxidative stress. The abundant expression of FGF-AS in neonatal liver and tissues such as adrenal, kidney and heart, suggests that the functional role of the antisense protein is likely related to the high metabolic function and/or oxidative stress inherent to these tissues.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| ABAE | adult bovine aortic endothelial |
| Abd-B | abdominal-B |
| altFGF-2 | alternatively spliced basic fibroblast growth factor |
| AMV | avian myeloblastosis virus |
| Ap4A | diadenosine 5', 5'''-P1, P4 tetraphosphate |
| bp | basepair |
| BLAST | basic local alignment search tool |
| BSA | bovine serum albumin |
| cAMP | cyclic adenosine monophosphate |
| cDNA | complementary DNA |
| CNS | central nervous system |
| cpm | counts per minute |
| CKII | casein kinase II |
| cRNA | complementary RNA |
| CYP | steroid 21 hydroxylase |
| ATP | adenosine triphosphate |
| CTP | cytidine triphosphate |
| DDPK | double-stranded RNA-dependent protein kinase |
| DHFR | dihydrofolate reductase |
| DMEM | Dulbecco's Modified Eagle's Medium |
| dNTP | deoxynucleoside triphosphate |
| DRADA | double-stranded RNA-specific adenosine deaminase |

| | |
|---|---|
| DSE | dyad symmetry element |
| dsRBP | double-stranded RNA binding protein |
| dsRNA | double-stranded RNA |
| DTT | dithiothreitol |
| dTTP | deoxythymidine triphosphate |
| ECM | extracellular matrix |
| EDTA | ethylenediaminetetraacetic acid |
| ENU | ethylnitrosurea |
| EST | expressed sequence tag |
| FMEN | familial multiple endocrine neoplasia |
| GAPD | glyceraldehyde-3-phosphate dehydrogenase |
| GBM | glioblastoma multiforme |
| GITC | guanidium isothiocyanate |
| GluR | glutamate receptor |
| GnRH | gonadotropin releasing hormone |
| GTBP | G/T mismatch-binding protein |
| GTP | guanosine triphosphate |
| GVBD | germinal vesicle breakdown |
| FBS | fetal bovine serum |
| FGF | fibroblast growth factor |
| FGFBP | high affinity fibroblast growth factor binding protein |
| FGFR | fibroblast growth factor receptor |
| HIV | human immunodeficiency virus |

| | |
|---|---|
| hnRNP | heterogeneous ribonucleoprotein |
| hr | hour |
| HS | horse serum |
| HSPG | heparan sulfate proteoglycan |
| Ig | immunoglobulin |
| IGF | insulin-like growth factor |
| IL | interleukin |
| IVF | *in vitro* fertilization |
| kDa | kilodalton |
| krpm | kilorevolutions per minute |
| LDL | low density lipoprotein |
| LPS | lipopolysaccaride |
| MAP | mitogen-activated protein |
| MAPK | mitogen-activated protein kinase |
| MB | Methylene blue |
| MEK | mitogen-activated protein kinase kinase |
| μM | micromolar |
| min | minute |
| MHC | myosin heavy chain |
| MMLV | murine moloney leukemia virus |
| MMR | mismatch repair |
| MOPS | 3-[N-morpholino]propanesulfonic acid |
| MPF | maturation promoting factor |

| | |
|---|---|
| mRNA | messenger RNA |
| MTH | mutT homolog |
| n | nano |
| NLS | nuclear localization signal |
| ORF | open reading frame |
| 8-oxo-dG | 8-oxo-7, 8-dihydro-2'-deoxyguanosine |
| 8-oxo-dGTP | 8-oxo-7, 8-dihydro-2'-deoxyguanosine 5'-triphosphate |
| p | pico |
| PAGE | polyacrylamide gel electrophoresis |
| PBL | peripheral blood leukocytes |
| PBS | phosphate buffered saline |
| PCNA | proliferating cell nuclear antigen |
| PCR | polymerase chain reaction |
| pgk | phosphoglycerate kinase |
| PIPES | 1, 4-piperazine-diethanesulfonic acid |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PLC | phospholipase C |
| PMA | phorbol 12-myristate 13-acetate |
| PMSG | pregnant mare serum gonadotropin |
| proGnrH | precursor gonadotropin releasing hormone |
| RACE | rapid amplification of cDNA ends |
| RED | RNA editase |

| | |
|---|---|
| rRNA | ribosomal RNA |
| RT | reverse transcription |
| SDS | sodium dodecyl sulfate |
| SH2 | src homology 2 |
| STAT | signal transducers and activators of transcription |
| STE | sodium chloride/Tris/EDTA |
| SSC | sodium chloride/sodium dodecyl sulfate/sodium citrate |
| TBE | Tris/boric acid/EDTA |
| TBS | Tris buffered saline |
| tcRNA | translational control RNA |
| TEMED | N, N, N', N'-tetramethylethylenediamine |
| TGF | transforming growth factor |
| TN-X | tenascin-X |
| TR | thyroid hormone receptor |
| UTP | uridine triphosphate |
| UTR | untranslated region |
| YC | yellow cresent |

# CHAPTER 1: THE FIBROBLAST GROWTH FACTOR (FGF) FAMILY

## I. Introduction

Fibroblast growth factor (FGF) was first identified as an activity from bovine pituitary that stimulated the proliferation of mouse NIH 3T3 fibroblasts (Gospodarowicz, 1974). The mitogenic activity was shown to co-purify with a 14-16 kDa protein having a basic isoelectric point (Gospodarowicz, 1975). Shortly afterward, another 3T3 cell mitogen was found in bovine brain but with an acidic isoelectric point (Maciag et al., 1979; Thomas et al., 1980). Based upon their difference in isoelectric points, these two mitogens were termed basic FGF and acidic FGF, respectively. It was later determined that native basic FGF protein is comprised of 155 amino acids with a predicted molecular weight of 18 kDa, and that the observed size heterogeneity resulted from cleavage of its $NH_2$-terminus (Gospodarowicz et al., 1987). FGFs were also found to be mitogenic for other cell types including endothelial cells and chondrocytes (Gospodarowicz et al., 1978). During the isolation of angiogenic factors, it was discovered that an endothelial cell mitogen with FGF-like properties bound strongly to heparin (Shing et al., 1984). The identification of this angiogenic activity led ultimately to the purification of FGFs, whereby bound acidic and basic FGF were eluted at 1.0 and 1.5 M NaCl, respectively (Gospodarowicz et al., 1984; Maciag et al., 1984; Klagsbrun and Shing, 1985). Since then, several heparin-binding FGF-like proteins have been identified and purified from a diverse selection of cell lines and tissues. Presently, the FGF family is comprised of ten structurally related polypeptides which share 30-80% homology, including acidic fibroblast growth factor (aFGF/FGF-1), basic fibroblast growth factor (bFGF/FGF-2), FGF-3 (INT-2), FGF-4 (HST/Kaposi's FGF), FGF-5, FGF-6, keratinocyte

growth factor (KGF/FGF-7), androgen-induced growth factor (AIGF/FGF-8), glia-activating factor (GAF/FGF-9), and FGF-10. FGF-3, FGF-4, and FGF-5 were first isolated as oncogenes (Dickson et al., 1984; Delli Bovi et al., 1987a; Zhan et al., 1988). The FGF-3 gene was originally identified as a frequent genomic site for insertion of the mouse mammary tumor virus (Dickson et al., 1984), whereas FGF-4 and FGF-5 were detected as dominant transforming genes following transfection of Kaposi's sarcoma DNA into NIH 3T3 cells (Sakamoto et al., 1986; Yoshida et al., 1987; Delli-Bovi et al., 1987a, b; Zhan et al., 1988). Using an FGF-4 probe for low stringency hybridization led to the isolation of FGF-6 (Marics et al., 1989). FGF-7 was isolated from a cultured mouse keratinocyte line based on its ability to stimulate the growth of these cells (Rubin et al., 1989). Unlike other FGF members, FGF-7 has little mitogenicity on mesenchyme-derived cells, which should not be surprising given that it is the most divergent family member. FGF-8 was purified as an androgen-induced growth factor from the conditioned medium of an androgen-dependent cell line (SC-3) derived from a mouse mammary carcinoma (Tanaka et al., 1992). FGF-9 was identified as a glia-activating factor in the culture supernatant of human NMC-G1 glioma cells (Miyamoto et al., 1993). The spectrum of activity of FGF-9 is distinct from other FGFs, in that it acts on fibroblasts, but does not affect human umbilical vein endothelial cells. The latest member of the FGF family, FGF-10, was isolated from rat embryos by homology-based PCR (Yamasaki et al., 1996). Additionally, four FGF homologous factors (FHFs) were recently identified as members of the FGF family (Smallwood et al., 1996). The FHFs show <30% amino acid sequence homology with other FGFs, and are primarily expressed in the developing and adult nervous systems. The majority of expression,

regulatory and functional studies have focused on FGF-1 and FGF-2, and therefore these are the best characterized members of the FGF family (see Chapter 2).

FGFs and their receptors has been found in a range of organisms including insects, birds, amphibia, and mammals (reviewed in Coulier et al., 1997). The genomic organization of FGF genes has been strongly conserved throughout evolution, which is consistent with their derivation from a common ancestral gene. All FGF genes are comprised of three exons that are separated by two introns of variable length, with the exception of FGF-8 which contains six exons. Generally, the second exon is very short and the third exon usually includes a long (~2-6 kb) 3' untranslated region. Comparison of the protein structure among FGF members reveals a family of polypeptides of varying lengths with a conserved ~120 amino acid core and substantial differences at the amino and carboxyl terminal regions (Figure 1). Interestingly, FGF-1, FGF-2, and FGF-9 lack the classical signal sequence and are released from cells by a route distinct from the endoplasmic reticulum-Golgi secretory pathway (Florkiewicz et al., 1995; Jackson et al., 1995).

**i. Regulation of FGFs**

An understanding of the regulation of FGF expression, particularly of FGF-1 and FGF-2, is now emerging through the identification of different cis- and trans-acting regulatory elements and factors, as well as the elucidation of posttranscriptional and translational control mechanisms. Inspection of the FGF-1 promoter sequence revealed purine-rich regions, which have previously been shown to serve as protein binding sites involved in transcriptional regulation of c-*myc* (Davis et al., 1989; Postel et

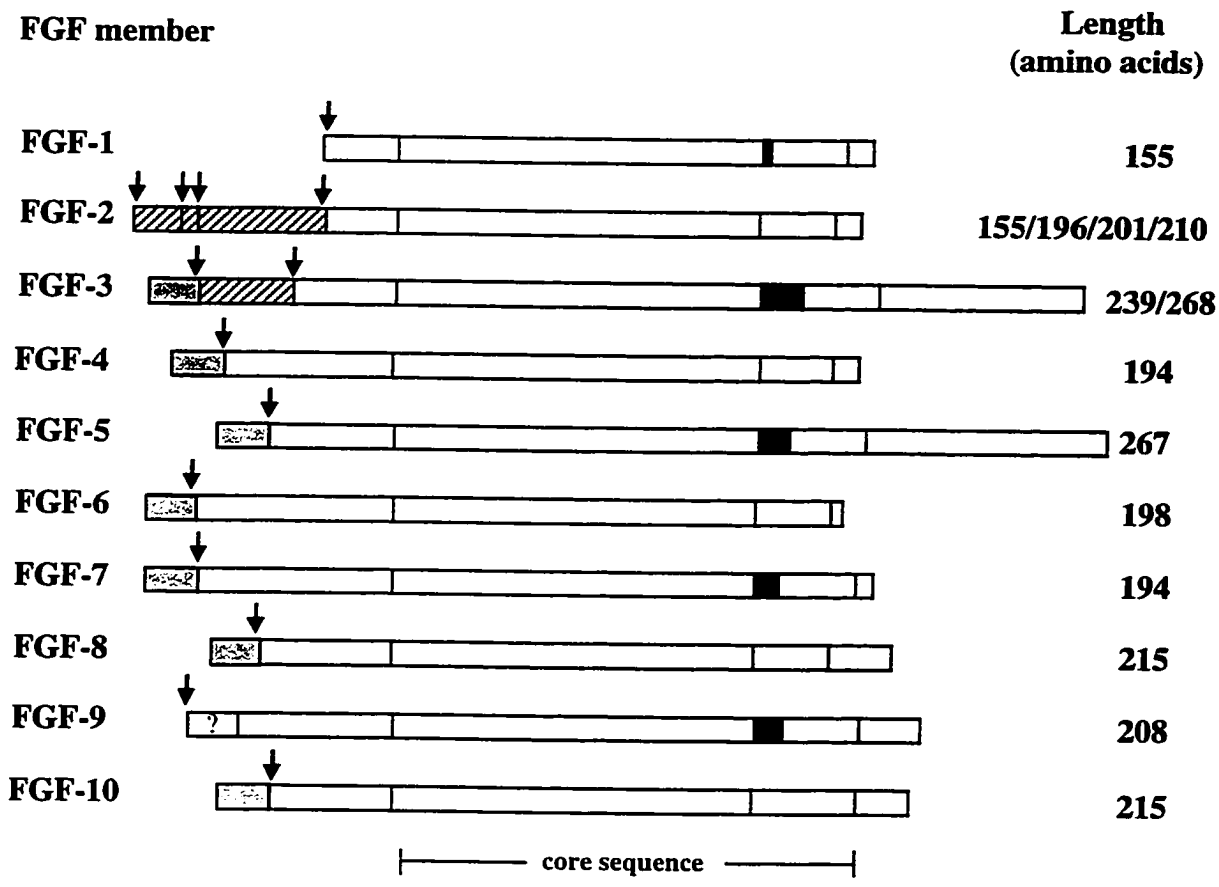



**Figure 1.** Schematic of the FGF protein family. All gene products are structurally related and have a core sequence that is highly conserved. Within this core sequence (open bar), some FGFs have a peptide insert (closed box) at a sequence which has been identified as a receptor binding domain. The FGF proteins show no homology at their carboxy termini. The stippled bar denotes the signal cleavage peptide for the FGF members (FGF-1 and FGF-2 have no signal peptide, and none have been identified in FGF-9). Both FGF-2 and FGF-3 include protein isoforms depending on alternative translation initiation (multiple arrows) as indicated by their variable amino acid length on the right. (Adapted from Baird, 1994).

al., 1989) and epidermal growth factor receptor (Johnson et al., 1988). Other putative protein binding sites within the FGF-1 promoter include an AP-1 site, and a CArG box which is believed to mediate promoter activation in response to serum (Chotani et al., 1995), similar to that shown for c-*fos* (Shaw et al., 1989). Winkles and Gay (1991) have reported that FGF-1 mRNA in vascular smooth muscle cells is upregulated by treatment with fetal bovine serum (FBS) or phorbol 12-myristate 13-acetate (PMA). Chotani et al., (1995) have complemented these findings by demonstrating that the expression of FGF-1 during serum or PMA treatment is under the control of alternative promoter usage. The FGF-1 gene contains four upstream noncoding exons designated 1A, 1B, 1C, and 1D, and alternative splicing of each of these exons to the first coding exon yields individual mRNAs termed 1A, 1B, 1C, and 1D which are expressed in a tissue-specific manner (Wang et al., 1989; Chiu et al., 1990; Wang et al., 1991a, b; Myers et al., 1993; Payson et al., 1993).

Analysis of the human FGF-2 gene has provided some insight to the control mechanisms of its promoter. For instance, this promoter does not contain a TATA box, but does have several putative Sp-1 binding sites and one AP-1 binding site (Abraham et al., 1986a), as well as, two negative regulatory domains located upstream from the promoter (Shibata et al., 1991). FGF-3 regulation appears to be mainly transcriptional, involving the use of three distinct promoters and two alternative polyadenylation sites (Smith et al., 1988; Grinberg et al., 1991). Although there is no evidence of posttranscriptional or translation control of FGF-4 expression, transcription is dependent on the presence of an enhancer element located in the 3' untranslated region of the third exon (Curatola and Basilico, 1990). This enhancer promotes FGF-4 transcription in undifferentiated

embryonic carcinoma (EC) cells, but not in their differentiated counterparts, indicating that specific developmentally regulated trans-acting factors may be necessary for activation of the FGF-4 enhancer.

Other transcriptional control mechanisms include transcriptional termination at alternative polyadenylation sites which generates multiple mRNA species from the FGF-1, FGF-2, and FGF-5 genes (Crumley et al., 1989; Abraham et al., 1986a; Kurokawa et al., 1987; Zhan et al., 1988). As mentioned earlier, the FGF-8 gene consists of at least six exons, and these exons encode at least seven protein isoforms due to alternative splicing of the primary transcript (MacArthur et al., 1995; Tanaka et al., 1992; Crossley and Martin, 1995). Differential translation initiation has also been shown for FGF-2 and FGF-3 mRNA which generates alternative isoforms (Florkiewicz and Sommer, 1989; Acland et al., 1990; Bugler et al., 1991; Florkiewicz et al., 1991). For human FGF-2 mRNA, an AUG codon in the proper context initiates translation of the 155 amino acid form, while three upstream CUG codons generate the 196, 201, and 210 amino acid forms. The primary translation product of the human FGF-3 mRNA is also initiated at an AUG codon that gives rise to a 239 amino acid protein, while an upstream CUG generates an alternative 271 amino acid form.

**ii. Expression Patterns of FGFs**

The patterns of expression of the FGF gene family are quite distinct, ranging from tightly controlled during development to broad expression in a variety of tissues and organs. The expression of FGF-1 displays a more limited distribution than FGF-2; however, both display widespread patterns of expression and are present at relatively abundant levels in most tissues. FGF-2 mRNA is expressed at much higher levels in the

adult brain than other tissues (Shimasaki et al., 1988), and is abundant in neuronal bodies of the CNS, whereas FGF-1 is localized to peripheral neurons (Pettmann et al., 1986; Emoto et al., 1989; Eckenstein et al., 1991).

The coordinated expression patterns of FGFs and their cognate receptors in mammalian embryos suggests an important role for FGFs in various developmental processes during gastrulation, limb-bud outgrowth and patterning, formation of the skeletal system and tissues such as lung, ear, and hair (Niswander and Martin, 1992; Wilkinson et al., 1988; Ohuchi et al., 1994; Crossley and Martin, 1995; Post et al. 1996). FGF-1 and FGF-2 are widely expressed during development and in adult tissues, whereas other FGF members are predominantly expressed in the embryo and in a restricted manner in specific adult tissues.

The specific spatiotemporal patterns of expression during murine embryonic growth suggest an important role for FGFs in development. *In situ* hybridization has revealed that FGF-3, FGF-4, FGF-5, and FGF-8 are expressed in a stringently regulated and complementary manner in the gastrulating mouse embryo (Wilkinson et al., 1988; Haub and Goldfarb, 1991; Hebert et al., 1991; Niswander and Martin, 1992; Ohuchi et al., 1994). In adult tissues, FGF-3 and FGF-4 are generally not found (Jakobovits et al., 1986; Wilkinson et al., 1988), FGF-5 is detected only in adult brain (Haub et al., 1990), and FGF-8 is seen only in adult testis (Lorenzi et al., 1995). FGF-6 has been shown to be restricted to cells in the skeletal muscle lineage during mouse embryogenesis and early fetal development (Han and Martin, 1993). However, it is also detected in the adult testis, heart and skeletal muscle (de Lapeyriere et al., 1990). FGF-7 is expressed in a very temporally restricted manner in normal tissues, and is strongly implicated as a mesenchyme-derived mediator of epithelial growth and development

(Herbert et al., 1990; Rubin et al., 1995). Analysis of various adult rat tissues reveals that FGF-9 mRNA is only detectable in brain and kidney (Miyamoto et al., 1993), whereas FGF-10 is predominantly expressed in the embryo and adult lung (Yamasaki et al., 1996).

**II. Biological Significance of FGFs**

The physiological roles of FGFs are not fully understood, yet these factors are involved in diverse cellular processes including cell growth, migration and differentiation, angiogenesis, wound healing, morphogenesis and development, and tumor formation (Folkman and Klagsbrun, 1987; Klagsbrun, 1989; Thomas, 1987; Sasada et al., 1988; Moscatelli et al., 1986a; Li and Bernard, 1992; Basilico and Moscatelli, 1992; Kimelman and Maas, 1992).

The most dramatic effect of FGF is the stimulation of proliferation of various cell types such as fibroblasts, endothelial cells, smooth muscle cells, myoblasts, chondrocytes, epithelial cells and glial cells (Burgess and Maciag, 1989; Klagsbrun, 1989). However, accumulating evidence suggests that FGFs, aside from their growth-promoting activities, also function as signaling molecules in developmental processes during vertebrate embryogenesis (see above). FGFs have been shown to inhibit the terminal differentiation of myoblasts (Clegg et al., 1987), and induce mesoderm formation in *Xenopus* (Kimelman and Kirschner, 1987; Slack et al., 1987). These studies, together with the spatiotemporal expression of FGFs, suggest a correlation of FGF function with stages of differentiation, rather than proliferation, implying that FGFs are both growth and differentiation mediators. Furthermore, it seems that the effects of FGF on cellular processes such as migration, proliferation, and differentiation, occur in a

concerted manner to accomplish a regulatory function during complex *in vivo* responses like angiogenesis, wound repair, and development.

Gene knockout by homologous recombination studies have shed considerable light on the functions of certain FGF members. For example, the FGF-4 homozygous mutant causes lethality by embryonic day 6.5, revealing its requirement in post-implantation development of the mouse (Feldman et al., 1995). Mice homozygous for an inactivated FGF-3 gene develop anatomical defects during embryogenesis, yet are viable, although with reduced survival rates (Mansour et al., 1993). Furthermore, those mice harboring a disrupted FGF-5 gene are viable and fertile, and the only apparent defect is abnormally long fur (Herbert et al., 1994). Targeted disruption of the FGF receptor-1 gene causes embryonic lethality, revealing the importance of FGF signaling through this receptor during pregastrulation development (Deng et al., 1994; Yamaguchi et al., 1994a). The evidence from gene knock-out studies suggests that functional complementation exists among members of the FGF family (Herbert et al., 1994). Therefore, the absence of one FGF member may be compensated by another member. The expression of various FGF and receptor isoforms imparts additional complexity to the functional overlap within the FGF family.

**i. Role of FGF in the Nervous System**

The importance of FGF in the ontogeny of the CNS is signified by the specific spatiotemporal expression of FGF-2 mRNA and protein in multiple regions of the brain (Giordano et al., 1992; Weise et al., 1993; Grothe and Meisinger, 1995). FGF-1 and FGF-2 protein are widely distributed throughout the CNS. Unlike peripheral tissues, the CNS

expresses high steady-state levels of FGF mRNA, possibly because FGFs play a continuous trophic function in the CNS. Numerous studies have implicated FGFs in the division, differentiation, and maintenance of a variety of neuronal cell types. FGF-1 and FGF-2 have been shown to be mitogenic for cultured glial cells including oligodendrocytes, Schwann cells, and astrocytes (Eccleston and Silberberg, 1985; Pettman et al., 1985; Davis and Stroobant, 1990). *In vitro* exposure of varying concentrations of FGF-2 has been shown to direct the fate of cortical progenitor cells which generate the three major CNS cell types; neurons, astrocytes, and oligodendrocytes (Davis and Temple, 1994; Qian et al., 1997). The role of FGF-2 in altering CNS progenitor cell fate suggests an important role for FGF in the normal patterning of cortical tissue. Direct effects of FGF-2 have also been observed in promoting neurite extension of cultured rat adrenal chromaffin precursor cells and their cell line counterpart, PC12 pheochromocytoma cells (Stemple et al., 1988; Sigmund et al., 1990). FGF-1, and to a lesser extent FGF-2, were also demonstrated to synergize *in vitro* with various catecholamines in the induction of differentiation of embryonic striatal neurons (Du and Iacovitti, 1995). Both FGF-1 and FGF-2 promote the *in vitro* survival of many peripheral sympathetic, parasympathetic, and central neurons (Eckenstein, 1994). *In vivo*, FGF-2 has been shown to be neurotrophic for ciliary ganglia of the chick embryo (Dreyer et al., 1989) and cholinergic neurons of the adult rat hippocampus (Grothe et al., 1989). Furthermore, FGF-2 produced by the rat GT1 gonadotropin-releasing hormone (GnRH) neuronal cell line is neurotrophic for these cells, implicating FGF in the regulation of postnatal neuroendocrine function (Tsai et al., 1995). The expression of different FGF receptor isoforms in specific neuronal cell populations (Werner et al.,

1992) further strengthens the functional significance of FGF in the nervous system.

**ii. Role of FGF in Angiogenesis**

Angiogenesis is a highly regulated and fundamental process by which new blood vessels are formed (Folkman and Shing, 1992). It is essential in normal processes related to reproduction, development, and wound repair, and in a variety of pathological states including diabetic retinopathy, rheumatoid arthritis, and tumor growth. Numerous studies have shown that FGF-1, FGF-2, FGF-4, and FGF-5 stimulate the proliferation of endothelial cells (Delli Bovi et al., 1988; Gospodarowicz et al., 1987). Further, both FGF-1 and FGF-2 are chemotactic for endothelial cells (Moscatelli et al., 1986b; Terranova et al., 1985) and have been demonstrated to induce angiogenesis *in vivo* in a variety of model systems (Lobb et al., 1985; Shing et al., 1985; Hayek et al., 1987). FGF-2 was shown to induce an invasive phenotype in cultured endothelial cells by increasing the production of plasminogen activator and collagenase, thereby enabling those cells to penetrate the basement membrane (Moscatelli et al., 1986b; Presta et al., 1986; Mignatti et al., 1989). The induction of growth plate cartilage ossification by FGF-2 (Baron et al., 1994), is presumed to involve the acceleration of vascular invasion (Iannoti et al., 1990), further supporting the chemotactic and mitogenic effect of FGF-2 on endothelial cells.

### III. FGF Receptors (FGFRs)

The original isolation and characterization of a high-affinity receptor for FGF revealed that it was a typical tyrosine kinase transmembrane receptor of the immunoglobulin IgG superfamily (Lee et al., 1989). To date, four high-affinity FGF receptor (FGFR) genes have been identified in mammals, including FGFR-1 (*fms*-like gene; *flg*), FGFR-2 (bek/K-*sam*), FGFR-3, and FGFR-4 (Lee et al., 1989; Ruta et al., 1989, Dionne et al., 1990; Avivi et al., 1991; Keegan et al., 1991; Partanen et al., 1991; Mansukhani et al., 1992). FGFR-1 was originally identified on the basis of sequence homology with the *fms* tyrosine kinase oncogene (Lee et al., 1989), while FGFR-2 (bek) was later found on the basis of its homology with K-*sam*, an oncogene amplified in stomach carcinoma cells (Hattori et al., 1990; Mansukhani et al., 1992). The FGFRs are a family of monomeric transmembrane receptors having two or three extracellular immunoglobulin-like domains and a split cytoplasmic tyrosine kinase domain (Figure 2). The extracellular domain determines ligand binding specificity and mediates ligand-induced receptor dimerization, which stimulates transphosphorylation and activation of the receptor (Schlessinger and Ullrich, 1992). Each FGFR binds ligands with varying affinity (Partanen et al., 1991; Ornitz et al., 1992; Werner et al., 1992; Mansukhani et al., 1992), and isoforms of FGFR-1 and FGFR-2, derived from alternative splicing, also have unique ligand binding properties (Werner et al., 1992; Miki et al., 1992; Chellaiah et al., 1994). The expression of secreted FGFR isoforms lacking the transmembrane domain has also been described (Givol and Yayon, 1992). The diversity of receptor isoforms and variable binding specificity is an essential mechanism for regulating FGF activity (Ornitz et al., 1996). Most of the FGFR binding

**Figure 2.** Schematic representation of the FGF receptor protein structures. The names of the variant receptor forms predicted from published cDNAs are shown. The variant which has not been isolated but whose mRNA transcript has been identified by PCR or Northern blotting is indicated by an asterisk. Furthermore, both 3 Ig and 2 Ig domain forms of this receptor mRNA appear to exist. The following structural features are identified in the figure: the 32 unique amino acids at the carboxy-terminus of the FGFR-1 1 Ig domain I secreted form (solid oval), acid box domains (open boxes), alternative sequences for the second half of Ig domain III labeled IIIa, IIIb, or IIIc (thick black line), transmembrane domains (solid boxes), kinase 1 and kinase 2 domains (shaded boxes), and the unique carboxy-tail domains of 2 FGFR-2 proteins (checkered and striped box). (Adapted from Johnson and Williams, 1993).

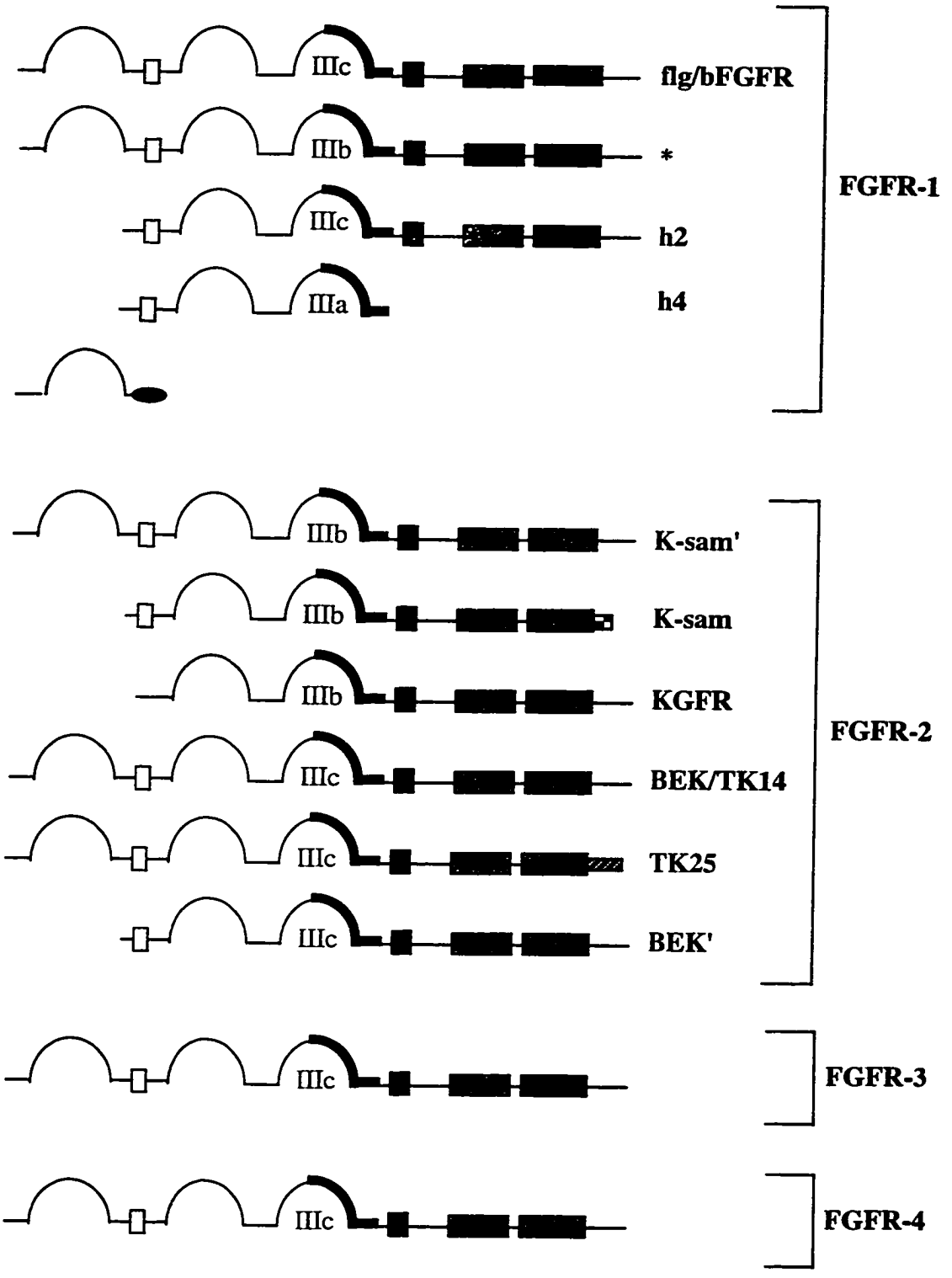

IIIc
flg/bFGFR
IIIb
*
IIIc
h2
IIIa
h4
FGFR-1
IIIb
K-sam'
IIIb
K-sam
IIIb
KGFR
IIIc
BEK/TK14
IIIc
TK25
IIIc
BEK'
FGFR-2
IIIc
FGFR-3
IIIc
FGFR-4

Figure 2

studies were performed with FGF-1 and FGF-2. The FGFR-1 isoform binds both FGF-1 and FGF-2 with comparable high affinities (Dionne et al., 1990; Johnson et al., 1990). Like FGFR-1, FGFR-2 has similar affinity for FGF-1 and FGF-2, and can also bind FGF-4 (Dionne et al., 1990). FGFR-3 and FGFR-4 bind preferentially with FGF-1 and show a 10 to 20-fold lower affinity for FGF-2 (Ornitz et al., 1992; Vainikka et al., 1992; Ron et al., 1993).

A role for FGF/FGFR interaction in limb and bone development was identified when it was discovered that mutations in the FGFR-1 and FGFR-2 genes were associated with human craniosynostotic syndromes, such as Pfeiffer (Muenke et al., 1994), Jackson-Weiss (Jabs et al., 1994), and Crouzon syndromes (Jabs et al., 1994; Reardon et al., 1994). Further, a missense mutation in the FGFR-3 gene was shown to be responsible for achondroplasia, the most common form of dwarfism (Shiang et al., 1994; Rousseau et al., 1994).

Like certain FGFs, FGFRs have been identified as oncogenes, suggesting their corresponding importance in tumorigenesis. In addition, differential expression of alternatively spliced isoforms of the FGFR, which in some cases appear to be mutually exclusive, have been implicated in the malignant progression of different human tumors including colon adenocarcinomas and astrocytomas (Yan et al., 1993a; Murgue et al., 1994; Yamaguchi et al., 1994b).

## IV. Heparan Sulfate Proteoglycans (HSPGs)

FGFs exert their biological effects by interacting with both high affinity FGFRs ($K_D$ = 2-20 X $10^{-11}$ M) and low affinity receptors, heparan sulfate proteoglycans ($K_D$ = 2 X $10^{-9}$ to 2 X $10^{-7}$ M) (Yayon et al., 1991; Ornitz et al., 1992; Pantoliano et al., 1994). The heparan sulfate proteoglycans (HSPGs) which serve as low-affinity receptors for FGF include syndecan, glypican, and perlecan. FGF-mediated signal transduction is believed to involve a complex that includes FGF, an FGFR dimer, and HSPGs (Figure 3) (Friesel and Maciag, 1995; Schlessinger et al., 1995). Defined carbohydrate sequences contained on heparan sulfate chains derived from fibroblasts have been shown to be specific for binding FGF-2 (Gallagher and Turnbull, 1992). Therefore, the state of glycosylation of HSPG may be an important determinant in the modulation of the extracellular activity of FGF.

Although HSPGs are not required for FGF-2/FGFR interaction, binding of FGF with HSPGs is suggested to induce FGF dimer formation, which facilitates binding to the high-affinity receptor (Schlessinger et al., 1995). Several possibilities have been proposed for the biological importance of heparan sulfate in FGF-2 signaling. FGF-2 associated with proteoglycans in the ECM or on the cell surface may allow sequestering of growth factor that can then bind to and activate receptors, thereby enabling an extended response to a transient exposure to FGF-2 (Flaumenhaft et al., 1989; Presta et al., 1989). Heparan sulfates have been suggested to induce conformational changes in FGF, and thereby protect it from thermal degradation (Gospodarowicz and Cheng, 1986) and/or from the action of proteases (Saksela et al., 1988; Sommer and Rifkin, 1989), which are present at high concentration on the surface of endothelial cells during angiogenesis (Moscatelli and Rifkin, 1988). Conversely, FGF may be

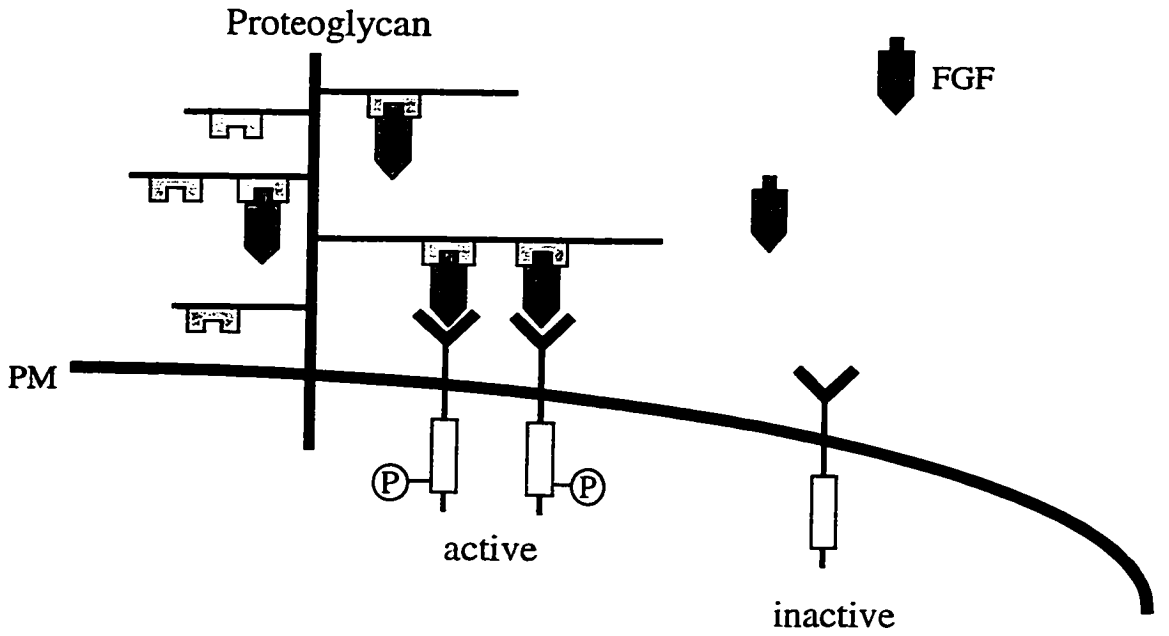



**Figure 3.** A general scheme for the activation of FGF receptor by interaction with multimeric FGF-heparan sulfate proteoglycan complexes. A cell surface proteoglycan molecule binds simultaneously to several FGF molecules, which in turn bind to the signaling FGF receptor with higher avidity as compared with monomeric FGF. Multivalent binding of the multimeric FGF complex stimulates FGF receptor dimerization and activation. P, phosphorylated tyrosine residue. (Adapted from Schlessinger et al., 1995).

released through mechanisms involving degradative enzymes, such as phospholipases (Bashkin et al., 1992), plasmin (Saksela and Rifkin, 1990), and heparanases (Ishai-Michaeli et al., 1990), which would solubilize the heparin-FGF-2 complex allowing it to diffuse and activate distally located FGF-Rs.

## CHAPTER 2: THE BASIC FIBROBLAST GROWTH FACTOR (FGF-2)

### I. Introduction

Basic fibroblast growth factor (bFGF; FGF-2) has been found in almost all tissues derived from the three primary germ layers (Baird et al., 1986; Rifkin and Moscatelli, 1989; Westermann et al., 1990). FGF-2 functions in a variety of processes such as mesoderm induction, neurite outgrowth, wound repair, hematopoiesis, cell migration, and angiogenesis (Basilico and Moscatelli, 1992).

### II. The FGF-2 Gene

#### i. Structure and Expression of the FGF-2 Gene

The highly conserved human FGF-2 gene (84-98% homology among vertebrates) consists of three exons widely separated by two large introns (Abraham et al., 1986a). The single promoter of FGF-2 does not contain a TATA box, but does have G/C rich sequences with several putative Sp-1 binding sites, and one AP-1 binding site (Shibata et al., 1991). The FGF-2 gene also contains two negative regulatory domains upstream from the promoter, which may account for the low level of transcription observed in serum-deprived cells (Goldsmith et al., 1991). Modulation of the FGF-2 promoter is one of the mechanisms of FGF-2 inhibition during direct cell-cell contact (Moffett et al., 1994; 1996). The region of the FGF-2 promoter which mediates cell density-dependent regulation contains a number of putative control sequences. The dyad symmetry element (DSE), which has been implicated in the negative regulation of c-*myc* promoter activity (Johnson and McKnight, 1989), may function in the density-dependent regulation of the FGF-2 promoter. The FGF-2 promoter also contains several A/T-rich regions with sequences similar to STAT (signal

transducers and activators of transcription) protein binding sites (Darnell et al., 1994; Ihle et al., 1994; Kotanides and Reich, 1993), which may regulate FGF-2 transcription during cell-cell contact (Moffett et al., 1996). Transcription from the FGF-2 promoter is also activated by FGF-2 and other growth factors and by neurotransmitters, hormones, cAMP, and protein kinase C (Stachowiak et al., 1994; Moffett et al., 1994), implying the presence of serum and hormone response elements.

Northern analysis reveals that transcription of the FGF-2 gene produces multiple polyadenylated transcripts in all species so far examined, including human (7.0, 3.7, 3.5, 2.8, 2.2, and 1.8 kb), rat (6.0, 3.7, 2.5, 1.8, 1.6, 1.4, and 1.0 kb), chicken (10.2, 7.8, 4.9, 2.8 and 1.5), and *Xenopus laevis* (4.2 and 2.3 kb) (Murphy et al., 1988a; Powell et al., 1991; Borja et al., 1993; Kimelman and Kirschner, 1989). Alternative expression of the various mammalian transcripts arises from differential lengths of the 3' untranslated region (UTR), however, their functional significance is not completely understood. FGF-2 mRNA expression is modulated by various factors including serum and cell density (Murphy et al., 1988b), thrombin (Weich et al., 1991), IL-1 (Gay and Winkles, 1991), TGF-β1 (Cook et al., 1990) and dexamethasone (Meisinger et al., 1996).

**ii. Translation of the FGF-2 mRNA**

In determining the translation product of the FGF-2 gene, analysis of the bovine and human cDNAs revealed an AUG codon in the proper context to initiate translation, with no additional in-frame AUG codons upstream of the putative start site (Abraham et al., 1986a, b). The predicted open reading frame generates a protein of 155 amino acids with a molecular weight of 18 kDa. The amino acid sequence of the 18 kDa FGF-2

isoform is highly conserved, with between 89-95% homology among human, cow, sheep, and rat (Abraham et al., 1986a, b; Kurokawa et al., 1988; Shimasaki et al., 1988; Simpson et al., 1987). The major structural features of the FGF-2 protein include PKA and PKC phosphorylation sites, FGFR and heparin binding sites, conserved cysteine residues and glycine-arginine repeats (Basilico and Moscatelli, 1992). Purification of larger FGF-2 forms (Moscatelli et al., 1987) revealed that translation of the FGF-2 gene also occurs by alternative initiation from three upstream CUG codons (Florkiewicz and Sommer, 1989; Prats et al., 1989). Translation initiated at these alternative CUG start codons in the human FGF-2 transcript generate proteins comprised of 196, 201 and 210 amino acids, corresponding to molecular weights 22, 22.5 and 24 kDa, respectively. These high molecular weight FGF-2 isoforms contain the same amino acid sequence as the 18 kDa form, but have additional $NH_2$-terminal extensions of varying lengths.

Interestingly, analysis of the chicken FGF-2 gene revealed expression of a novel, alternatively spliced FGF-2 (altFGF-2) mRNA which encodes an FGF-2 isoform with a divergent amino terminal (Borja et al., 1993). The altFGF-2 isoform is predominantly targeted to the endoplasmic reticulum and displays a more temporally and spatially restricted pattern of expression during chicken embryogenesis than the other FGF-2 isoforms (Borja et al., 1996).

### III. Distribution and Function of FGF-2 Isoforms

Alternative initiation of translation is believed to determine the cytoplasmic or nuclear localization of FGF-2 (Bugler et al., 1991). The use of chimeric gene products for generating FGF-2 isoforms initiated at either of the two CUG codons or at the downstream AUG codon, demonstrated that isoforms harboring the additional $NH_2$-terminal sequence were detected exclusively in the nucleus, while the shorter isoforms were found predominantly in the cytoplasm. It was therefore proposed that the presence of a nuclear localization signal (NLS) in the extended $NH_2$-termini of CUG-initiated isoforms selectively directs their translocation to the nucleus, while the shorter AUG-initiated isoforms lacking the putative NLS remain in the cytoplasm. Interestingly, when the 18 kDa isoform is exogenously added to cells it is internalized and specifically translocated to the nucleoli during the G1 phase of the cell cycle despite the lack of an $NH_2$-terminus signal for targeting (Bouche et al., 1987; Baldin et al., 1990). This suggests that alternative pathways exist for nuclear or nucleolar translocation depending on whether FGF-2 is produced by or provided to the cells.

The use of point mutations to selectively prevent the synthesis of either cytoplasmic or nuclear isoforms has shown that they have distinct cellular effects. FGF-2 in the cytoplasm is capable of increasing cell migration, inducing upregulation of integrins or downregulation of FGFR, and growth in soft agar, whereas the nuclear isoform only induces growth in soft agar (Mason et al., 1994). Further, transfection of cardiac myocytes with FGF-2 constructs which selectively express the high molecular weight isoforms affected the nuclear morphology (Parsumarthi et al., 1994) and binucleation (Parsumarthi et al., 1996) of these cells.

Different FGF-2 isoforms may also have discrete functions during development of the CNS, as shown by western blot analysis of FGF-2, which revealed the differential expression of FGF-2 isoforms among subregions of the developing rat CNS (Giordano et al., 1992). Therefore, differential subcellular localization may determine the specialized function(s) of multiple FGF-2 isoforms during various cellular processes. Indeed, the presence of FGF-2 in different subcellular compartments correlates with changes in cell proliferation (Renko et al., 1990; Florkiewicz et al., 1991) and the state of cell differentiation (Quarto et al., 1991; Hill and Logan, 1992). High molecular weight FGF-2 isoforms do not seem to associate with the plasma membrane, are not detected extracellularly (Florkiewicz et al., 1991; Bikfalvi et al., 1995), and are localized to the nucleus (Florkiewicz et al., 1995). In addition, the large FGF-2 isoforms stimulate proliferation independent of their cell surface receptors (Bikfalvi et al., 1995; Joy et al., 1996). Moreover, transfection of cells with only the nuclear-translocated FGF-2 isoform results in serum-independence and immortalization characteristics through as yet unknown mechanisms (Couderc et al., 1991).

Immunohistochemistry performed by Joy et al., (1996) has revealed the nuclear accumulation of FGF-2 in reactive astrocytes of the adult human brain and in neoplastic cells of human glioblastoma multiforme (GBM). Nonreactive astrocytes contained immunoreactive FGF-2 only in the cytoplasm, while in glioma cells and large reactive astrocytes immunoreactive FGF-2 is present in the nuclei, nucleoli, and cytoplasm, which suggests that the nuclear accumulation of FGF-2 is a regulated process which accompanies cell proliferation. Furthermore, the nuclear accumulation of FGF-2 in reactive astrocytes or glioma cells may be

induced by the release from direct cell-contact inhibition (Moffett et al., 1996; Joy et al., 1996). Therefore, cell proliferation may be controlled by the nuclear content of FGF-2 in a cell-density dependent manner, whereby glioma cells generate a constitutive level of nuclear FGF-2 nonresponsive to the inhibitive mechanism of cell-cell contact.

High affinity FGF binding proteins (FGFBPs) of 46-48 kDa and immunologically related to the FGFR-1 are present in isolated fetal growth plate chondrocytes and undergo a perinuclear localization during the G1 of the cell cycle (Kilkenny and Hill, 1996). Incubation of cells with protamine sulfate to prevent the binding of endogenous, cell membrane-associated FGF-2 to high affinity receptors and their subsequent internalization, did not alter the perinuclear accumulation of the FGFBP in late G1 (Kilkenny and Hill, 1996), indicating that this protein does not derive predominantly from intact internalized receptor, and may represent a distinct nuclear transport route for the intracrine functions of FGF-2.

Further diversity of FGF-2 isoforms is achieved through posttranslational modifications such as nucleotidylation, phosphorylation, ADP-ribosylation, and methylation of nuclear FGF-2 isoforms (Baird and Klagsbrun, 1991; Mason, 1994; Boulle et al., 1995). These posttranslational events occur normally inside the cell, and provide a means to modulate FGF-2 activities such as during the maintenance of differentiated function.

## IV. Function of Nuclear FGF-2

The specific function of FGF-2 in the nucleus has not been determined. However, several studies support a direct role for FGF-2 in regulating gene expression. Bouche et al., (1987) have performed both *in vitro* and *in vivo* studies which demonstrate the cell-cycle dependent nuclear uptake of FGF-2 by adult bovine aortic endothelial (ABAE) cells. This nuclear accumulation of FGF-2 occurs only in late G1 and correlated with an increase in transcription of ribosomal RNA (rRNA) genes (Baldin et al., 1990). The ability of FGF-2 to bind DNA (Almaric et al., 1994) suggests that it may elicit this function by direct interaction with the genome. Furthermore, the exogenous addition of FGF-2 to ABAE cells leads to the phosphorylation of nucleolin, a casein kinase II (CKII) substrate (Bouche et al., 1987), and addition of either CKII or FGF-2 to isolated nuclei from quiescent cells results in enhanced transcription of rRNA genes (Baldin et al., 1990). Other CKII substrates involved in transcriptional activation of rRNA genes include RNA polymerase I and topoisomerase I. *In vivo* demonstration of an FGF-2-CKII complex (Almaric et al., 1994) implies that as an alternative to the direct binding of FGF-2 to DNA, it may combine with nuclear proteins to form a trans-acting complex for achieving gene regulatory function(s). Using a cell-free system, Nakanishi et al., (1992) have demonstrated that FGF-2 augments expression of the phosphoglycerate kinase-2 (pgk-2) gene, and inhibits the phosphoglycerate kinase (pgk-1) gene, and therefore may directly regulate transcription in a specific manner.

## V. FGF-2 Secretion

Given the pluripotent effect of FGF-2 on cellular functions, it is somewhat surprising that FGF-2 does not possess a typical hydrophobic leader sequence to direct its secretion (Abraham et al., 1986a). However, several studies refute the general assumption that FGF-2 release occurs only during cell death or when the integrity of plasma membrane is compromised (McNiel et al., 1989; Muthukrishnan et al., 1991). Soluble FGF-2 can be detected in the culture medium of fibrosarcoma tumor cell lines (Kandel et al., 1991), in the serum of patients with familial multiple endocrine neoplasia type-1 (FMEN-1) (Zimering et al., 1990), and in the urine of patients with certain cancers (Nguyen et al., 1993). Mignatti et al., (1992) have demonstrated that FGF-2 isoforms are not secreted through the endoplasmic reticulum-Golgi apparatus, the multidrug resistance pathway or by exocytosis. Interestingly, Florkiewicz et al., (1996) have shown that the release of FGF-2 from COS-1 cells is inhibited >75% by cardioglycoside ouabain which is believed to target the plasma membrane translocation of FGF-2 by interfering with the electrochemical gradients maintained by ouabain-sensitive Na/K ATPases. The latter workers speculated that endogenous oubain-like compounds may control the release of FGF-2 during injury repair, inflammation and angiogenesis.

## VI. Signal Transduction Pathways Initiated by FGF-2

FGF-2 stimulation of the FGFR activates a number of intracellular enzymes including phospholipase C-γ (PLC-γ), Raf-1 kinase, and mitogen-activated protein (MAP) (MacNicol et al., 1993). Of the various potential substrates for tyrosine phosphorylation, only PLC-γ has been demonstrated to interact directly with the FGFR (FGFR-1), and is phosphorylated on a tyrosine residue, which is essential for its activation (Mohammadi et al., 1991). However, it is apparent that not all FGF-2 activities are mediated by PLC-γ, since a mutant FGFR-1 unable to activate PLC-γ is still capable of transducing cell growth- and differentiation-promoting signals (Spivak-Kroizman et al., 1994).

The growth-promoting and differentiation effects of FGF-2 appear to be mediated in part through activation of p42 and p44 mitogen-activated protein kinases (MAPKs/ERKs) (Heasley and Johnson, 1992; Qiu and Green, 1992). The stimulation of MAPKs is dependent on activation of Ras by the GTP/GDP exchange factor SOS (Friesel and Maciag, 1995). Small adapter proteins Grb2 and Shc may provide a link between FGFRs and Ras, via the nucleotide exchange factor Sos. Grb2 has been shown to interact directly with growth factor receptors, and also indirectly via Shc (Pawson, 1995), and a number of other proteins which bind to the Grb2 SH2 domain (Pendergast et al., 1993; Schlaepfer et al., 1994; Sieh et al., 1994). However, no direct interaction between Grb2 and the FGFR has been demonstrated (Vainikka et al., 1994), therefore Ras activation in response to FGFR stimulation may occur through assembly of a Shc-Grb2-Sos complex. In support of this, Shc is phosphorylated on tyrosine residues in response to FGF-2 stimulation of FGFR-1-expressing cells (Vainikka et al., 1994; Wang et al., 1994; Klint et al., 1995). Klint et al. (1995) have characterized the

coupling of FGFR-1 to Ras via Grb2 and Shc, and found that Shc and a novel 89 kDa component couple to the Grb2-Sos complex upon stimulation by FGF-2 of both FGFR-1-transfected L6 myoblasts and murine brain endothelial cells.

Ras interacts with the serine kinase c-Raf-1 in a GTP-dependent manner, and this complex phosphorylates and activates MAPK/ERK kinase (MEK), which in turn activates p42mapk and p44mapk, members of the MAPK family (Marshall, 1994). Raf-1 serine/threonine protein kinase mediates FGF-2 induction of the *Xenopus* mesoderm, as demonstrated by the use of dominant-negative Raf-1 constructs (MacNicol et al., 1993). Also, the expression of active forms of MEK or MAP kinases are necessary and sufficient for ventral mesoderm induction of the kind elicited by FGF (Umbhauer et al., 1995), suggesting that mesoderm induction by FGF-2 involves Ras and Raf which act through the MAP kinases. Besser et al. (1995) have implicated Ras, Raf-1, MEK, and ERK-2 in the signaling pathway of FGF-2 induced urokinase-type plasminogen activator (uPA) gene transcription in NIH 3T3 fibroblasts. Downstream of ERK-2, FGF-2 effects primarily the phosphorylation of the transcription factor JunD, completing an intracellular cascade occurring in the following order; Ras, Raf-1, MEK, ERK-2, and JunD.

Another important signal transduction pathway involving the mitogenic stimulation of receptor tyrosine kinases, is the breakdown of phosphatidylinositol 4, 5-bisphosphate into diacylglycerol which activates PKC (Ullrich and Schlessinger, 1990). FGF-2 has been shown to enhance PKC activity in aortic endothelial cells and Swiss 3T3 cells (Presta et al., 1991; Nanberg et al., 1990). In addition, FGF-2 activation of LDL receptor

transcription in smooth muscle cells was demonstrated to result, in part, from activation of PKC (Hsu et al., 1994).

Multiple signal transduction pathways are induced by FGF-2 stimulation of its receptor, however the occurrence of overlapping phosphorylation events in pathways activated by different second messengers or "cross-talking" (Verma and Sassone-Corsi, 1987; Sassone-Corsi et al., 1990), further suggests that the diverse effects of FGF-2/FGFR interaction on cellular events involve a variety of downstream cytoplasmic and nuclear regulatory factors.

## VII. Role in Tumorigenesis

The abnormal synthesis and release of autocrine-acting FGF-2 underlies the *in vivo* transforming ability of FGF-2. The importance of FGF-2 in tumorigenesis is clearly illustrated by numerous studies which have examined its expression in neoplastic tissues and cell lines. FGF-2 mRNA has been shown to be abundantly expressed in >90% of human glioma tissues (Takahashi et al., 1990), and FGF-2 protein is reported to be abundantly produced *in vivo* (Maxwell et al., 1991; Gross et al., 1993). Also, FGF-2 mRNA and immunoreactive protein are abundantly expressed in glioma-derived cell lines (Okumura et al., 1989; Morrison et al., 1990), melanomas (Halaban et al., 1988), rhabdomyosarcomas (Schweigerer et al., 1987) and schwannomas (Murphy et al., 1989). In addition, transfection of mouse BALB/c3T3 cells with FGF-2 cDNA leads to transformation (Sasada et al., 1988). Further, antisense RNA against FGF-2 mRNA induces a loss of the transformed phenotype of human SK-Hep1 hepatoma cells (Maret et al., 1995), and antisense oligonucleotides against FGF-2 inhibit the growth of malignant melanomas (Becker et al., 1989) or glioma cells (Morrison et al., 1991; Murphy et al., 1992). Inappropriate expression of the FGF-2 gene in cancer may arise from gene amplification, or rearrangement, such as the insertion of enhancer sequences or deletion of negative regulatory sequences, like that which has been suggested for activation of the FGF-4 gene (Curatola and Basilico, 1990). FGF-2 may also be indirectly involved in tumorigenesis through the inappropriate expression of FGFR or its ligand-independent activation (reviewed in Wilkes, 1993).

The majority of evidence supports an autocrine mechanism of FGF-2 in the growth of glial tumors. For instance, neutralizing antibodies

against human FGF-2 inhibited the growth of glioma cells (Takahashi et al., 1991), indicating that FGF-2 acts in an autocrine manner to elicit the autonomous growth and tumorigenesis of these cells. The detection of FGF-2 and FGFR mRNA in a series of human glioma cell lines (Murphy and Knee, 1995), is consistent with a recent report that increased expression of FGFR, and autocrine stimulation of these cells, is associated with malignant progression of astrocytic tumors (Yamaguchi et al., 1994b). Furthermore, the degree of malignancy of brain tumors was demonstrated to be proportional to the level of FGF-2 expression, indicating that FGF-2 plays a key role in tumor progression (Gross et al., 1990; Takahashi et al., 1990; Paulus et al., 1990; Stefanik et al., 1991; Takahashi et al., 1992). However, the mechanism leading to FGF-2 overexpression and its role in the progression of malignant tumors remains to be determined. One possible pathway involving FGF in tumor progression is its activation by the p53 tumor suppressor gene, a nuclear phosphoprotein and cell-cycle regulator (Yin et al., 1992). In support of this, mutant p53 is detected during tumor progression and has been demonstrated to activate the FGF-2 promoter (Ueba et al., 1994).

Interestingly, the secretion potential of FGF-2 seems to be modulated by an undetermined mechanism, as cells undergo transition from benign to malignant and highly vascularized states (Kandel et al., 1991). FGF-2 was largely cell-associated in mild fibromatosis, but was released in the culture medium in aggressive fibromatosis and in particular fibrosarcoma. Transfection of NIH 3T3 cells with an FGF-2 cDNA harboring an immunoglobulin signal peptide sequence at the $NH_2$-terminal can transform these cells (Yayon and Klagsbrun, 1990), suggesting that defective secretion of FGF-2 may be the fundamental event in its autocrine

stimulation of tumor growth. A secreted FGF binding protein (BP) which interacts with FGF-2 in non-covalent reversible manner (Wu et al., 1991), and positively modulates its activity (Czubayko et al., 1994), is believed to serve as an extracellular carrier molecule for FGF-2 by solubilizing the extracellular-bound FGF and allowing it to reach its receptor. Moreover, expression of BP in non-tumorigenic FGF-2-expressing SW-13 cells, leads to an angiogenic and tumorigenic phenotype of these cells (Czubayko et al., 1994).

Angiogenesis is necessary for both normal and pathological processes such as wound healing, tumor growth and metastasis (Folkman and Shing, 1992). FGF-2 acts as a "direct" angiogenic factor by virtue of its ability to stimulate endothelial cell migration and tube formation *in vitro* (Montesano et al., 1986), as well as to enhance the production of proteases and plasminogen activator (Moscatelli et al., 1986; Presta et al., 1986). This, together with the requirement for angiogenesis in the growth of solid tumors (Folkman, 1990), has implicated FGF-2 as a key factor in the development of malignant tumors. Okimoto et al., (1997) have shown that FGF-2 mRNA is detected in ethylnitrosurea (ENU)-induced gliomas, but not in normal glia, indicating that FGF-2 plays an *in vivo* role in mediating glioma tumor growth. *In vivo* evidence supporting the proposed role for FGF-2 in solid tumors was also provided by Stan et al., (1995), who demonstrated that treatment of intracerebrally-implanted U87-MG glioma cells with antibodies against FGF-2 results in the inhibition of angiogenesis and growth of these cells.

**i. Overexpression of FGF-2 in the Human U87-MG Glioma Cell Line**

Although biologically active FGF-2 is present in significant amounts in many tissues, its mRNA levels are generally below the limits of northern detection (Abraham et al., 1986a). This differs from the readily detectable levels of FGF-2 mRNA transcripts in various tumor-derived cell lines. In view of this, it was postulated that FGF-2 mRNA may have a short half-life in most normal tissues. The human U87-MG malignant glioblastoma cell line is characterized by a high level of FGF-2 mRNA that correlates with high concentrations of FGF-2 both intracellularly and in the culture medium of these cells (Sato et al., 1989). The elevated levels of FGF-2 mRNA in U87-MG glioma was demonstrated by Murphy et al., (1990) to be attributable to an increased stability of the transcripts in these cells, however, the mechanism of increased FGF-2 mRNA stability has not been elucidated. Since these cells also express the FGFR (Murphy and Knee, 1995), the high level of FGF-2 protein, likely acting in an autocrine manner, presumably underlies the tumorigenic state of these cells. This was confirmed by the demonstration that antisense oligonucleotides against FGF-2 inhibited U87-MG cell growth in culture (Murphy et al., 1992).

## VIII. Is FGF-2 mRNA Stability Regulated by an FGF-2 Antisense RNA?

In the African clawed toad *Xenopus laevis*, the FGF-2 gene generates three mRNA transcripts (4.5, 2.3 and 1.5 kb in length), the largest of which encodes the FGF-2 polypeptide. The 1.5 kb mRNA transcript, originally believed to represent a fragment of FGF-2 pre-mRNA (Kimelman and Kirschner, 1987) was later identified as an antisense mRNA splice variant transcribed from the opposite strand of the FGF-2 gene (Kimelman and Kirschner, 1989). In *Xenopus* oocytes, the FGF-2 sense and antisense transcripts coexist as RNA duplexes in the cytoplasm of the immature oocyte, and these duplexes have been shown to be unwound and modified by double-stranded RNA-specific adenosine deaminase (DRADA) (Kimelman and Kirschner, 1989). The destabilization of RNA duplexes by DRADA modification is believed to target the transcripts for rapid degradation by dsRNA-specific RNases (See Chapter 3, Section III). Aside from the putative posttranscriptional role of the FGF-2 antisense gene in regulating the stability of FGF-2 sense RNA, the *Xenopus* antisense mRNA also contains an ORF which predicts a 24 kDa protein (Volk *et al.*, 1989), whose function has not yet been determined.

It is only recently that the prevalence of natural antisense gene expression in eukaryotes has been demonstrated, yet the occurrence of this phenomenon in prokaryotes has been studied for almost 20 years. The next section of this thesis provides a detailed review of natural occurring antisense RNA expression in eukaryotes and its potential role in the regulation of gene expression.

## CHAPTER 3: NATURALLY OCCURRING ANTISENSE RNA

### I. Introduction

Transcription of the double-stranded DNA template involves the positive or sense DNA strand. The negative or antisense strand may also be transcribed but in the opposite direction. Bidirectional transcription of the sense/antisense gene locus, depending on the position of their respective promoters, generates RNA messages which may share regions of complementary overlap. Expression of RNA from the antisense strand of a gene has been a well known phenomenon in prokaryotes for many years, and is being encountered with increasing frequency in eukaryotes. Gene regulation by natural antisense RNA is supported by mounting evidence which suggests that it may be a conserved feature within the genomes of all species from archaebacteria to humans (Stolt and Zillig, 1993; Merino et al., 1994). Antisense RNA in prokaryotes has been implicated in the control of gene expression at many levels including regulation of transcriptional termination, RNA processing, transcript stability, ribosome binding, and inhibition of translation (reviewed by Simons, 1993). However, in contrast to prokaryotic antisense RNA, several eukaryotic antisense RNAs have been identified which contain open reading frames (ORFs) and therefore potentially encode functional proteins.

That antisense transcription is not simply attributable to occasional accidental read-through of adjacent genes is evidenced by the formidable array of molecules and pathways designed specifically to bind, inactivate, modify, degrade or otherwise respond to the formation of double-stranded RNA (dsRNA) hybrids (Figure 4). For example, dsRNA-dependent protein kinases (DDPKs) such as the vertebrate serine/threonine kinase

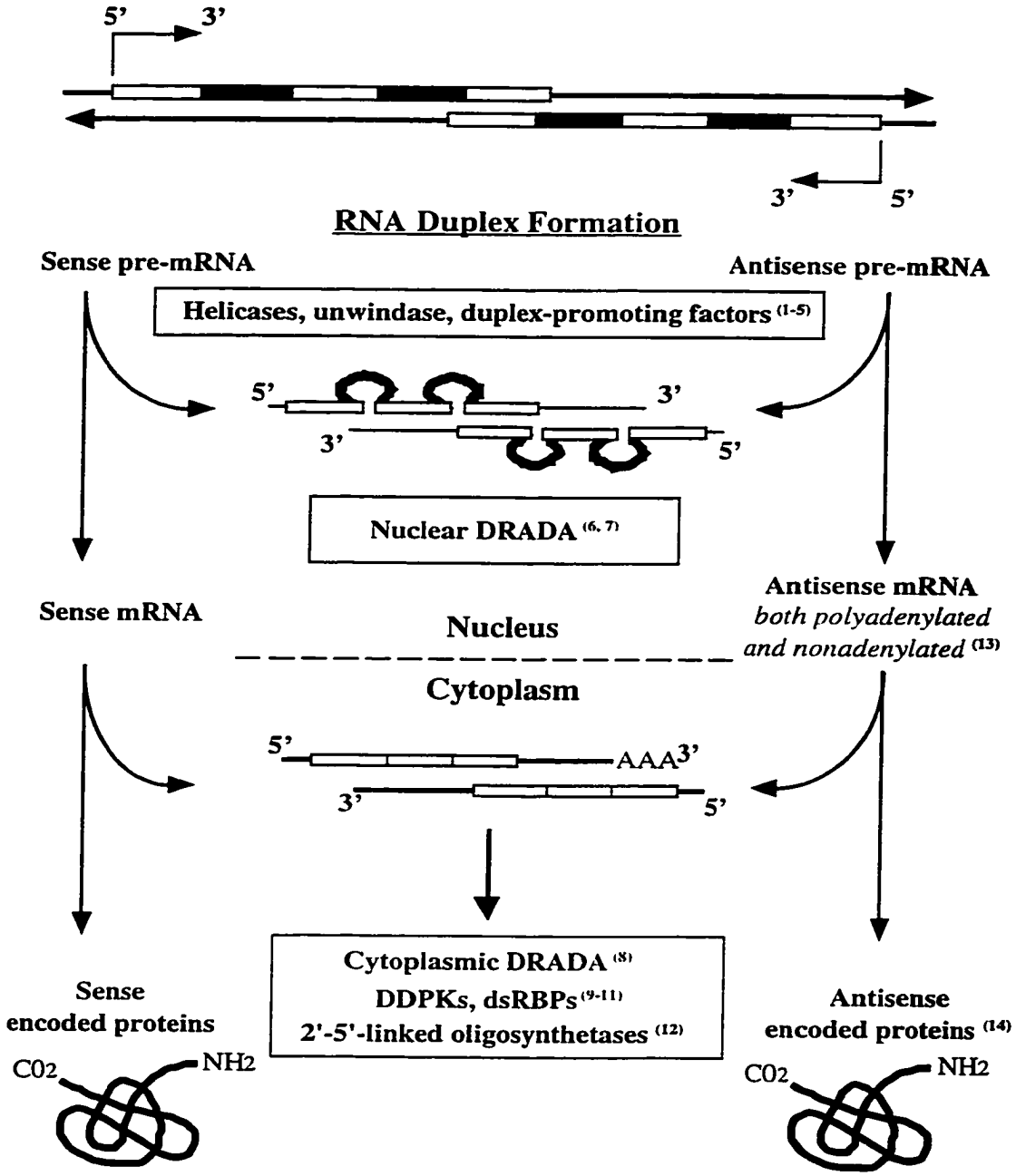



**Figure 4.** Regulatory proteins involved in sense: antisense RNA interaction. Abbreviations; dsRNA adenosine deaminase (DRADA), dsRNA-dependent protein kinases (DDPKs), dsRNA binding proteins (dsRBPs). (*Numbers in parentheses correspond to references*; **1**, Ray *et al.*, 1985; **2**, Hirling *et al.*, 1989; **3**, Bass and Weintraub, 1987; **4**, Rebagliati and Melton, 1987; **5**, Pontius and Berg, 1990; **6**; **7**, Bass and Weintraub, 1987; 1988; **8**, Patterson and Samuel, 1995; **9**, Haines *et al.*, 1991; **10**, Manche *et al.*, 1992; **11**, Saccomanno and Bass, 1994; **12**, Nellen and Lichtenstein, 1993; **13**, Krystal *et al.*, 1990; **14**, Harding and Lazar, 1993).

DDPK (p68), which specifically responds to the formation of RNA duplexes and may provide a sensitive means of regulating cellular signal transduction pathways (Haines et al., 1991; Manche et al., 1992). The highly sophisticated array of dsRNA-specific proteins suggests a diversity of modes of action of natural antisense RNA and that, at least in some cases, interaction of these sense and antisense transcripts is a physiologically relevant phenomenon.

**II. Prokaryotic and Viral Antisense RNA**

Natural antisense control was first demonstrated in the plasmid ColE1, in which DNA replication is controlled by an antisense RNA that regulates maturation of the ColE1 primer (Lacatena and Cesareni, 1981). Sense-antisense RNA interactions are initiated by the sequence-specific association of loops (called the kissing complex) in the highly ordered stem-loop structure of the two RNAs. Initiation of the kissing complex and subsequent formation of a stable dsRNA complex induces conformational changes in the sense transcript which prevent primer formation and plasmid replication. Many of the known prokaryotic antisense RNAs fit this general model; they are short, highly structured, untranslated, and contain at least one stem-loop secondary structure in which the loop regions serve as the primary recognition site and the stems often determine its stability (Hjalt and Wagner, 1995). Although nucleotide sequence and loop size are key determinants in sense-antisense interaction, hybrid formation may also be regulated by a variety of accessory proteins which may facilitate (Eguchi et al., 1991) or inhibit (Masters et al., 1990) hybridization. Proteins which may serve similar functions in eukaryotes have also been described (Pontius and Berg, 1990).

Recently, antisense RNA has been implicated in the regulation of HIV-1 gene expression. A naturally occurring antisense RNA transcript complementary to the HIV-1 *env* gene was isolated from HIV-infected T cell lines by RT-PCR (Vanhee-Brossollet et al., 1995). The simultaneous expression of *env* sense and antisense transcripts in cells chronically infected with HIV-1 supports the potential formation of a nuclear and/or cytoplasmic dsRNA complex, which may serve in regulating the expression of these genes (Vanhee-Brossollet et al., 1995).

## III. Eukaryotic Antisense RNA Expression

### i. Introduction

The prevalence of eukaryotic gene loci which transcribe both sense and antisense RNA species has only recently been recognized (reviewed by Simons, 1993). The sense/antisense transcription units identified to date represent a complex variety of structures; regions of overlapping transcription have been described at both the 5' (Mitchell et al., 1986; Krystal et al., 1990) and 3' ends (Kimelman and Kirschner, 1989; Miyajima et al., 1989; Lazar et al., 1989; van Duin et al., 1989; Williams and Fried, 1986) of the transcriptional units, and in some instances the sense and antisense transcripts share exonic regions of DNA (Miyajima et al., 1989; Lazar et al., 1989; Adelman et al., 1987). In other cases, antisense transcriptional units are localized entirely within the introns of expressed genes (Henikoff et al., 1986; Levinson et al., 1990; Wallace et al., 1990; Chen et al., 1987) and are unlikely to be involved in mutual regulation. Similarly, although antisense RNAs arising from gene rearrangements are known to exert regulatory control of sense transcript expression (Tosic et al., 1990), they are clearly not physiologically significant in natural antisense regulatory mechanisms. The high frequency of in-phase ORFs in the antisense strands of recognized eukaryotic gene sequences (Merino et al., 1994) suggests that the number of reported natural antisense RNAs will continue to grow. It must be recognized that many of these naturally occurring antisense RNAs are likely not involved in the regulation of sense RNA expression, but simply represent fortuitous use of minus strand DNA for coding purposes. Eukaryotic genes reported with the ability to generate endogenous antisense RNA transcripts include the thyroid receptor gene c-*erb*Aα (Miyajima et al., 1989), N-*myc* (Kindy et al.,

1987), GnRH (Adelman et al., 1987), IGF-II (Taylor et al., 1991), and dihydrofolate reductase (Mitchell, 1986).

### ii. Role of Natural Antisense RNA in Gene Expression

#### a. Introduction

The extensive sequence homology in eukaryotes between sense and antisense genes either across exon/intron junctions or among 5' or 3' regions of overlap in the transcription unit, suggests that antisense transcripts may regulate gene expression at many of the same levels as in prokaryotes.

Convincing evidence has been provided for post-transcriptional regulation of c-*erbA*$\alpha$ (Munroe and Lazar, 1991), N-*myc* (Krystal et al., 1990) and FGF-2 (Kimelman and Kirschner, 1989) mRNAs by antisense transcripts. Circumstantial evidence supports the possibility of regulation in other bi-directionally transcribed loci including p53 (Khochbin et al., 1992), Hoxa-11 (Hsieh-Li et al., 1995), IGF-II (Baccarini et al., 1993), TGF-$\beta$ (Huang et al., 1995), and GnRH (Kelly et al., 1991). A summary of the genes for which clear evidence of regulation, or potential regulation by natural antisense RNA is presented Table 1.

**Table 1. Known and putative actions of natural antisense RNA.**

***Prokaryotes and viruses***

(I) *Control of plasmid replication*
Premature transcriptional termination: *Staphylococcal* plasmid pT181; antisense RNA suppresses RepC expression (Novick *et al.*, 1989)

(II) *Control of bacteriophage development*
Degradation by dsRNase: Bacteriophage λ; OOP RNA regulates stability of cII RNA (Krinke and Wulff, 1987)

(III) *Inhibition of bacterial gene expression*
Translational regulation: *Escherichia coli, dicF* RNA blocks ribosome binding site in *ftsZ* RNA (Tetart and Bouche, 1992)

(IV) *Regulation of viral expression*
Regulation of HIV expression:*env* (envelope) antisense, *ASO1* encodes protein which potentially regulates HIV life cycle (Micheal *et al.*, 1994)

***Eukaryotes***

(I) *Regulation of transcription*
N-*myc*: regulation of transcriptional elongation by N-*cym* antisense RNA (Krystal *et al.*, 1990)

(II) *Regulation of RNA editing*
Nuclear DRADA: modifies reading frame of glutamate receptor subunit RNA, alters channel properties (Higuchi *et al.*, 1993)

(III) *Inhibition of mRNA splicing*
Thyroid hormone receptor (*c-erbA*): Rev-erbAα prevents splicing of α2 pre-mRNA (Munroe and Lazar, 1991)
Gonadotropin-releasing hormone (GnRH): SH antisense transcripts may block GnRH pre-mRNA processing (Wilson *et al.*, 1995)

(IV) *Modulation of RNA transport*
Myelin deficient mice (*mld* gene): antisense RNAs block *mld* mRNA transportation to the cytoplasm (Tosic *et al.*, 1990)

(V) *Inhibition of mRNA half-life*
Basic fibroblast growth factor (bFGF): Antisense RNA may target bFGF sense RNA for rapid degradation (Kimelman and Kirschner, 1989)

(VI) *Regulation of dsRNA induced enzymes*
dsRNA-dependent protein kinase (DDPK): dsRNA may modulate cellular signal transduction pathways by activating DDPKs (Haines *et al.*, 1991; Manche *et al.*, 1992)

(VII) *Regulation of translation*
Chicken myosin heavy chain (MHC): translational control RNA (tcRNA) is an antisense RNA complementary to MHC mRNA (Heywood, 1986)

**b. Antisense Regulation by RNA Editing**

DsRNA-specific adenosine deaminase (DRADA) was originally called RNA unwindase because of its ability to specifically unwind RNA duplexes (Bass and Weintraub, 1987; Rebagliati and Melton, 1987). More recently it has been recognized as a ubiquitously expressed nuclear enzyme which modifies selected adenosine residues in dsRNA to inosines by hydrolytic deamination, replacing A-U with mismatched I-U base pairs (Kim and Nishikura, 1993). (The inosine residues are read equivalent to cytosine by reverse transcription, so that edited adenosine residues are detected as guanosine in cDNA). This enzyme has been shown to mediate the post-transcriptional editing of glutamate-gated ion channel gene transcripts in mammalian brains (Higuchi et al., 1993; Melcher, et al., 1995; Sommer et al., 1991). A-to-I editing of the channel subunit *GluR-B* pre-mRNA results in the substitution of a glutamine (CAG) by an arginine (CGG) codon (Sommer *et al.*, 1991). This modification, at the so-called Q/R site, occurs prior to splicing on a dsRNA structure formed between complementary intron and exon sequences, and causes a marked reduction in the calcium permeability of the channel.

RNA editing by this mechanism, directed by interaction of sense and antisense RNA sequences could, for example, be used to suppress translational termination or other coding changes that may lead to translation of non-functional protein products. It has been postulated that the formation of dsRNA hybrids between sense and antisense FGF-2 transcripts targets these mRNAs for modification by DRADA (Kimelman and Kirschner, 1989) possibly resulting in altered transcript stability (discussed below). The recent report of a cytoplasmic form of DRADA, and of a cytoplasmic dsRNA binding protein which protects dsRNA from

modification by DRADA (Saccomanno and Bass, 1994) lends further support to the suggestion that sense-antisense RNA interactions are physiologically significant phenomena.

### c. Antisense Regulation of pre-mRNA Splicing

*c-erbA/Rev-erbA Gene Locus*

The thyroid hormone receptor/*c-erbA* gene locus is the most concrete example of gene regulation by a natural antisense RNA. Rat and human thyroid hormone receptors (TRs) are encoded by two genetic loci, c-*erbA*$\alpha$ and c-*erbA*$\beta$ (Weinberger et al., 1986; Nakai et al., 1988; Murray et al., 1988; Hodin et al., 1989). The c-*erbA*$\beta$ gene gives rise to two $\beta$ receptors, encoded by two mRNAs which have a tissue-specific, developmentally regulated pattern of expression. The c-*erbA*$\alpha$ locus generates three different mRNAs, of which the first, $\alpha$1, encodes the authentic TR and the second, $\alpha$2, encodes a TR which lacks thyroid hormone binding ability. A third mRNA, Rev-ErbA$\alpha$, is transcribed from the c-*erbA*$\alpha$ locus, but in the opposite direction from $\alpha$1 and $\alpha$2 (Miyajima et al., 1989; Lazar et al., 1989). The 3' end of this mRNA overlaps the 3' end of $\alpha$2, but not $\alpha$1, mRNA. The complementary sequence in Rev-ErbA$\alpha$ has been shown to inhibit $\alpha$2 pre-mRNA splicing in HeLa cell nuclear extracts (Munroe and Lazar, 1991). This inhibition of splicing was also observed with a shorter antisense RNA complementary only to the 3' exon of $\alpha$2 mRNA, indicating that splicing is sensitive to relatively limited RNA duplex formations. *In vitro*, $\alpha$2 splicing is extremely efficient, raising the possibility that formation of $\alpha$2 mRNA is favored over $\alpha$1 in the absence of specific regulation. These results implicate naturally occurring

antisense RNA as having a physiologically important role in the regulation of mRNA splicing.

The Rev-ErbAα transcript, in addition to its regulatory role at the RNA level, encodes a nuclear hormone receptor-related protein which has been demonstrated to be a sequence-specific DNA-binding protein and transcriptional activator (Harding and Lazar, 1993). Therefore, the *erb-A* gene is a unique example of natural antisense in which two different polypeptides encoded by RNAs transcribed from opposite ends of the gene share related functions.

*N*-myc/*N*-cym *Gene Locus*

The Myc nuclear protooncogene family includes c-*myc*, L-*myc*, and N-*myc* (DePinho et al., 1987). C-*myc* is the cellular homologue of v-*myc*, originally isolated from an avian myelocytomatosis virus. The N-*myc* and L-*myc* protooncogenes are homologues to c-*myc* and are amplified in human neuroblastomas (Kohl et al., 1983; Schwab et al., 1983) and small cell lung cancer (SCLC) (Nau et al., 1985), respectively. All the *myc* genes contain three exons, wherein the exon 2 and 3 coding regions are highly conserved and the 5' end structurally distinct (Krystal et al., 1990).

The N-*myc* antisense gene, termed N-*cym*, originates within intron 1 and extends back through exon 1 of the N-*myc* gene, with extensive overlap between the 5' ends of the transcriptional units (Krystal et al., 1990). Transcription initiation of the antisense gene occurs in a similar pattern to that of the N-*myc* sense gene, with multiple trans-activating sites existing over a few hundred bases. Conceivably, the entire region may serve as a bidirectional promoter transcribing both the sense and antisense gene. This possibility is supported by the observations that transcription in both orientations appears to be co-regulated (Armstrong

and Krystal, 1992) and that relative expression of sense to antisense transcription across exon 1 remains balanced (Krystal et al., 1990). Bidirectional promoters have been reported for other eukaryotic genes. For example, the murine α1(IV) and α2(IV) collagen genes, oriented in opposite directions, are transcribed by a bidirectional promoter and shared enhancer (Burbelo et al., 1988). The mammalian *dhfr* gene also contains a bidirectional promoter, and like N-*myc* and the mouse collagen genes, lacks the TATA consensus and has multiple SP1 binding sites in a GC-rich region (Linton et al., 1989).

Transcription of the N-*cym* antisense gene produces distinct 1.0 and 1.8 kb polyadenylated RNA transcripts, and also smaller, more abundant nonadenylated species by initiation at multiple sites predominantly within intron 1 (Krystal et al., 1990). Given the limited amount of antisense RNA available to engage in duplex formation, a potential function of the RNA duplex in regulating N-*myc* translation does not seem likely (Krystal et al., 1988). However, different RNase sensitivities among the multiple N-*myc* sense RNAs suggests that not all forms anneal with the antisense transcript to the same degree (Krystal et al., 1990). RNA hybrid analysis has demonstrated that N-*cym* RNA transcripts selectively form *in vivo* RNA duplexes with a subpopulation of N-*myc* sense transcripts. Generally, each RNA complex formed contains sequences which correspond to the region extending from the 5' end of N-*myc* to the 5' end of N-*cym* in intron 1, so N-*myc* sense transcripts which retain intron 1 selectively form RNA duplexes with N-*cym* antisense transcripts. Translation of the N-*myc* intron 1-containing RNAs may therefore potentially be regulated by N-*cym* antisense RNA. This RNA species contains an in-frame AUG which is preferentially used for generating a

longer N-*myc* protein *in vitro* (Makela et al., 1989). The N-*cym* antisense transcript seems to prevent translation from the intronic AUG because this longer N-*myc* protein is not detected *in vivo*. In addition, the antisense transcript may inhibit N-*myc* pre-mRNA splicing (Krystal et al., 1990). Regulation of splicing patterns by intermolecular RNA duplexes has previously been suggested (Konarska et al., 1985).

*GnRH/SH Gene Locus*

The decapeptide gonadotropin-releasing hormone (GnRH) modulates reproductive endocrine functions via the central nervous system (CNS) and is generated from a larger precursor peptide termed proGnRH (Adelman et al., 1986). Both the rat and human GnRH genes display the conserved feature of antisense transcription. Antisense RNA transcripts derived from the opposite strand of the GnRH gene were first detected in rat heart (Adelman et al., 1987), and subsequently in the preoptic area of the hypothalamus (Bond et al., 1989) There are four alternatively spliced antisense RNAs transcribed in the opposite direction relative to the GnRH gene, each with similar length sharing a common 3' sequence and a unique 5' end (Adelman et al., 1987). The human GnRH gene gives rise to antisense transcripts which are expressed in all placental cell types at a level three-fold higher than GnRH sense transcripts (Kelly et al., 1991). The apparent localization of GnRH antisense RNA in the nucleus suggests that it may play a role in GnRH mRNA processing.

In rat brain a relatively large pool of proGnRH pre-mRNA exists which may arise from a high rate of transcription and/or slow RNA processing. Blockade of proGnRH pre-mRNA processing or regulation of stability by interaction with SH transcripts in this tissue does not seem likely because of the very low levels of these antisense transcripts compared to that of the

proGnRH RNA species. The functional significance of SH transcripts in the regulation of GnRH gene expression remains to be determined.

**d. Antisense Regulation of Translation**

*IGF-II Gene Locus*

Transcription of insulin-like growth factor-II (IGF-II) antisense RNAs has been identified in a broad distribution of species from chicken to human, suggesting an evolutionary conservation of the antisense gene. The human and rat IGF-II genes are quite complex, transcribing multiple RNAs by alternative promoter usage, alternative splice sites, and polyadenylation signals (Sussenbach, 1989). The first exon of the IGF-II gene is located approximately 1.5 kb downstream of the insulin gene (Sussenbach, 1989; Rotwein and Hall, 1990). In an attempt to characterize the exonic organization of the mouse IGF-II gene, a novel antisense gene was found oriented in the opposite direction, and located between the insulin and IGF-II loci (Rivkin et al., 1993). Developmental expression analysis of the IGF-II antisense gene revealed that it is transcribed most abundantly in various tissues of the mouse embryo at day 18, but at much lower levels than IGF-II RNA. The antisense transcript became progressively less abundant at the postnatal stage in all tissues examined and undetectable after day 10.

A study of IGF-II expression during chick embryonic development allowed the isolation of a clone with homology to the second coding exon of the human, mouse, and rat IGF-II genes (Taylor et al., 1991). Sequence comparison of this clone to that of the corresponding genomic clone indicated that it was derived from a spliced antisense transcript of the chicken IGF-II gene. Similar to the expression pattern in mouse, the

chicken IGF-II antisense transcripts were also most abundant in the late stages of embryonic development.

A possible regulatory role for the antisense IGF-II transcript is suggested by the observation that human fetal IGF-II protein levels are not as high as might be predicted from the observed level of IGF-II mRNA (Hill, 1990). Such disproportionate levels of expression between IGF-II RNA and protein also exists in childhood nephroblastoma, or Wilms' tumor (Haselbacher et al., 1987; Irminger et al., 1989). The presence of IGF-II antisense transcripts in Wilms' tumor (Baccarini et al., 1993) as well as during fetal development, may explain the lack of IGF-II protein despite high levels of IGF-II sense RNA.

**e. Antisense Regulation of mRNA Stability**

*PCNA/YC Gene Locus*

Recently, natural antisense transcription of the proliferating cell nuclear antigen (PCNA) gene was demonstrated in the ascidian *Styela clava* (Swalla and Jeffery, 1996). PCNA is an auxiliary protein of DNA polymerase-γ, which is required for DNA processivity in eukaryotic cells (Bravo et al., 1987; Shivji et al., 1992). The long 3' UTR of PCNA mRNA shares a 521 nt region of overlap at its 3' end with the noncoding yellow cresent (YC) RNA, whose function is unknown. The antisense YC RNA has been suggested to function in limiting the localization of maternal PCNA to cellular regions during ascidian development. The temporal upregulation of YC RNA coincides with PCNA mRNA attenuation in the myoplasm following fertilization. Since this occurs at a time when dsRNAases are activated, YC RNA may play a role in the control of mRNA stability to facilitate PCNA localization during embryogenesis.

*Hox Gene Locus*

The *Abdominal-B* (*Abd-B*) type Hox genes of the *Antennapedia*-like homeobox class display overlapping domains of expression in developing limbs and are therefore believed important in specifying regional identity (Dolle et al., 1989; Yokouchi et al., 1991). Targeted mutations of the murine *Abd-B* type *Hoxa-11* are shown to result in both axial and appendicular skeletal defects (Small and Potter, 1993) and sterility (Hsieh-Li et al., 1995). Analysis of the murine *Hoxa-11* gene structure led to the finding that the antisense strand gives rise to elaborately spliced mRNAs, suggesting a possible regulatory role for the antisense transcript (Hsieh-Li et al., 1995). The temporal expression of sense and antisense transcripts in mouse embryos indicate that the antisense transcripts are more abundant than *Hoxa-11* sense transcripts (Hsieh-Li et al., 1995). Moreover, *in situ* hybridization demonstrated a distinct complementary pattern of sense and antisense transcript expression in the developing limbs with an increase in antisense RNA abundance in regions where sense RNAs are diminishing. This pattern of expression is consistent with an antisense role in regulating *Hoxa-11* sense transcript stability.

*Transforming growth factor-β*

The transforming growth factor-β (TGF-β) gene family, like the FGF gene family, includes a large number of genes (TGF-β1-5) which share at least 70% homology at the nucleotide level, as well as a series of less closely related genes including the activins, *Vg1*-related genes, and bone morphogenetic proteins. The TGF-β genes are conserved throughout evolution from amphibians to humans. While screening developing cardiac tissues for TGF-β3 RNA expression, Runyan and coworkers (1992) fortuitously observed the presence of an antisense TGF-β3 RNA transcript.

When a TGF-β3 antisense strand-specific riboprobe was used for northern hybridization, the transcript was shown to be 4 kb in length, longer than the 3 kb sense transcript (Potts et al., 1992). The temporally controlled appearance of RNA complementary to the TGF-β3 sense transcript suggests that this molecule may play a role in the regulation of TGF-β3 production in heart tissue. The exact nature of the sense/antisense transcription unit has not been defined. However, the region of overlap is reported to be at least 120 bp in length, sufficient to confer some regulatory capacity (Huang et al., 1995). This possibility is supported by the fact that the TGF-β3 antisense transcript is present in excess of the sense transcript in stage 20 avian cardiac cells. Although no direct evidence yet exists, it has been suggested that, similar to FGF-2 (see below), the TGF-β3 antisense RNA functions to target the sense transcript for inactivation by DRADA (Huang et al., 1995).

*FGF-2 Gene Locus*

Bidirectional transcription of the *Xenopus laevis* FGF-2 gene locus generates multiple sense mRNA and a single 1.5 kb antisense RNA (Kimelman and Kirschner, 1989). In *Xenopus* oocytes, the FGF-2 sense and antisense transcription units share a 900 bp region of overlap at their 3' ends (Volk et al., 1989). The antisense transcript is present in the oocyte at a 20-fold excess; a sufficient quantity to bind all the FGF-2 sense RNA present. The sense and antisense transcripts coexist as RNA duplexes in the cytoplasm of the immature oocyte, and these duplexes have been shown to be unwound and modified by dsRNA-specific adenosine deaminase (DRADA) at the time of germinal vesicle breakdown (Kimelman and Kirschner, 1989). Given the rapid attenuation of FGF-2 RNA stability following this reaction, it has been suggested that the

destabilization of RNA duplexes by DRADA modification targets the transcripts for rapid degradation by dsRNA-specific RNases (Kimelman and Kirschner, 1989). Inhibition of translation does not appear to be natural antisense mechanism since synthetic duplexes formed between FGF-2 sense and antisense RNAs does not prevent *in vitro* translation of sense mRNA.

Two antisense FGF-2 transcripts of approximately 2.3 and 2.6 kb in size have been reported to be expressed during chicken embryogenesis (Borja et al., 1993). Adult chicken organs were found to express inversely proportional levels of FGF-2 sense and antisense transcripts, supporting the suggestion (Kimelman and Kirschner, 1989) that FGF-2 sense transcripts are negatively regulated by antisense transcripts. Both of the antisense transcripts contained sequences complementary to exon 3 of the FGF-2 sense gene, suggesting evolutionary conservation of the sense/antisense transcription unit. The two transcripts may arise by differential polyadenylation (Borja et al., 1993). Savage and Fallon (1995) have also demonstrated FGF-2 antisense RNA expression in developing chick tissues. The sense and antisense transcripts were shown to colocalize in some tissues and both transcripts were expressed in a developmentally regulated manner. Tissue levels of FGF-2 protein were shown to be inversely proportional to the level of antisense mRNA expression, indirectly supporting the possibility that the antisense RNA regulates FGF-2 protein levels. Interestingly, Savage et al., (1993) have reported that cellular FGF-2 protein levels in developing chick mesoderm were lowest during the G1 phase of the cell cycle, and suggest that the antisense message may act at each cell cycle to ensure the turnover of FGF-2 mRNA (Savage and Fallon, 1995).

The genomic organization of FGF genes has been strongly conserved throughout evolution, raising the possibility that a comparable family of antisense genes may exist. To date however, antisense expression has only been reported for FGF-6 (Coulier et al., 1991). Strand-specific cRNA probes demonstrate an abundant 0.85 kb transcript in the testes of sexually mature mice, but not prepubertal animals, suggesting that the antisense transcript may be expressed in mature germ cells.

## IV. Thesis Proposal

The potential function of the FGF-2 antisense gene in regulating the stability of FGF-2 sense mRNA led us to speculate that a defective antisense gene may underlie the overexpression of FGF-2 in U87-MG glioma cells and possibly other tumors. Strand-specific FGF-2 cRNA probes have been reported to detect FGF-2 antisense transcripts in a variety of species, but antisense RNAs have not previously been characterized. It was therefore of interest to clone and characterize the mammalian FGF-2 antisense mRNA.

## CHAPTER 4: MATERIALS AND METHODS

### I. Chemicals

Radioisotope-labeled products for probe synthesis ($^{32}$P-αrUTP, $^{32}$P-αCTP) and DNA sequencing ($^{35}$S-αATP) were purchased from Amersham, Oakville, ON. Bacto-tryptone, yeast extract, and agar for propagation of bacterial cultures were obtained from Difco Laboratories, Detroit, MI. Gel electrophoresis reagents; Ultra-Pure agarose, acrylamide, N, N'-methylene-bis-acrylamide, ammonium persulfate, and N, N, N', N'-tetramethylethylenediamine (TEMED) were purchased from Life Technologies, Grand Island, NY. Guanidium isothiocyanate (GITC) and phenol for RNA isolation were also obtained from Life Technologies. All other chemicals were from Sigma Chemical Company, St. Louis, MO, unless otherwise specified.

### II. Cell Culture

Liquid cell culture reagents; Dulbecco's Modified Eagle's Medium (DMEM), MEM-α medium, RPMI 1640 medium, Ham's F10 medium, horse serum (HS), fetal calf serum (FCS), penicillin and streptomycin, were purchased from Life Technologies.

All cell lines were from American Type Culture Collection (ATCC) Rockville, MD, these included the human lung carcinoma cell lines, A549 and A427; human colon adenocarcinoma cell lines, HT-29 and T84; the human glioblastoma cell line, U87-MG; rat C6 glioma and PC12 pheochromocytoma cell lines; and the mouse Hepa1 hepatoma cell line. Frozen cell lines were removed from storage in liquid nitrogen, rapidly thawed at 37°C, transferred to 5ml DMEM in a 15ml tube and centrifuged at 2200 rpm (1000 xg) at 4°C for 10 min in a GPR counter-top centrifuge

(Beckman Instruments, Inc., Palo Alta, CA). Media was decanted, cells resuspended in fresh media, and seeded at 5000 cell/$cm^2$ in T75 flasks and grown in 12 mL DMEM supplemented with 10% FBS, 100 IU/mL penicillin and 100 µg/mL streptomycin under 5% $CO_2$ at 37°C. All cell lines were grown under these conditions except rat PC12 cells which were grown in RPMI medium 1640 containing 10% HS, 5% FCS, 100 IU/mL penicilin and 100 µg/mL streptomycin.

**i. Oxidation and Nucleotide Treatments**

*Hydrogen Peroxide*

Rat C6 glioma cells were grown to 70% confluency and treated with 1 mM hydrogen peroxide in culture media for increasing duration (0.5, 1, 2, 4, and 8h). Total RNA was then isolated from cultures at each of the time points and used for RT-PCR analysis.

*Methylene Blue plus Visible Light*

Mouse hepatoma Hepa1 cells were grown to 70% confluency prior to photosensitization essentially as described by Floyd et al., 1989. Media was removed and cells were washed twice with PBS. Methylene blue (MB) (4 µM) in PBS was added to cultures which were placed at 37°C under dark conditions for 10 min to allow for MB uptake. MB plus light cultures then received light (100W tungsten) at 10 cm for 5 min while MB only cultures were stored under dark conditions during this time. Cultures were then incubated under dark conditions at 37°C for the following times; 0, 0.5, 1, 2, 4, and 8 hrs. Total RNA was isolated from MB only and MB plus light cultures at the indicated times for RT-PCR analysis.

*Ap5A (diadenosine 5', 5''''-P1, P5-pentaphosphate)*

Rat PC12 pheochromocytoma cells were grown to approximately 70% confluency prior to treatment. Ap5A at a final concentration of 1 mM was added to the media and cultures were then incubated for the following times; 0, 1, 3, 6, 12, and 24 hr. Total RNA was then isolated from cultures at the indicated times for RT-PCR analysis.

**III. Animals**

Tissues for RNA and protein isolation were surgically removed from Sprague-Dawley rats (Charles River, St. Constante, QC), immediately snap-frozen in liquid nitrogen and stored at -70°C until processing. For development studies; brain, heart, kidney, and liver tissue were obtained from embryos (prenatal d17) and neonates (postnatal d10) of timed-pregnant rats. Whole liver, primary hepatocytes and Kuppfer cells were obtained from male CFW mice.

Ovarian tissue from *Xenopus laevis* for RT-PCR was obtained from Dr. Richard Wassersug, Department of Anatomy, Dalhousie University. Total RNA was isolated as described (see below).

Animal maintenance and the $CO_2$ euthanasia procedure were in compliance with the guidelines of the Canadian Council on Animal Care (CCAC).

**IV. Primary Cells**

*Human oocytes and Granulosa Cells*

Human oocytes and granulosa cells were obtained from the Grace Maternity Hospital In Vitro Fertilization Clinic. Oocytes discarded from the IVF clinic after being determined unfertilized by the absence of

pronuclei at 72 hrs post-insemination were snap-frozen and stored at -70°C until being subjected to RNA isolation and RT-PCR analysis. Granulosa cells were obtained after follicular aspiration, and subsequently purified by Percoll gradient centrifugation (Yan et al., 1993). In following this procedure, follicular aspirates were diluted in Ham's F10 medium containing heparin (30 U/mL) and centrifuged at 500 xg at 4C for 20 min. The pelleted cells were resuspended in 2 mL medium and layered on a 10 mL cushion of 70% Percoll (Phamacia Fine Chemicals, Dorval, QC). The sample was centrifuged at 2000 xg for 30 min to remove contaminating blood cells, and the supernatant and 2 mL of the underlying Percoll cushion were collected to a fresh tube and recentrifuged. Granulosa cells were collected at the interface, then washed 3 times in 5 mL of Ham's F10 medium and stored at -70°C until processing.

*Mouse Liver Cells*

Hepatic cells were isolated essentially as described by Renton et al., (1978). Dissociated cells were placed on ice and allowed to settle by gravity for 20 min. The supernatant (non-parenchymal cells) was carefully decanted and the pellet (hepatocytes) was resuspended in 5 mL serum-free RPMI medium 1640. Centrifugation was then performed at 500 xg for 3 min (non-parenchymal fraction (supernatant)) or 50 xg for 1 min (hepatocyte fraction), cell pellets resuspended and recentrifuged again before resuspending in 5 mL/liver of RPMI 1640 containing 10% FCS (non-parenchymal fraction) or 10 mL/liver of MEM-α containing 10% FCS (hepatocyte fraction). The non-parenchymal fraction was examined under light microscope to ensure the absence of hepatocytes and viability of the cells was determined by addition of trypan blue to an aliquot of each fraction. Hepatocytes were counted and plated at 1 X $10^6$ cells/mL MEM-α

containing 10% FCS, 100 IU/mL penicilin and 100 µg/mL streptomycin in 5 mL dishes. Media was changed after 6 hrs and the incubation was continued up to 48 hr before harvesting for RNA and protein isolation.

**V. RNA Isolation**

Total RNA was isolated from cells and tissue by homogenization (Polytron PT 3000, Brinkman Instruments, Rexdale, ON) at 20 krpm for 5 sec in guanidium isothiocyanate (GITC) buffer and sequential extraction in phenol and chloroform (Chomcynzski and Sacchi, 1987). RNA samples were quantified by absorbance reading at 260 nm using the Beckman DU640 spectrophotometer (Beckman). Oligo-dT cellulose chromatography (Aviv and Leder, 1972) was performed for the isolation of poly(A)+ in northern analysis. Poly(A)+ RNA for semi-quantitative RT-PCR was isolated using the QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech, Baie D'urfe, QC).

**VI. Complementary DNA (cDNA) Libraries**

For isolation of the full-length FGF-2 antisense cDNA three different libraries were screened; the human brainstem cDNA library (ATCC #37432), human adult liver cDNA library (ATCC #77402), and a developmental-specific cDNA library derived from rat neonatal liver, which was constructed using a kit supplied by Stratagene, La Jolla, CA.

**i. Rat Neonate Liver cDNA Library**

*RNA Preparation*

Liver tissue for cDNA library construction was obtained from day 10 neonatal Sprague-Dawley rats and poly(A)+ RNA was isolated using the

Messenger RNA Isolation Kit from Stratagene. Tissue (500 mg) was homogenized in denaturing solution (5 mL) containing 50 μL β-mercaptoethanol. Elution buffer (10 mL) was added and mixed by inversion, then centrifuged at 10,000 rpm (12,100 xg force) for 10 min in a JA20 rotor using the J2-21M/E floor model centrifuge (Beckman). Supernatant was transferred to a 50 mL tube containing 5 mL of 40 mg/mL oligo(dT) cellulose and gently shaken for 15 min at room temperature. The cellulose was centrifuged at 3000 rpm (2060 xg force) for 3 min using the GPR counter-top centrifuge (Beckman). Once the supernatant was removed, the packed resin was resuspended in 5 mL of a high-salt buffer and washed an additional two more times. After the final high-salt wash, the pelleted cellulose was resuspended in 5 mL of low-salt buffer and transferred to a push column. Using 2X 400 uL of elution buffer heated to 68°C, the poly(A)$^+$ RNA was washed from the column and precipitated in 40 μL of 3M sodium acetate and 1.1 mL of 100% ethanol at -70°C.

*Library Construction*

First- and second-strand synthesis of liver poly (A)$^+$ RNA (5 μg) was performed using the ZAP Express cDNA Synthesis Kit (Stratagene). Insert termini were blunted by addition of 23 μL of blunting dNTPs (2.5 mM each) and 2.0 uL of Pfu DNA polymerase (2.5 U/μL), followed by incubation at 72°C for 30 min. The reaction was extracted with 200 μL phenol-chloroform (1:1 (v/v)) and then 200 μL of chloroform, prior to precipitation with 20 μL of 3 M sodium acetate and 400 μL of 100% ethanol at -20°C overnight. After centrifugation at 14,000 rpm (25,500 xg) for 60 min, the cDNA pellet was washed with 500 μL of 70% ethanol and resuspended in 9.0 μL of EcoR1 adapters (0.4 μg/μL) together with 1.0 μL of 10X ligation buffer, 1.0 μL of 10 mM rATP, and 1.0 μL of T4 DNA ligase

(4U/μL), then placed in a cooling water bath overnight at 8°C. The *Eco*R 1 ends were kinased by addition of 1.0 μL of 10X ligation buffer, 2.0 μL of 10 mM rATP, 6.0 μL of sterile water, and 1.0 μL of T4 polynucleotide kinase (10 U/μL), and incubating at 37°C for 30 min. The sample was then digested with 3.0 μL of Xho 1 (40 U/μL) and 28 μL of Xho 1 buffer supplement for 1.5 hrs at 37°C. Following Xho 1 digestion, 5 μL of 10X STE buffer (100 mM NaCl, 20 mM Tris-HCl pH 7.5, 10 mM EDTA) was added for size fractionation of cDNA inserts. Using a Sephacryl S-500 spin column, the cDNA sample was centrifuged at 1400 rpm (400 xg) for 2 min. The first fraction was discarded and 60 μL of 1X STE added to the column and recentrifuged. This was repeated two more times and each fraction (5 μL) was electrophoresed in a 5% nondenaturing acrylamide gel to check the size of cDNA. Fractionated inserts ranging between 0.8-2.0 kb in length were sequentially extracted with an equal volume of phenol-chloroform [1:1 (v/v)] and chloroform. To each extracted sample, a 2X volume of 100% (v/v) ethanol was added and stored overnight at -20°C. Samples were then microfuged for 60 min at 18,000 rpm (42,100 xg) at 4°C, washed with 200 μL of 80% (v/v) ethanol, and recentrifuged for 2 min.

Pelleted cDNA was resuspended in 10.5 μL of sterile water and quantitated prior to ligation into the ZAP Express Vector Arms (pBK-CMV Phagemid Vector, Stratagene). The ligation reaction consisted of 0.5 μL of 10X ligase buffer, 0.5 μL of 10 mM rATP, 1.0 μL of the ZAP Express Vector (1 μg/μL), 0.5 μL of T4 DNA ligase (4 U/μL) and 100 ng of cDNA in a total reaction volume of 5 μL. Incubation was carried out overnight at 12°C.

After the ligation was complete, 1 μL (20 ng) was added on ice to the Gigapack II Gold Packaging Extract (Stratagene) and incubated for 2 hrs at room temperature. SM buffer (500 μL) and chloroform (20 μL) were added,

and the phage solution was stored at 4°C. For plating and titering, 1 uL of packaging reaction was added to 200 µL of XL1-Blue MRF' cells ($OD_{600}$ of 0.5), and incubated at 37C for 15 min. This mixture was then added to 3 mL of NZY top agar (0.1 M NaCl, 20 mM $MgSO_4$, 0.5% (w/v) yeast extract, 1% (w/v) casein hydrolysate, 1.5% (w/v) agar, 0.7% (w/v) agarose), 15 µL of 0.5 M IPTG, and 50 µL of X-gal (250 mg/mL), which was then dispensed onto NZY agar plates and placed at 37°C overnight. Recombinant plaques appeared as clear colonies, approximately 100-fold above the blue background plaques. The primary library was then amplified to make a large, stable quantity of high-titer stock. The host strain, XL1-Blue MRF' cells (600 µL/150-mm plate), was added to 10 aliquots of the library suspension (~50,000 pfu), and the tubes were incubated for 15 min at 37°C. Melted NZY top agar (6.5 mL) was mixed with each aliquot of infected bacteria and spread onto 150-mm NZY agar plates, then incubated overnight at 37°C. The plates were then overlaid with 10 mL SM buffer and stored at 4°C overnight. The pooled bacteriophage suspension was centrifuged at 500 xg for 10 min, the supernatant was recovered and chloroform added to a final concentration of 0.3% and the suspension stored at 4°C. After determining the titer of the amplified library, twenty 150-mm plates were prepared, each containing ~50,000 pfu for screening of approximately 1 X $10^6$ recombinants.

*Library Screening*

Libraries were titered by serial dilutions (1:10) prior to screening. Phage (20,000 pfu) were diluted in 0.1 mL of sterile SM buffer (100 mM NaCl, 20 mM $MgCl_2$, 50 mM Tris-HCl pH 7.5, 0.01% (w/v) gelatin), dispensed to 15 mL polypropylene tubes, followed by addition of 0.3 mL of SM buffer and 0.6 mL of host cells (*E. coli* strain Y1090), then incubated at 37°C for 20 min.

Samples were added to 3 mL of molten LB soft agar (47°C), and spread on prewarmed LB-ampicillin (100 µg/mL) plates, then incubated at 37°C for 16 hrs. Plates were then chilled for 2 hrs at 4C and the recombinant plaques transferred in duplicate to nitrocellulose membranes (Plaque Screen Hybridization Transfer Membranes, NEN Research Products, Boston, MA). Membranes were placed on the top agar for 2 min (duplicate membranes for 4 min), then denatured by submerging in 1.5 M NaCl and 0.5 M NaOH for 2 min and neutralized in 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0) for 5 min. The membranes were rinsed in 0.2 M Tris-HCl (pH 7.5) and 2X SSC for 30 sec, blotted briefly on Whatman 3MM paper, and DNA crosslinked with 0.12J of UV energy for 20 sec using the UV Stratalinker 2400 (Stratagene).

Conventional filter hybridization screening was performed on 2 X $10^6$ recombinants (human brainstem library), 5 X $10^6$ recombinants (human liver library), and 1 X $10^6$ recombinants (rat neonate liver library). Prehybridization was carried out at 42°C for 2 hrs in the following solution; 2X 1, 4-piperazine-diethanesulfonic acid (PIPES) buffer, 50% formamide, 0.5% (w/v) SDS, and 100 µg/mL of denatured salmon sperm DNA. After overnight hybridization with 2 X $10^6$ cpm/mL of radiolabeled probe at 42°C, membranes were washed with 2X SSC, 0.1% SDS twice at 55°C and 60°C, followed by 0.1X SSC, 0.1% SDS twice at 60°C, and then exposed to X-ray film (Reflection film, NEN Research Products) at -70°C for 4 days. Putative positive clones were isolated using a Pasteur pipette to remove the agar piece containing the plaque identified by autoradiography. The agar piece was transferred to a microfuge tube containing 1 mL of SM buffer and 20 uL chloroform, and stored at 4°C. Resultant eluted phage were diluted and titered so that each plate would

have ~500 well-isolated plaques. Following secondary screening, putative positive clones were again isolated and tertiary screening was performed.

*Clone Isolation and Sequencing*

For single-clone excision of the pBK-CMV phagmid vector, overnight cultures of XL1-Blue MRF' cells, supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$, and XLOLR cells in LB broth were prepared. The XL1-Blue MRF' and XLOLR cells were then resuspended in 10 mM $MgCl_2$ at an absorbance ($OD_{600}$) of 1.0. In 15 mL polypropylene tubes, 200 µL of XL-1 Blue MRF' cells, 250 µL of phage stock, and 1 µL of the ExAssist helper phage were incubated for 15 min at 37°C. Three mL of LB broth (200 mM NaCl, 1% (w/v) tryptophan and 0.5% yeast extract) was added to the tubes and incubation was continued for 3 hrs. The tubes were heated at 65°C for 20 min and then centrifuged at 2200 rpm (1000 xg) for 15 min. The supernatant containing the excised pBK-CMV vector was transferred to a sterile 15 mL tube and stored at 4°C. For plating of the excised vector, 200 µL of freshly grown XLOLR cells was added to 100 µL of phage supernatant and incubated for 15 min at 37°C. Following addition of 300 µL of LB broth and incubation for 45 min at 37°C, 200 µL of the cell mixture was plated on LB-kanamycin agar plates (50 µg/mL) and incubated overnight at 37°C. The Wizard Miniprep DNA Purification System (Promega) was used for isolation of each recombinant plasmid from bacterial cultures. Plasmid clones were then subjected to restriction enzyme analysis for insert size determination, subcloning, and sequencing.

The Sequenase Kit Version 2.0 (USB Corp., Cleveland, OH) was purchased for Sanger dideoxy chain-termination sequencing of the various cDNA clones. Denaturing 6% acrylamide sequencing gels (8 M urea, 5.7% acrylamide, 0.3% bis acrylamide) were prepared, samples added

and electrophoresed for 3 hrs at 50 W in 1X TBE (0.1 M Tris-HCl pH 7.4, 0.5 M boric acid, and 1 mM EDTA). Gels were dried at 80°C for 1 hr (Gel Dryer, Labconco, Kansas City, MS) then subjected to autoradiography (NEN Reflection Film, NEN Research Products) for 2 days.

**VII. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

Polymerase chain reaction (PCR) in combination with reverse transcription (RT) was utilized as a sensitive technique for the detection of specific RNA transcripts. RT-PCR methodology and optimization was performed similar to that described in *PCR Protocols, A Guide to Methods and Applications*, Ed. M. A. Innis et al., 1990.

First-strand cDNA synthesis was performed on 1 μg of total RNA using 200 U of Murine Moloney Leukaemia Virus (MMLV) reverse transcriptase (Promega, Madison, WI) in a 25 μL reaction containing 1X RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT), 40 U of RNasin ribonuclease inhibitor (Promega), 100 μM dNTPs (Pharmacia Biotech), and 40 pM random hexamers (pd(N)6) (Pharmacia Biotech) for 60 min at 42°C. One microliter of the RT reaction was added to the standard PCR mixture containing 1X buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, and 0.1 % Triton X-100), 100 μM dNTPs, 1.5 mM $MgCl_2$, 50 pmols of each primer, and 0.5 U of Taq DNA polymerase (Promega) in a final volume of 25 μL. The Ericomp TwinBlock Thermal Cycler (Ericomp Inc., San Diego, CA) was used for PCR analysis. The thermal profile included an initial denaturation at 94°C for 2 min, followed by 30-35 (for FGF-2 sense and antisense) or 20 (glyceraldehyde-3-phosphate dehydrogenase (GAPD)) cycles of 94°C for 45 sec, annealing at 65°C for 45 sec, and elongation at 72°C for 1 min. Preliminary experiments confirmed that under these

conditions all products remained within the exponential phase. Samples were overlaid with filtered mineral oil (50 µL) to prevent evaporation during repeated cycling. Following addition of 5X loading dye, samples were electrophoretically separated in 1X TAE (40 mM Tris-HCl pH 7.5, 1 mM EDTA, and 0.1% acetic acid) in a 1.5% agarose gel at 100V for 60 min and UV transilluminated for photographing (Polaroid Type 53 Instant film).

For cloning PCR products a T-vector was constructed essentially as described by Marchuk et al., (1991). Three µg of blunt-end restriction digested pUC118 plasmid (Stratagene) was added to a 50 µL reaction containing 1X PCR buffer, 2 mM dTTP, and 5 U Taq DNA polymerase, then incubated at 72C for 3 hrs. The T-vector was purified by phenol-chloroform extraction and precipitation, and quantitated prior to use.

**i. Oligonucleotides**

Selection of sequences for synthesizing PCR oligonucleotides was determined using Amplify (v1.0). All primer pairs were chosen to span at least 1 intron-exon splice boundary to eliminate the possibility of amplification of genomic DNA. Oligonucleotides were synthesized by Life Technologies (Grand Island, NY) and a 10X stock solution was prepared at 1 nmol/µL. The concentration of each oligonucleotide was determined by measuring the absorbance at 260 nm and calculating the mass with the equation; $\mu g/OD_{240}$ = molecular wt (ammonium salt)/extinction coefficient. PCR oligonucleotides for amplification of the FGF-2 sense-specific PCR fragment were based on the sequence for human FGF-2 mRNA reported by Kurokawa et al. (1987), hFGF-A: GCTCTTAGCAGACATTGGAAGA, complementary to nucleotides 783-

804, and hFGF-B: GGCTTCTTCCTGCGCATCCA, corresponding to nucleotides 451-470, which generated a PCR fragment of 352 bp. For amplification of the 3' end FGF-2 antisense-specific PCR fragment, primer HUM-1: ATGTGGAAGTTTCCAGGAGGCCTGTCA, complementary to nucleotides 6179-6205 of the human FGF-2 3' UTR, and primer HUM-2: GCCTAGCAACTCTGCTGGTGATGGGAG corresponding to nucleotides 1509-1535 of the FGF-2 3' UTR, were used which generated a 301 bp fragment. Following cDNA cloning of the rat FGF-2 antisense RNA, an additional set of PCR primers were designed for amplification of a 5' end specific fragment, As 1: ACTGCCTCGAGCGGCCTGGAGATCA corresponding to nucleotides 115-139, and As 2: CTGGTGCTAACATCAAATACGGCA, complementary to nucleotides 491-468, and generated a 379 bp product. The oligonucleotide, P2A: GAACCGCTGTGTCTCCAATATCTTCTTCAG, specific to the FGF-2 antisense exon III-exon IV splice junction (complementary to nts 426-455 in the *Xenopus* FGF-2 antisense mRNA), was synthesized for use as an internal control. Primers specific for the *Xenopus laevis* FGF-2 antisense were Xas1, TTAGCAACTCTGCTTGTGATG; and Xas2, TGGAAGTTTCCTGGAGGGCTTTC, which generated a 297-bp product. For normalization of the input RNA in RT-PCR studies, primers were designed for amplification of GAPD RNA, GAPD-1 : TGATGACATCAAGAAGGTGGTGA which corresponds to nts 830-852 in the rat GAPD cDNA and GAPD-2 : TCCTTGGAGGCCATGTAGGCCAT which is complementary to nts 1047-1069, which produced a fragment of 242 bp.

Oligonucleotides specific for bacteriophage λgt11 to be used in the anchor PCR procedure were λgt11 forward primer:

TGGCGACGACTCCTGGAGCCCG and λgt11 reverse primer: TGACACCAGACCAACTGGTAATGG.

**ii. Anchor PCR**

The use of anchor PCR for cloning the full-length FGF-2 antisense cDNA relies on a region of known sequence in the cDNA of interest and sequences in bacteriophage λgt11 which flank the cloning site. An aliquot of the human liver cDNA library (1 μL) was boiled for 10 min prior to addition of the PCR components. PCR conditions and cycling parameters were the same as those previously described (see above). Oligonucleotides used in the first round of amplification were lamba-forward and P1, and lamba-reverse and P2, for detection of the 5' and 3' end products, respectively. After the first PCR amplification (35 cycles), samples were diluted 1:100 and 1:500, then subjected to a second round of amplification (30 cycles). In the second PCR, nested primers were used for additional specificity, lamba-forward together with reverse P2A for amplifying the 5' end fragment, and lamba-reverse with P2A for amplifying the 3' end fragment. PCR samples were electrophoretically separated in a 1.5% agarose gel, and DNA fragments of the appropriate size were subsequently cloned for sequencing.

**iii. 5' Rapid Amplification of cDNA Ends (5' RACE)**

Total RNA from human U87-MG glioma cells was used for 5' RACE with the 5' AmpliFINDER RACE kit (Clontech, Palo Alto, CA). First-strand cDNA was synthesized by addition of 5 U of Avian Myeloblastosis Virus (AMV) reverse transcriptase in 1X buffer, 50 U of RNase inhibitor, 5 mM dNTPs, 300 nM of gene-specific cDNA primer, and 2 μg of poly(A)+

RNA which were incubated at 52°C for 30 min. RNA was then hydrolyzed by adding 2 µL of 6 M NaOH to the reaction and incubating at 65°C for 30 min, followed by neutralization with 2 µL of 6 M acetic acid. For purification of cDNA, 80 µL of 6 M NaI was added to the microfuge tube together with 8 µL of GENO-BIND (Clontech) and placed on ice for 10 min. The cDNA was then centrifuged at 15,000 rpm (12,000 xg) for 10 sec, resuspended with 500 µL of 80% ethanol and this step was repeated. The supernatant was removed and pellet resuspended in 50 µL of DEPC-treated $H_2O$. The cDNA was then precipitated by addition of 5 µL of 2 M sodium acetate, 100 µL of 95% ethanol and 15 µg of glycogen as carrier, followed by incubation at -20°C for 30 min. The sample tube was then centrifuged as before and the resultant pellet washed with 40 µL of 80% ethanol, and centrifuged again. Once the supernatant was removed, the cDNA was resuspended in 6 µL of DEPC-treated $H_2O$. The AmpliFINDER anchor was then ligated onto the single-stranded cDNA in 1X RNA ligase buffer, 0.8 pmol of anchor, 10 U of RNA ligase, with 2.5 µL of cDNA, made up to a final volume of 10 µL and incubated at 22°C for 20 hrs. PCR amplification (30 cycles) was performed using a nested 3' gene-specific primer and the AmpliFINDER anchor primer. The amplified 5' cDNA fragments were then analyzed by agarose gel electrophoresis.

**VIII. Northern Analysis**

**i. Radiolabeled Probe Synthesis**

For complementary RNA (cRNA) probe synthesis, restriction enzyme-digested plasmid was initially treated with proteinase K (50 µg/mL), then purified by phenol:chloroform (1:1) extraction and ethanol precipitation. *In vitro* transcription was then performed on 1 µg plasmid in a 25 µL

reaction containing 5X transcription buffer (200 mM Tris, pH 8.0, 40 mM $MgCl_2$, 10 mM spermidine, 250 mM NaCl), 400 μM rATP, rCTP, and rGTP, 30 mM DTT, 40 U RNasin, 5 μL of 800 Ci/mmol $\alpha$-$^{32}$P-rUTP, and 10 U of T7 RNA polymerase for 30 min at 37°C. Probes were purified from unincorporated radiolabeled nucleotides using NucTrap Probe Purification Columns (Stratagene).

The glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNA and human FGF-2 cDNA were radiolabeled by random priming (Rediprime Labeling Kit, Amersham). Oligonucletides were 3'-end radiolabeled using terminal transferase (3'-End Labeling Kit, Amersham).

**ii. Northern Gel Preparation**

Approximately 3 μg of poly(A)$^+$ RNA was resuspended in 10 μL of RNA buffer (1X MOPS, 50% formamide, 6.5% formaldehyde, 50 μg/mL ethidium bromide) (Sambrook et al., 1989) and denatured at 65°C for 15 min. RNA samples were then electrophoresed in 2% formaldehyde-containing agarose gels in 1X MOPS for 4 hr at 120V and transferred to nylon membranes (Hybond-N+, Amersham) by downward alkaline transfer in 3 M NaCl, 8 mM NaOH, and 2 mM sodium lauryl sarcosine (Munoh, 1995). For size estimation, 5 μg of RNA molecular size standards (Life Technologies) were also loaded on gels. Human multiple tissue northern blots were purchased from Clontech.

**iii. Northern Hybridization**

Prehybridization of cRNA probes was performed at 65°C for 2 hrs in 50% formamide, 5X SSC, 1X PE (50 mM Tris-HCl pH 7.5, 0.1% w/v sodium pyrophosphate, 1% SDS, 0.2% polyvinylpyrolidone, 0.2% ficoll, and 5 mM

EDTA) and 150 µg/mL denatured salmon sperm DNA. The prehybridization was replaced with fresh solution for hybridization at 65°C overnight using 2 X $10^6$ cpm probe/mL buffer. Prehybridization/hybridization with cDNA probes was performed at 42°C for 2 hrs in 50% formamide, 5X SSC, 0.1% SDS, 5X Denhardts, 50 mM $Na_2HPO_4$, and 100 µg/mL denatured salmon sperm DNA. Washings were performed 2 X 15 min at 60°C in 2X SSC, 0.1% SDS, followed by 2 X 15 min at 65°C, and finally 2 X 15 min in 0.1X SSC at 65°C. Blots were exposed to film (NEN Reflection Film, NEN Research Products) at -70°C for varying times.

**IX. Preparation of Antisera**

Antisera were raised against synthetic peptides corresponding to three distinct regions of the deduced FGF-2 antisense protein; the $NH_2$-terminus peptide, RARRRTASSGLEITGS, corresponding to residues 23-38; the mutT domain peptide, SEPGEDIGDTAVREVFEET, corresponding to residues 175-193; and the COOH-terminus peptide, KLYHRGLPERYKAEMGTD, corresponding to residues 296-313. Synthetic multi-antigen peptides (MAPs) containing eight copies of the each peptide (mutT domain or $NH_2$-terminus) sequence on a branched lysine core and KLH-coupled peptide (COOH-terminus) were synthesized by Research Genetics Inc., (Huntsville, AL).

**X. Western Analysis**

Protein isolation was performed by addition of ice-cold buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.4, 1.5 mM $MgCl_2$, 1% Triton X-100, 10% glycerol, 2 mM phenylmethylsulfonylfluoride, and 3 µg/mL pepstatin A,

leupeptin and antipain), followed by cellular disruption (Sonifier Model W185, Heat Systems-Ultrasonics Inc., Plainview, NY) twice for 5 sec. Protein extracts were then centrifuged at 1000 xg for 10 min at 4°C and the supernatants were aliquoted at -70°C until SDS-PAGE. Protein concentrations were determined using the method described by Lowry et al. (1951). Bovine Serum Albumin (BSA) at 1 mg/mL in 0.1 M NaOH was diluted for preparing the standard curve. Standard and experimental protein samples in 4.5 mL polystyrene cuvettes were made up to 1.5 mL volume with 0.7 M NaOH, followed by the addition of 1.5 mL of a solution containing 13% $Na_2CO_3$, 4% Na tartrate, and 2% cupric sulfate. Reactions were mixed and incubated for 10 min at room temperature. Folin Ciacalteau reagent (0.5 mL) was added to each cuvette, mixed and incubated for 30 min, after which time the absorbance ($OD_{625}$ nm) was determined. Protein samples (50 µg) were diluted 1:5 in ImmunoPure Lane Marker Reducing Buffer (Pierce Chemical Co., Rockford, IL) and boiled for 5 min prior to SDS-PAGE. Individual protein medleys of normal human tissues were purchased from Clontech (Palo Alta, CA).

Using the Mini-Protean II Dual Slab Cell (Bio-Rad), samples were electrophoresed in 4-20% linear gradient gels (Bio-Rad) for 45 min at 150 V, and transferred to nitrocellulose membranes (Hybond ECL, Amersham) using the Trans-Blot cell (Bio-Rad) in the appropriate buffer (39 mM glycine, 48 mM Tris-HCl pH 7.4, 20% v/v methanol), for 1.5 hrs at 200 mA. Nitrocellulose membranes were subsequently blocked by shaking incubation for 1 hr at room temperature in Tris-buffered saline (TBS [10 mM Tris-HCl, 150 mM NaCl]) containing 5% (w/v) non-fat skim milk powder and 1% Tween-20 (Bio-Rad). Membranes were then incubated in TBS-5% milk-Tween-20 containing 7 µg/mL of anti-$CO_2$H-terminus

antibody for 1-2 hrs, followed by 6 X 5 min washes in TBS-1% Tween 20. Secondary antibody (Anti-rabbit Ig, horseradish peroxidase linked F(Ab')2, Amersham) at 1:5000 dilution in TBS-milk-Tween 20 was subsequently added for 1 hr, followed by 5 X 5 min washes in TBS-1% Tween-20. Immunoreactive bands were detected by enhanced chemiluminscence (SuperSignal CL-HRP Substrate System, Pierce Chemical Co.). An equal volume of luminol/enhancer solution was combined with stable peroxide solution and added to the surface of membrane for 5 min. The membrane was then blotted briefly and exposed to autoradiographic film (Pierce Chemical Co.) for 5 to 30 min. For size estimation of protein bands, prestained low-range molecular weight markers were used (Bio-Rad) .

**XI. Computer Hardware and Software**

Image capturing of RT-PCR photographs and northern/western autoradiograms was performed using the Abaton Scan 300/GC densitometer on a Power Macintosh Performa 5200CD computer. Nucleotide and amino acid analysis were done using DNA Strider version 1.1. Homology searches of the nucleotide database (Human Genome Center, Baylor College of Medicine, Houston, TX) were performed using the Basic Local Alignment Search Tool (BLAST) program (Altschul et al. 1990). Other software programs used include Adobe Photoshop Version 1.0.7, Aldus Pagemaker Version 4.0, NIH Image Version 1.59, MacDraw II Version 1.1, Microsoft Word Version 5.1a, and Endnote Plus Version 1.0.

## CHAPTER 5: ISOLATION OF THE HUMAN PCR-DERIVED PARTIAL FGF-2 ANTISENSE cDNA CLONE

### I. Introduction

In *Xenopus laevis*, transcription of the FGF-2 gene yields three mRNA transcripts (4.5, 2.3 and 1.5 kb in length), the largest of which encodes the FGF-2 polypeptide. The 1.5 kb mRNA transcript, originally believed to represent a fragment of FGF-2 pre-mRNA (Kimelman and Kirschner, 1987) was later identified as an antisense mRNA splice variant transcribed from the opposite strand of the FGF-2 gene (Kimelman and Kirschner, 1989). The FGF-2 sense and antisense transcription units share a 900 bp region of overlap at their 3' ends (Volk et al., 1989) and coexist as RNA duplexes in the cytoplasm of the immature oocyte. These RNA duplexes were shown to be unwound and modified by dsRNA-specific adenosine deaminase (DRADA) at the time of germinal vesicle breakdown (GVBD) (Kimelman and Kirschner, 1989). Given the rapid attenuation of FGF-2 RNA stability following GVBD, it has been suggested that the destabilization of RNA duplexes by DRADA modification targets the transcripts for rapid degradation by dsRNA-specific RNases (Kimelman and Kirschner, 1989).

Our main focus of research has been the analysis of FGF-2 gene expression and its role in tumor growth. The identification of a natural FGF-2 antisense transcript in *Xenopus* with a potential regulatory function in FGF-2 gene expression prompted us to determine whether the FGF-2 antisense gene is also expressed in human. Recently, FGF-2 antisense mRNA has been shown to be expressed in avian tissues (Borja et al., 1993), and given that the FGF-2 gene locus is highly conserved throughout

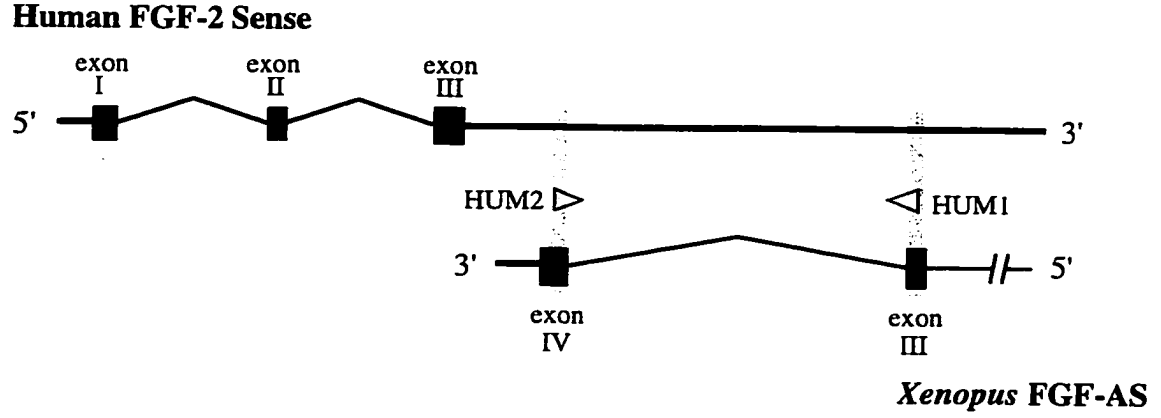
evolution, it is conceivable that this antisense gene is expressed in higher vertebrates.

## II. Results

### i. Prediction of Mammalian FGF-2 Antisense Gene Expression

Comparison of the published full-length human FGF-2 cDNA sequence (6.7 kb) with the reported *Xenopus* FGF-2 antisense cDNA sequence revealed two regions sharing 72-74% homology (Figure 5A). These two regions are found in exons III and IV of the *Xenopus* antisense sequence and the distal (6160-6202 nt) and proximal (1386-1763 nt) 3' UTR of the human FGF-2 sense sequence, respectively. The two regions of homology are separated by 4300 bp of 3' UTR in the human FGF-2 sense sequence, which may represent intronic sequence in the putative human FGF-2 antisense sequence. To examine the possibility that the antisense gene is expressed in human cells, we employed RT-PCR to specifically amplify a fragment of the antisense mRNA corresponding to this region. PCR oligonucleotides were designed to hybridize to regions flanking the putative intron. Primer HUM 1 was complementary to nucleotides 6179-6205 of the human FGF-2 3' UTR, and primer HUM 2 corresponded to nucleotides 1509-1535 of the FGF-2 3' UTR. A 301 bp amplification product is predicted with the HUM 1/HUM 2 primer pair only if the appropriately spliced antisense transcript is expressed, if not, a 4699 bp product is predicted from the FGF-2 mRNA transcript. As an internal control, the HUM P2A primer which was designed to span the putative human FGF-2 antisense exon III-exon IV junction (Figure 5B), was used in Southern analysis to confirm the identity of the PCR product.

A.




B.

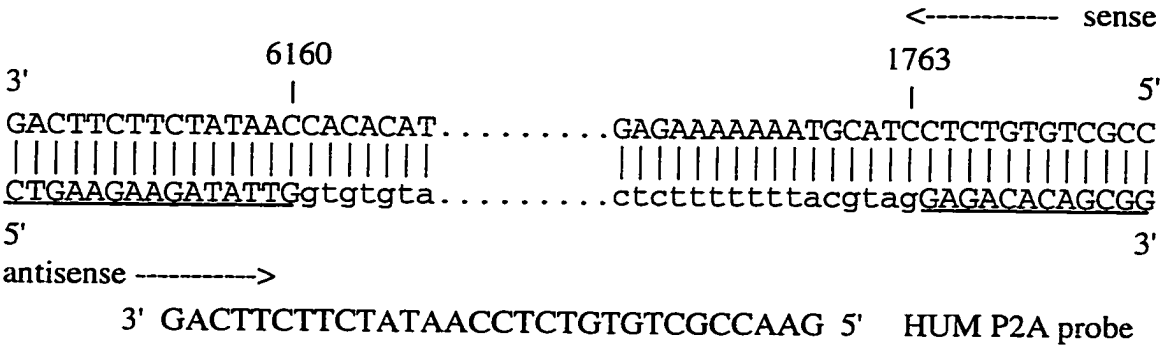



**Figure 5.** Sequence homology between the human FGF-2 and *Xenopus laevis* antisense genes predicts a human FGF-2 antisense splice variant. (**A**) Schematic comparison of the human FGF-2 gene (top) with the *Xenopus* FGF-2 antisense gene. The shaded bars indicate regions of ~73% sequence homology. The locations of the PCR primers used to amplify the human antisense transcript are also indicated. The HUM1/HUM2 primer pair predict a 301 bp amplification product from the expected antisense transcript, and a 4699 bp product from the 3' untranslated region of the FGF-2 sense transcript. (**B**) Sequence of the human FGF-2 gene at the predicted antisense splice junction. The sequence of the sense (upper) and antisense (lower) strands are shown. Numbering of the nucleotides is based on the 6757 bp human FGF-2 cDNA sequence (GenBank accession number J04513). Intron sequence in the antisense strand is indicated by lower case letters. The underlined nucleotides in the antisense strand represent the sequence recognized by the HUM P2A oligonucleotide probe.

**ii. Identification of the Human FGF-2 Antisense cDNA**

RT-PCR amplification of total RNA from the human U87-MG glioma cell line using the HUM 1/HUM 2 primer pair generated a single cDNA fragment of the predicted size (301 bp, termed *gfg-1*), revealing the presence of the predicted antisense mRNA transcript (Figure 6A). As a positive control for RT-PCR experiments, the 352 bp FGF-2 cDNA was also amplified from the same RNA sample using primers specific for exon I and III of the human FGF-2 sense mRNA. Southern analysis of the PCR products using a $^{32}P$ end-labeled HUM P2A oligonucleotide probe hybridized to the 301 bp product, but not to the 352 bp FGF-2 amplification product, confirming the identity of the antisense mRNA (Figure 6B). As an additional positive control, reverse-transcribed RNA from ovarian tissue of *Xenopus* was amplified with species-specific primers derived from the FGF-2 antisense transcript, which generated the predicted PCR product of 297 bp (Figure 6C). The *Xenopus* antisense product was not detected in Southern analysis with the HUM P2A oligonucleotide under the high stringency conditions used in these experiments, reflecting the differences between human and *Xenopus* in this region of the transcript.

**iii. Cloning and Sequence Analysis of the Human FGF-2 Antisense cDNA fragment**

A sufficient supply of the 301 bp FGF-2 antisense cDNA was amplified from the human U87-MG glioma cell line for purification and cloning into the constructed T-vector. The recombinant vector was isolated and subjected to dideoxy chain-termination sequencing.

Nucleotide sequence of the *gfg-1* cDNA was analyzed for complementarity to known sequences contained in GenBank using the

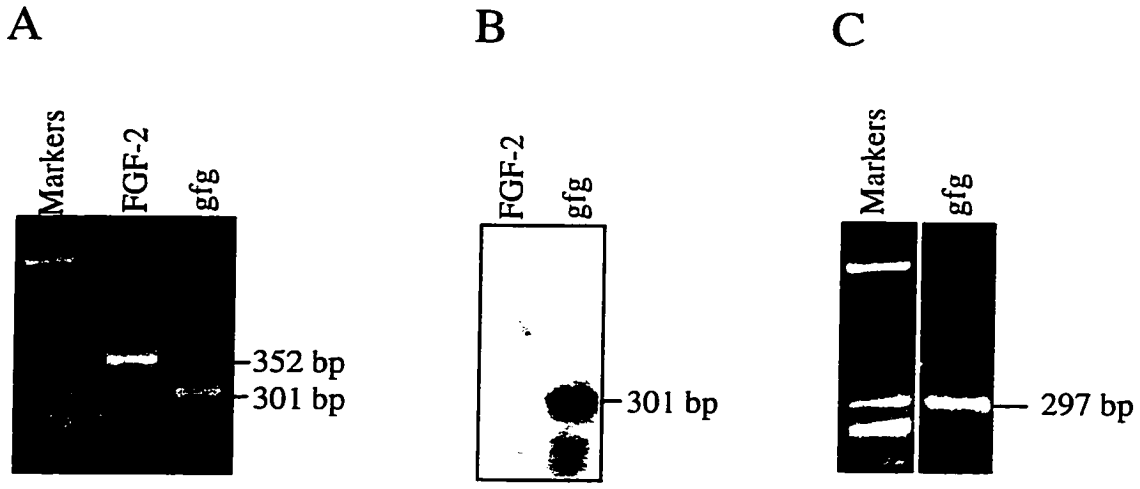



**Figure 6.** Detection of FGF-2 sense and antisense mRNA transcripts by RT-PCR. **(A)** PCR amplification of reverse transcribed mRNA from human U87-MG glioma cells using primers specific for the sense (FGF-2) and antisense (*gfg*) transcripts. Total RNA (200 ng) was reverse transcribed and subjected to 30 cycles of PCR amplification. The expected product sizes were 352 bp (FGF-2) and 301 bp (*gfg*). Actual product sizes were determined by comparison to ϕX Hae III DNA markers. **(B)** Southern hybridization with a nested oligonucleotide probe confirms the identity of the antisense transcript. The gel shown in (A) was transferred to nitrocellulose and hybridized with a $^{32}$P-end labeled internal oligonucleotide probe (HUM P2A). **(C)** RT-PCR amplification of the *Xenopus laevis* FGF-2 antisense transcript. Total RNA from *Xenopus* ovary was reverse transcribed and amplified exactly as described in (A) except that species-specific PCR transcripts (Xas1 and Xas2) were used.

Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al., 1990). The *gfg-1* cDNA was later identified as being derived from rat sequence (see page 99) and found to share 73% homology to regions of exon III and IV in the 3' region of the *Xenopus* antisense transcript (Figure 7). Sequence analysis revealed a conserved splice junction and open reading frame (ORF), whose deduced amino acid sequence has 74% identity with the deduced *Xenopus* antisense protein and 84% similarity, allowing for conservative changes. The FGF-2 antisense cDNA (*gfg-1*) was deposited in the GenBank database (NCBI) and has been assigned the accession no. L31408.

### iv. Attempts to Isolate the Full-length Human FGF-2 Antisense cDNA

The partial antisense cDNA (*gfg-1*) corresponds to the 3' end of the FGF-2 antisense mRNA. Using the sequence data derived from the *gfg-1* clone, we attempted to isolate the remaining 5' end portion of this transcript.

#### a. 5' Rapid Amplification of cDNA Ends (5' RACE)

The methodology of 5' RACE involves the use of a gene-specific 3' primer and a second primer designed to match the 5' end anchor which together will amplify a cDNA fragment that extends from known 3' end sequence through to the unknown 5' end. This technique was employed for isolation of the 5' end of the FGF-2 antisense transcript which contains the putative exon I and II regions, upstream of the *gfg-1* sequence. Total RNA from human U87-MG glioma cells was subjected to the 5' RACE protocol described in Methods and Materials. However, using total RNA from human U87-MG glioma cells, we did not detect 5' RACE product for

```
  4  TGGAAGTTTCCAGGAGGCCTGTCAGAGCCTGGAGAAGATATTGGAGACAC  53
273  TGGAAGTTTCCTGGAGGGCTTTCTGATCAAGGGGAAGACATAGGAGCTAC 322

 54  AGCAGTCCGAGAGGTGTTTGAAGAGACTGGTGTCAAGTCAGAATTCAGGT 103
323  AGCAGTTAGGGAAGTTCTTGAAGAGACTGGTATTCATTCGGAGTTTAAGT 372

104  CTCTGTTGAGCATCCGGCAGCAACACAGGAGCCCTGGGCGCTTTGGGATG 153
373  CCTTATTAAGCATAAGACAGCAGCATAATCACCCTGGGGCCTTTGGGAAG 422

154  TCAGACATGTACCTGATCTGCCGCCTGCAGCCGCGTTCCTTCACCATCAA 203
423  TCTGATCTGTACATCATTTGTCGCTTAAAGCCATTGTCATACACTATAAA 472

204  CTTCTGCCAGCAGGAATGCTTGAAGTGTGAATGGATGGATCTAGAAAGCC 253
473  CTTCTGCCATCAGGAATGCTTGAAGTGTGAATGGATGGATCTACAAGAGC 522

254  TGGCCAGGACTAAACACACAACTCCCATCACCAGCAGAGTTGCTAGGC    301
523  TTGCCTATTGTAGTAATACAACCATCATCACAAGCAGAGTTGCTAAGC    570
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**Figure 7.** Nucleotide sequence alignment of the rat and *Xenopus laevis* FGF-2 antisense cDNAs. Top and bottom strands correspond to the rat and *Xenopus* sequences, respectively. Using the BLAST algorithm, the rat 301 bp *gfg-1* insert sequence (upper) shows 73% homology with exons III and IV of the reported *Xenopus* antisense sequence (lower). The *gfg-1* sequence has been deposited in the GenBank database (accession number L31408).

the FGF-2 antisense transcript. This may be due in part to the level of FGF-2 antisense mRNA in these cells, which may be below the level of 5' RACE detection. Preparation of poly(A)$^+$ RNA from U87-MG cells or an alternative tissue source which exhibits a more abundant expression of FGF-2 antisense mRNA may have proved successful with this technique.

**b. Screening the Human Fetal Brain cDNA library**

A human fetal brainstem cDNA library was selected based on northern and RT-PCR analysis of rat brain and U87-MG glioma cells, which revealed the expression of FGF-2 antisense mRNA. Approximately 2 X $10^6$ recombinants of this library were screened using the *gfg-1* PCR clone as a $^{32}$P-labeled probe. Several putative positive clones were identified in the primary screening, but were not detected following amplification in the subsequent plaque screenings.

**c. Screening of the Human Liver cDNA Library**

Due to the difficulty of detecting a full-length cDNA clone in the brain derived library, the *gfg-1* cDNA was used to probe multiple human tissue northerns (see Figure 11). Evidently heart, liver or testis would have been a more favorable tissue choice in selection of the corresponding cDNA library because of the higher level of antisense expression in comparison to brain. A human adult liver cDNA library was selected for screening based on the apparent low ratio of FGF-2 sense to antisense RNA levels, which would decrease the possibility of cross-hybridization with a sense cDNA clone given that the probe used for library screening contains regions of the FGF-2 sense 3' UTR.

*Anchor PCR*

The use of anchor PCR was employed as a direct means of cloning the full-length FGF-2 antisense cDNA from the brainstem cDNA library by sequential amplification. This technique involved two rounds of PCR amplification using nested oligonucleotides derived from a region of known sequence in the cDNA of interest and oligonucleotides corresponding to bacteriophage λgt11 sequences which flank the cloning site. Seven PCR products, 600, 550, 500 bp corresponding to the forward anchor product and 450, 325, 300, 250 bp corresponding to the reverse anchor product, were cloned into the constructed T-vector and dideoxy sequenced. Nucleic acid database searches with these sequences showed no homology with the reported *Xenopus* antisense sequence and no significant matches with any known mammalian sequences.

*Conventional Plaque Hybridization*

Approximately 5 X $10^6$ recombinants were screened in duplicate using both a 98 bp fragment of the *gfg-1* PCR clone and the HUM P2A oligonucleotide. Following tertiary screening, a single plaque was isolated and amplified for restriction analysis. Electrophoretic separation of the EcoRI digested sample was visualized under UV and a single cDNA fragment was observed at approximately 1.5 kb. The cDNA insert was subcloned and sequenced for comparison with known sequences deposited in the GenBank database. The clone matched overlapping regions of exonic and intronic sequences of the human FGF-2 gene and therefore was believed to represent a partially processed FGF-2 transcript.

### v. Expression of the FGF-2 Sense and Antisense mRNAs

#### a. Strand-specific Analysis of FGF-2 Sense and Antisense mRNA Expression

Northern analysis of human or rat mRNA using a bovine FGF-2 double-stranded cDNA probe detects multiple transcripts ranging in size from 7-1.8 kb (Murphy et al., 1988a; Powell et al., 1991). We attempted to determine if one or more of these transcripts is derived from the antisense strand of the FGF-2 gene locus. As shown in Figure 8, northern hybridization with a cDNA probe corresponding to exon I-III of the bovine FGF-2 sequence detects four major FGF-2 mRNA transcripts in the human U87-MG glioma cell line, but not in T47D breast tumor cells, which do not express detectable levels of FGF-2 mRNA (Li and Shipley, 1991). In contrast, the HUM P2A oligonucleotide probe, which is specific for the antisense RNA transcript, hybridizes to an abundant 1.5 kb transcript in T47D cells that was not detected in U87-MG glioma cells. The HUM P2A oligonucleotide did not detect any of the multiple FGF-2 sense transcripts in U87-MG cells.

The relative abundance of FGF-2 sense and antisense mRNAs in these cells is indicated by the differences in autoradiographic exposure times (see Figure 8 legend). This also demonstrates an apparent reciprocal relationship between FGF-2 sense and antisense mRNA abundance. The 1.5 kb antisense mRNA transcript was abundant in human T47D cells, which contain low or undetectable levels of FGF-2 mRNA. In contrast, in U87-MG cells, which overexpress FGF-2, the antisense transcript was below the limit of detection by northern hybridization with the antisense *gfg-1* cRNA probe (not shown) or with the P2A oligonucleotide probe (Figure 8).

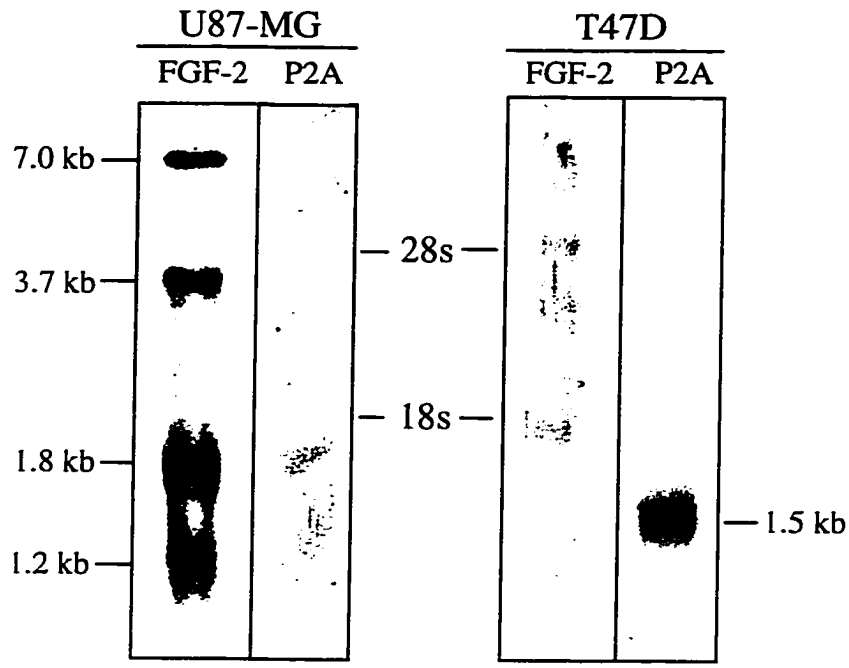



**Figure 8.** Northern hybridization with the *gfg*-specific oligonucleotide probe (HUM P2A) detects a 1.5 kb RNA transcript in human T47D breast cancer cells but not in U87-MG glioma cells. Blots contained 2 μg of poly(A)$^+$ RNA. Autoradiographic exposures for FGF-2 were 24 hours (U87-MG) or 10 days (T47D). Exposures for *gfg* were 2 days (T47D) or 7 days (U87-MG).

To provide further evidence of antisense transcription from the FGF-2 gene, single-stranded complementary RNA (cRNA) probes were generated by subcloning the *gfg-1* PCR cDNA into pBluescript II SK+ vector to give the pB*gfg-1* construct shown in Figure 9A. The vector was then linearized for *in vitro* transcription from the T3 or T7 promoter (Figure 9B). northern blots of total RNA from various rat tissues were then analyzed with the strand-specific riboprobes (Figure 9C). Both riboprobes showed nonspecific hybridization to 28S ribosomal RNA, and no specific transcripts were detected with the sense-specific riboprobe. The antisense-specific riboprobe hybridized specifically to an approximately 1.5 kb transcript in all tissues examined. Minor RNA species both larger and smaller than the predominant 1.5 kb transcript were also detected with the antisense-specific probe. However, only the 1.5 kb transcript was detected in poly(A)$^+$ RNA (see below), suggesting that the other transcripts may not be polyadenylated. Analysis of poly(A)$^+$ RNA from rat kidney using a double-stranded bovine FGF-2 cDNA corresponding to the coding region, detected multiple FGF-2 mRNA transcripts ranging in size from 1-6 kb (Figure 10). This probe also detected a minor transcript at 1.5 kb, which was abundantly detected with the *gfg-1* cRNA probe, confirming the antisense orientation of this transcript.

**b. FGF-2 Sense and Antisense mRNA Expression in Normal Human Tissues**

We next examined FGF-2 sense and antisense mRNA expression using northern blots which contained poly(A)$^+$ RNA from a variety of human tissues. The multiple tissue northern blots were sequentially hybridized with cDNA probes corresponding to FGF-2 sense, FGF-2 antisense and

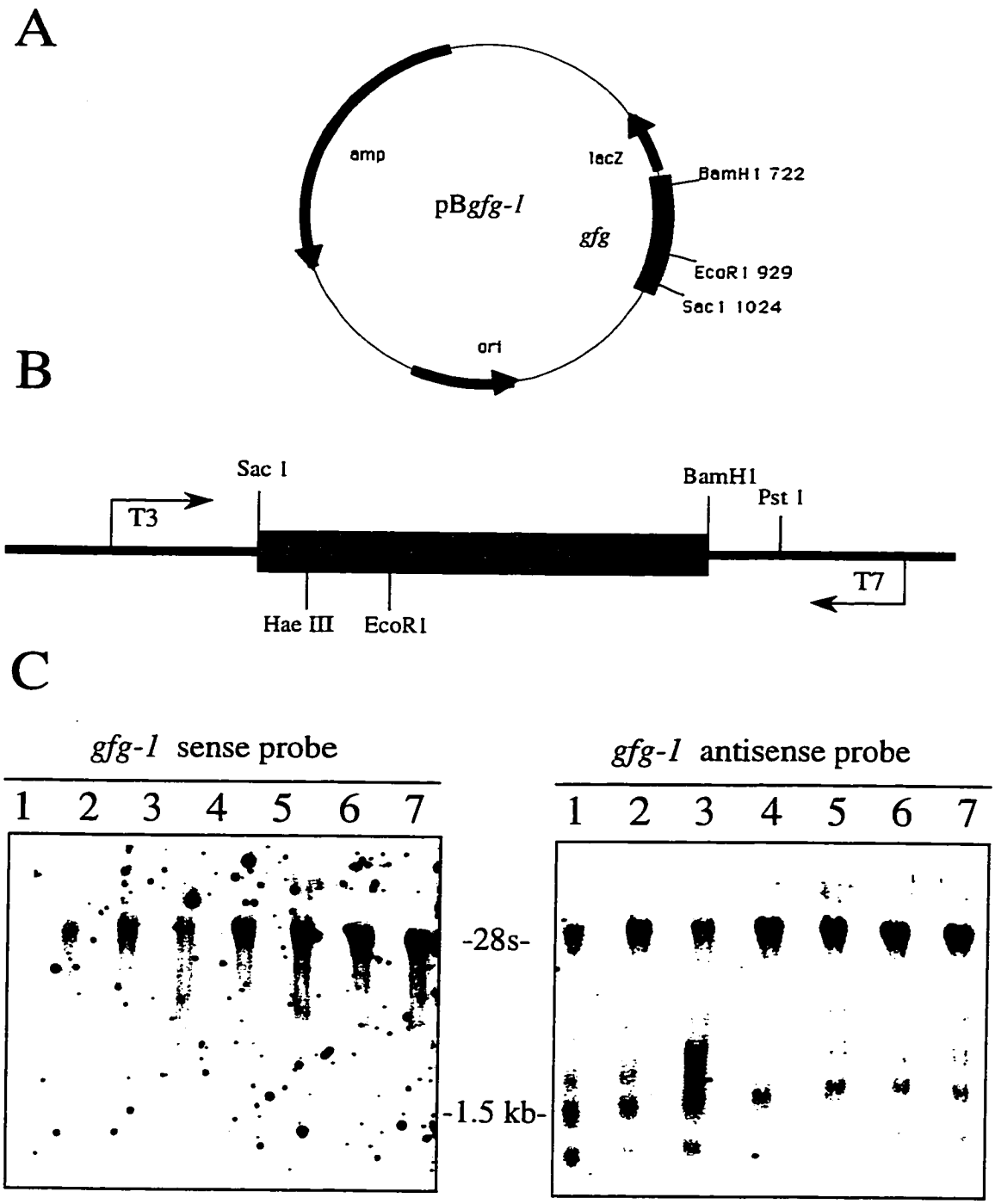



**Figure 9.** Utilization of strand-specific riboprobes for Northern analysis of rat tissues. **(A)** Map of recombinant vector containing the PCR-derived *gfg-1* cDNA; **(B)** Orientation of the subcloned cDNA and promoter utilization for strand-specific ribroprobe synthesis; **(C)** Antisense-strand specific riboprobe detects a 1.5 kb RNA transcript in total RNA from rat tissues, lanes: (1) brain; (2) heart; (3) kidney; (4) liver; (5) skeletal muscle; (6) spleen; (7) thymus.

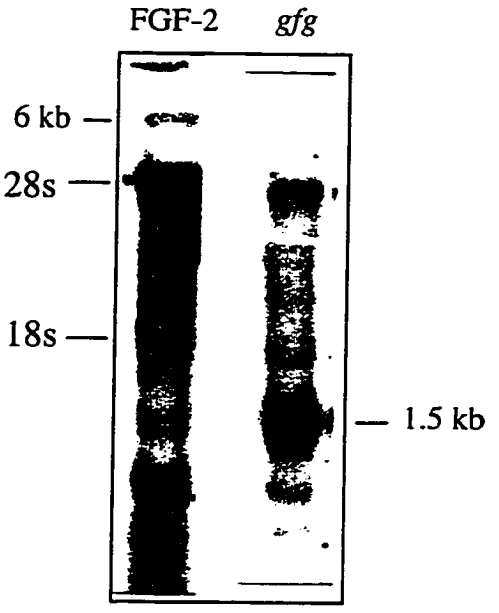



**Figure 10.** Northern hybridization with the *gfg-1* antisense cRNA probe detects a 1.5 kb RNA transcript in normal rat kidney. Blot contained 5 μg of poly(A)$^+$ RNA. Autoradiographic exposures for FGF-2 were 24 hours and for *gfg* were 2 days.

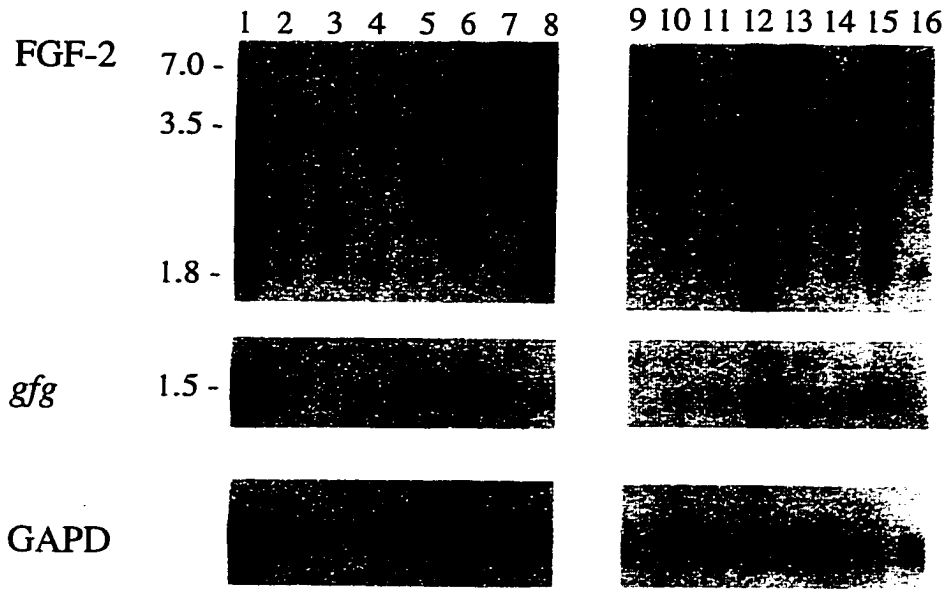
GAPD transcripts (Figure 11A). Hybridization with the FGF-2 sense probe detected 3 predominant mRNA transcripts of 7, 3.7 and 1.8 kb at various levels of abundance in most tissues. Intermediate size (2-2.5 kb) transcripts observed in placenta, skeletal muscle and pancreas may result from partial degradation of the larger mRNA species.

Using the 301 bp *gfg-1* fragment for hybridization detected a discrete 1.5 kb transcript in nearly all tissues examined, but most abundantly in heart, kidney, liver, skeletal muscle, and testis (Figure 11A). Comparison of the relative levels of FGF-2 antisense mRNA with that of sense mRNA illustrates that sense RNA was highest in brain, placenta, lung, pancreas, thymus, prostate, ovary and small intestine, while antisense RNA was more abundant than sense RNA in heart, liver, and testis. Sense and antisense transcripts were essentially equal in abundance in kidney and colon. The ratio of FGF sense to antisense transcripts was markedly tissue specific as shown in Figure 11B. Furthermore, the tissue-dependent expression of human FGF-2 sense and antisense RNA observed here is in agreement with expression reported in avian tissues (Borja et al. 1993).

We have shown that the FGF-2 antisense transcript does not correspond to any of the sense multiple transcripts detected in rat tissues by using strand-specific riboprobes or FGF-2 sense cDNA probes. The calculated size of transcripts detected by the antisense-specific probe in northern analysis of human tissues is markedly similar to that of *Xenopus*, further supporting the expression of an equivalent mammalian FGF-2 antisense RNA transcript. The double-stranded *gfg-1* cDNA probe used in this experiment also hybridized weakly to the 7 kb but not to the 3.7 or 1.8 kb FGF-2 sense transcripts. This is not surprising since the *gfg-1*

**Figure 11.** Expression of FGF-2 sense and antisense mRNA in human tissues. **(A)** Northern analysis of FGF-2 sense and antisense mRNA transcripts in human tissues. Northern blots containing 2 μg of polyA$^+$ RNA from normal adult human tissues were probed sequentially for FGF-2, FGF-2 antisense (*gfg*), and glyceraldehyde-3-phosphate dehydrogenase (GAPD) expression. *Lane 1*, heart; *lane 2*, brain; *lane 3*, placenta; *lane 4*, lung; *lane 5*, liver; *lane 6*, skeletal muscle; *lane 7*, kidney; *lane 8*, pancreas; *lane 9*, spleen; *lane 10*, thymus; *lane 11*, prostate; *lane 12*, testis; *lane 13*, ovary; *lane 14*, small intestine; *lane 15*, colon; *lane 16*, peripheral blood leukocytes. **(B)** Ratio of FGF-2 sense/antisense mRNA expression in human tissues. Relative abundance of FGF-2 (sense) and *gfg* (antisense) mRNAs were determined by densiometric scanning of a range of exposures of the Northern blots shown in (B). Expression of those samples too low to reliably estimate the level for either transcript is represented by an asterisk.

**A.**


1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16
FGF-2
7.0 -
3.5 -
1.8 -
gfg
1.5 -
GAPD

**B.**

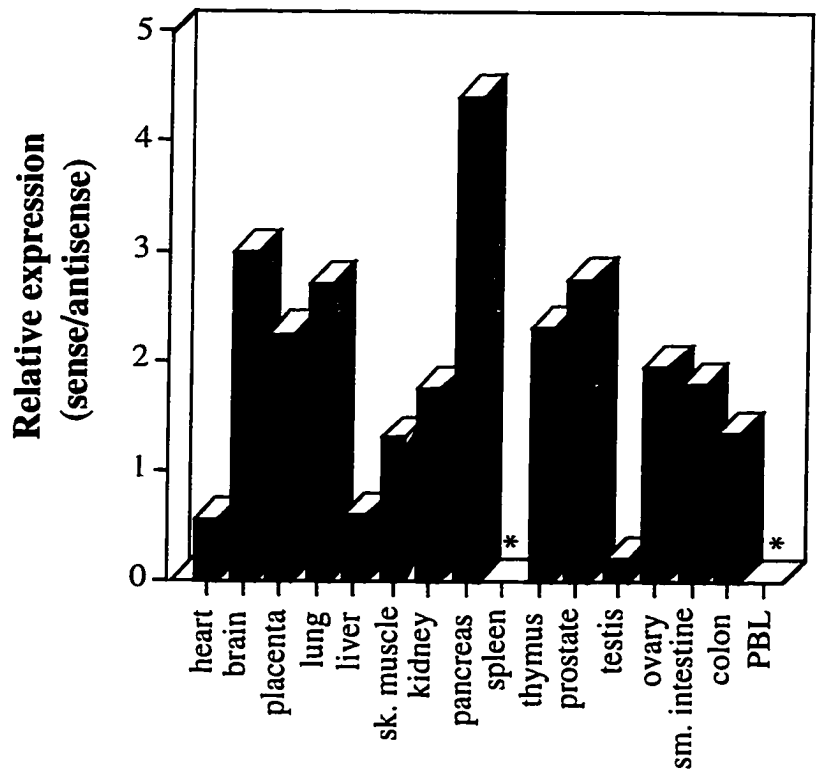

Relative expression
(sense/antisense)
5
4
3
2
1
0
*
*
heart
brain
placenta
lung
liver
sk. muscle
kidney
pancreas
spleen
thymus
prostate
testis
ovary
sm. intestine
colon
PBL

Figure 11

probe contains sequences common to the coding region of the antisense transcript and the distal 3' UTR of the FGF-2 sense transcript. Failure to detect either the 3.7 or 1.8 kb transcripts indicates these smaller transcripts are derived by differential polyadenylation site usage, as previously suggested (Bost and Hjelmeland, 1993).

**c. Expression in Human Oocytes and Granulosa Cells**

Given that the FGF-2 antisense gene is expressed in *Xenopus laevis* oocytes, we decided to determine if it is also expressed in human oocytes. Total RNA isolated from unfertilized oocytes and granulosa cells was subjected to RT-PCR for amplification of FGF-2 sense and antisense PCR fragments. RT-PCR analysis revealed similar abundance of the *gfg-1* PCR product (301 bp) and the FGF-2 sense product (352 bp) in granulosa cells (Figure 12A). The oocytes displayed an abundant level of FGF-2 antisense product and a significantly lower level of sense product (Figure 12B). Repetition of the RT-PCR experiment with different pools of oocyte RNA confirmed the observed pattern of FGF-2 sense and antisense mRNA expression. Restriction enzyme analysis and Southern hybrization with $^{32}$P-labeled internal probes specific for the FGF-2 sense and antisense RNA derived amplification products confirmed the identity of the two cDNA fragments (Figure 12C). Negative control reactions containing all components except input RNA, did not produce any detectable PCR products.

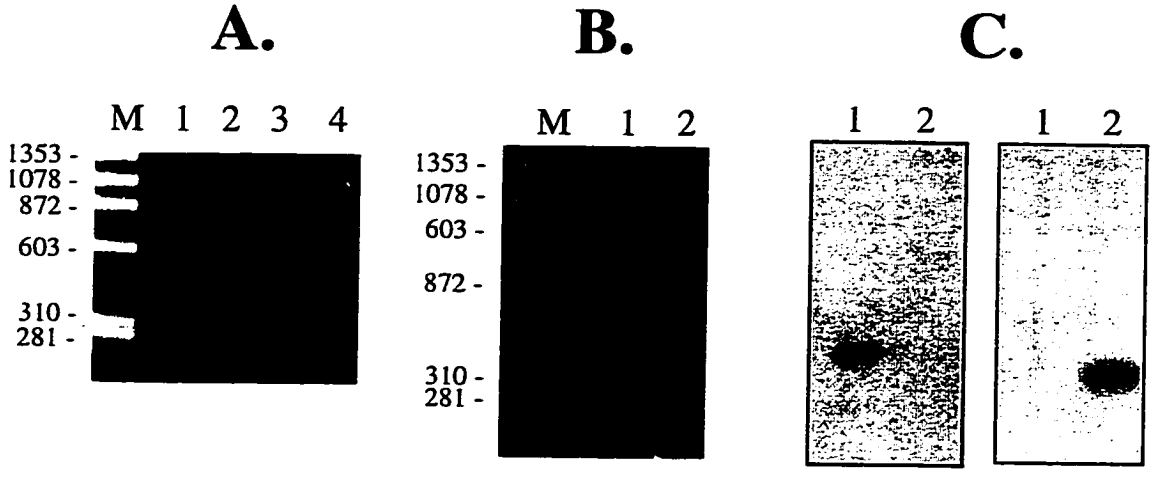



**Figure 12.** RT-PCR analysis of human oocyte and granulosa cells. (**A**) Detection of FGF-2 and *gfg* mRNA transcripts in human granulosa cells. Total RNA (300 ng) was reverse transcribed and amplified for 30 cycles using primer pairs specific for *gfg* (lane 1) or FGF-2 (lane 3). Negative control reactions (lanes 2 and 4) contained all reaction components except RNA. Product sizes were estimated by comparison with ϕX Hae III markers (M).(**B**) Detection of FGF-2 and *gfg* mRNA transcripts in unfertilized human oocytes. Ethidium bromide-stained gel of RT-PCR reactions for FGF-2 (lane 1) and *gfg* (lane 2) (arrowhead points to the faint FGF-2 band). Total RNA from pooled oocytes was reverse transcribed and amplified for 30 cycles.(**C**) Southern hybridization of the gel shown in (B) with $^{32}$P-labeled FGF-2 probe (left) and with *gfg*-specific probe (right).

## III. Discussion

Isolation of the *gfg-1* PCR-derived cDNA demonstrated for the first time that the opposite strand of the mammalian FGF-2 gene is transcribed into an antisense RNA. The rat FGF-2 antisense mRNA fragment has extensive structural homology (73%) to the previously identified *Xenopus laevis* FGF-2 antisense transcript (Kimelman and Kirschner, 1989; Volk et al., 1989). The rat FGF-2 sense/antisense transcription unit is highly homologous to the *Xenopus* gene, and as in *Xenopus*, the rat FGF-2 sense and antisense transcripts overlap at their 3' ends. Given the high degree of homology between the *gfg-1* fragment and *Xenopus* antisense transcript, the rat transcript is predicted to contain additional 5' sequence, corresponding to exons I and II contained in the *Xenopus* antisense RNA.

The *Xenopus* antisense mRNA contains an ORF which predicts a putative 217 amino acid protein with a calculated mass of 24 kDa (Volk *et al.*, 1989). The *gfg-1* cDNA sequence contains a conserved ORF which predicts a peptide with 84% similarity to the corresponding amino acid sequence of the *Xenopus* product. Antibodies raised against a synthetic peptide based on the deduced human FGF-2 antisense ORF detect both a 28 and 35 kDa protein in rat tissue, and a single 24 kDa protein in *Xenopus* (Li et al., 1996b). Although the function of this putative protein remains unknown, its deduced amino acid sequence is conserved throughout vertebrate evolution (Volk *et al.*, 1989). Interestingly, the antisense protein sequence contains an amino acid motif characteristic of the MutT-related family of DNA repair enzymes present in species from eubacteria to eukaryotes (Koonin, 1993; see Chapter 6).

In the human ovary, FGF-2 mRNA is expressed in granulosa and cumulus cells (Watson et al., 1992) and a paracrine role for FGF-2 in

follicular development and oocyte maturation has been proposed (Lapolt et al., 1990). Normal embryonic development relies on mesoderm induction and dorsal-ventral patterning directed by growth factor signaling from the underlying endoderm and FGF-2 is a necessary component of the induction process (Kimelman and Kirschner, 1987). In *Xenopus* oocytes, FGF-2 sense mRNA stability has been suggested to be regulated posttranscriptionally by dsRNA complex formation with the antisense RNA. Developing oocytes produce the FGF-2 antisense transcript in 20-fold excess over the sense transcript suggesting that all of the sense transcript in the unfertilized oocyte may exist in heteroduplexes with the antisense transcript (Kimelman and Kirschner, 1989). Sense: antisense heteroduplexes are destabilized by DRADA which causes these transcripts to become susceptible to degradation by dsRNA-specific RNases following germinal vesicle breakdown (Kimelman and Kirschner, 1989). DRADA modifies selected adenosine (A) residues in dsRNA to inosines (I) by hydrolytic deamination, replacing A-U with mismatched I-U base pairs (Kim and Nishikura, 1993). Sequencing of several FGF-2 sense cDNA clones isolated 4 hr after GVBD revealed that within the region of complementary overlap, approximately half (47%) of all A residues were converted to I. The replacement of A by I (coding equivalent of G) would not cause termination but may result in the suppression of termination or translational changes resulting in a nonfunctional protein. However, the validity of these results has been called into question because maturation promoting factor (MPF) used to induce germinal vesicle breakdown was suspected to be contaminated with DRADA activity (Bass, 1992). The suggestion that RNA duplex formation in *Xenopus* targets the FGF-2 sense mRNA (Kimelman and Kirschner, 1989) has received some further

skepticism in light of the recent report that oocyte cytoplasm contains a protective factor which blocks the action of DRADA (Saccomanno and Bass, 1994). Yet the fact that the sense and antisense RNAs were present in the *Xenopus* oocyte cytoplasm in the form of a dsRNA complex is not disputed and the possibility of FGF regulation by interaction with the antisense transcript cannot be ruled out. Duplex formation does not appear to prevent translation, as demonstrated by the presence of immunoreactive FGF-2 in oocytes and stage 17 embryos (Kimelman et al., 1988) and by *in vitro* translation of FGF-2 from synthetic dsRNA hybrids (Kimelman and Kirschner, 1989). Further, it is possible that the function(s) of sense:antisense pairing may vary at different stages of oocytes development. Our RT-PCR results indicate that, as in *Xenopus*, human oocytes express FGF-2 antisense transcripts in excess relative to sense transcripts. The presence of maternal FGF-2 sense and antisense transcripts in unfertilized human oocytes supports the possibility that this functional relationship is conserved in higher vertebrates.

Northern hybridization with strand-specific cRNA probes demonstrates the presence of a 1.5 kb polyadenylated antisense RNA transcript in a variety of tissues from both humans and rat. Expression of FGF-2 antisense RNA in rat revealed a widespread pattern among different adult tissues, implying a physiological importance for this transcript. The antisense transcript was abundantly expressed in adult rat tissues, in which FGF-2 sense transcripts are very low, and in human T47D breast tumor cells, in which FGF-2 expression is undetectable (Li and Shipley, 1991). In contrast, the expression of the antisense transcript appears to be lowest in human U87-MG glioma cells, which express an unusually stable FGF-2 sense RNA transcript (Murphy *et al.*, 1990). It is

reasonable to speculate that the extremely low level of antisense RNA expression in U87-MG cells may account for the stability of FGF-2 transcripts observed in these cells. Potential disruption of the relative levels of sense and antisense transcripts could lead to overexpression of FGF-2 in tumor cells. The reciprocal abundance of sense and antisense transcripts in a variety of human and rat tissues and cell lines supports the possibility of a regulatory relationship.

Analysis of FGF-2 sense and antisense mRNA expression in both chicken and rat has provided additional support for the proposed regulatory role of the antisense transcript. The FGF-2 antisense gene of chicken transcribes two individual mRNA species; 2.3 and 2.6 kb. Cloning and sequencing of partial cDNAs of these transcripts revealed that the 3' UTR of the chicken antisense transcript overlaps with the 3' UTR and the entire exon III of FGF-2 mRNA (Borja et al., 1993). It is not clear if this overlap also occurs in rat and human. Expression of FGF-2 antisense mRNA in chicken has been studied in adult tissues and during embryonic development using northern and RNase protection assay (Borja et al., 1993). A significant level of antisense mRNA was detected at embryonic stages 16 to 24, and comparison between sense and antisense message levels in adult liver revealed a striking inverse proportion of high antisense to low sense mRNA abundance. Grothe and Meisinger (1995) have shown that FGF-2 antisense mRNA is expressed in rat embryonic CNS, and also in the adult CNS, albeit at much lower levels. This low level of antisense mRNA is in reciprocal context to the abundant expression of FGF-2 mRNA in the adult CNS. The inverse levels of abundance between FGF-2 sense and antisense mRNAs suggests a developmental- and tissue-specific role for the antisense transcript, which

may involve heteroduplex formation with sense transcripts and its subsequent targeting for degradation.

The attenuation of PCNA mRNA in ascidian between fertilization and the first cleavage is coordinate with the localization of its antisense RNA (YC) in the myoplasm during this time (Swalla and Jeffery, 1996). These observations led to the suggestion that the developmental-specific localization of myoplasmic PCNA mRNA may involve a targeted degradation facilitated by sense-antisense duplex formation between the PCNA/YC RNA pair. Similarly, the interaction between FGF-2 sense and antisense RNA may be important in the spatial and temporal pattern of FGF-2 activity during its developmental requirement in mesoderm induction and postnatal tissue differentiation. The colocalization of sense and antisense transcripts in normal adult tissues, together with their tissue-specific inverse patterns of expression, suggests a functional relationship between FGF-2 sense and antisense mRNAs.

# CHAPTER 6: ISOLATION OF THE FULL-LENGTH RAT FGF-2 ANTISENSE cDNA

## I. Introduction

The isolation of a full-length FGF-2 antisense cDNA would have several advantages in the study of the regulatory capacity of its RNA. The antisense cDNA could be transfected into U87-MG cells to establish the role it may play in the regulation FGF-2 expression. Transfection could possibly lead to a reduction in the level of FGF-2 transcripts and restore a normal growth pattern to these cells. The full-length clone would allow direct examination of possible heteroduplex formation between FGF-2 sense and antisense RNA using a double RNase protection assay.

The translational capacity of the mammalian FGF-2 antisense RNA may also be examined. The *Xenopus* antisense cDNA contains an ORF which specifies an unknown 24 kDa protein. A full-length clone would allow analysis of the complete ORF as well as *in vitro* translation. Further, we could select specific regions of the deduced amino acid sequence for synthesis of peptides for antisera production. These antisera may be used for immunoprecipitation and expression analysis of FGF-2 antisense protein *in vivo*. The complete ORF of the antisense mRNA would also permit the production of recombinant protein for functional analysis.

The reason our initial attempts at isolating a full-length mammalian FGF-2 antisense cDNA were unsuccessful was believed to be due to the choice of cDNA library. Subsequent northern analysis of developing rat tissues, using our human *gfg-1* clone, revealed an abundant expression of FGF-2 antisense RNA in early postnatal liver (Li et al., 1996a).

## II. Results

### i. Construction and Screening of the Rat Neonatal Liver cDNA library

Utilizing rat liver at day 12 of postnatal development as a rich source of FGF-2 antisense RNA, we constructed the corresponding cDNA library using a modified λgt11 unidirectional cloning vector. Approximately 1 X $10^6$ recombinants were screened in duplicate for the primary round using the human *gfg-1* cDNA probe. Sixteen potential positives were detected in the primary screening, which were then selected for amplification and screening in the secondary round. Following tertiary screening 4 positives were detected, each of which yielded a 1.1 kb insert when subjected to restriction enzyme digestion. The autoradiographic results for plaque hybridization of a putative positive clone are shown in Figure 13. One of these clones were then selected for generating restriction fragments, which was subcloned into the pBluescript vector and sequenced bidirectionally using the universal forward and reverse primers.

### ii. Sequence Analysis of the Rat FGF-2 Antisense cDNA

Dideoxy sequencing revealed that the rat FGF-2 antisense cDNA comprised 1099 bp and contained a consensus polyadenylation signal 13 nts upstream of the 18 nts polyA-stretch (Figure 14). The complete sequence for the FGF-2 antisense cDNA clone was submitted to GenBank and has been assigned the accession number U58289. The rat cDNA has 67% overall homology to the *Xenopus* FGF antisense cDNA. The rat cDNA also contains a long ORF whose deduced amino acid sequence has 70% identity with the *Xenopus* hypothetical 24 kDa protein (see below). With the exception of its homology to the *Xenopus* antisense cDNA and human *gfg-1* cDNA, and its complementarity with FGF-2 sequences, the

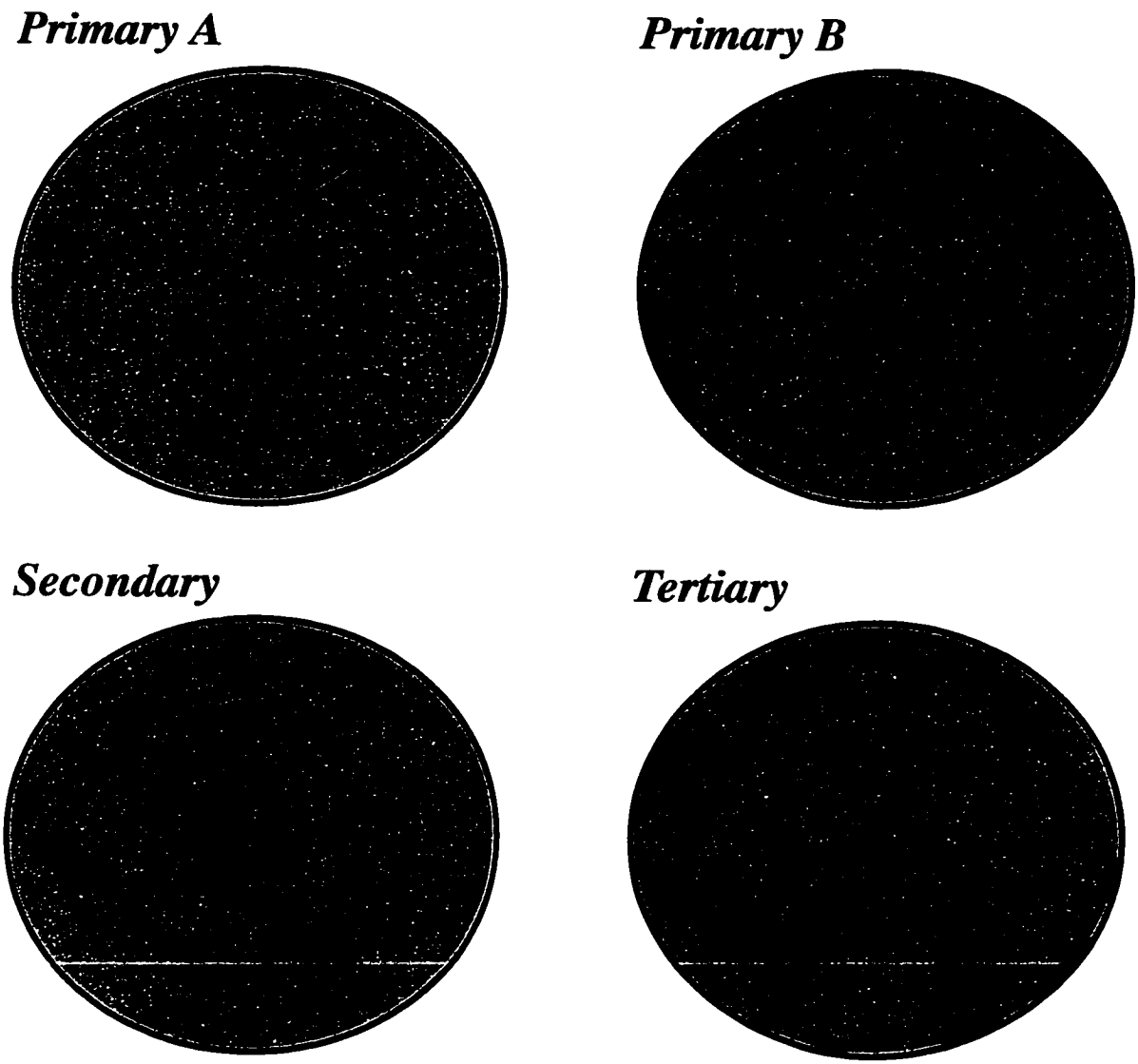



**Figure 13.** Hybridization screening of a "positive" cDNA clone from the rat neonate cDNA library. Primary plaques detected in duplicate were isolated from agar plates and amplified for secondary screening. Secondary plaques were again isolated and amplified for tertiary screening. Plaque lifts were subjected to autoradiography for 4 days at -70C.

AATTCGGCAC GAGCTGAAAG GCAAGGTGCA ACAATGTGGT GGGCGAGCAG 50

AGCGCGATGG TTGTTCTCGG CTTTGCTCGA CGTGGGCGGA GTCGGACTCC 100

GGGCTAGGCG GCGCACTGCC TCGAGCGGCC TGGAGATCAC GGGCTCCTGC 150

GGGGGCGAGC TACAAGGCGA GCTGGACAGA TTTGGGGGCA TCTCGGTGCA 200

CTTGTCGCGG CACCGCACCC TGCACCGGCT GGATGCCGCC GCCTTCCGGA 250

GGCTCTTGCA GGCCGCCATT CAGCAGTGGC GAGCGGAAGG AAGGATCGCT 300

GCATGGCTGC ACATCCCCAT CCTGCAGAGC CACTTCATCG CCCCTGCTGC 350

CTCTCTGGGC TTCTGCTTCC ACCACGCAGA ACCGCACTTG TCAACGCTGA 400

CTCTGTGGTT GGGAGAAGGA CCCAGCAGAC TCCCGGGGTA TGCCACACAC 450

CAAGTAGGGG TTGCAGGTGC CGTATTTGAT GTTAGCACCA GGAAAGTACT 500

GGTTGTACAA GATCGAAATA AATTGAAAAA CATGTGGAAG TTTCCAGGAG 550

GCCTGTCAGA GCCTGGAGAA GATATTGGAG ACACAGCAGT CCGAGAGGTG 600

TTTGAAGAGA CTGGTGTCAA GTCAGAATTC AGGTCTCTGT TGAGCATCCG 650

GCAGCAACAC AGGAGCCCTG GGGCCTTTGG GATGTCAGAC ATGTACCTGA 700

TCTGCCGCCT GCAGCCGCGT TCCTTCACCA TCAACTTCTG CCAGCAGGAA 750

TGCTTGAAGT GTGAATGGAT GGATCTAGAA AGCCTGGCCA GGACTAAACA 800

CACAACCCCC ATCACCAGCA GGGTGGCTAG GCTACTACTA TACGGACACA 850

GGGAAGGGTT TGACAAGATT GACCTCAGCA TGGAGGAACT CCCTGCAGTA 900

TACACAGGCC TGTTCTACAA GCTCTACCAC AGGGGACTGC CTGAGAGGTA 950

CAAGGCTGAA ATGGGAACAG ATTGAATGCC AGCCTTCCTC ACATACTTGT 1000

GTTTCCAGGG TAGAAATTCA CAAATGTGAA TTTTGTGAAT ATATGTTTAA 1050

ATCCTCTTTT CAAATAAAGG AAATACGTGA CAAAAAAAAA AAAAAAAAA 1099

**Figure 14.** Nucleotide sequence of the rat FGF-2 antisense cDNA. The translation start codon (ATG) and the stop codon (TGA) are denoted by the thick overline. The consensus polyadenylation signal sequence (AATAAA) is denoted by a double overline. The region of complementarity with the FGF-2 mRNA is underlined. This cDNA has been deposited in GenBank and assigned the accession number U58289.

rat FGF-2 antisense sequence has no striking homology with any other nucleotide sequences presently deposited in the GenBank database.

The rat antisense cDNA sequence was however, found to match with a number of sequences in the dbEST GenBank database. dbEST is a division of GenBank that contains sequence data from "single-pass" cDNA sequences, or Expressed Sequence Tags, which are being use to develop transcript maps for different organisms.

NhHMPu S1 human EST clone (accession no. AA193558) is a 448 nt cDNA isolated from a mixed library derived from melanocyte (2NbHM), pregnant uterus (NbHPU), and fetal heart (NbHH19W), which contains 82% homology (1-370 nt) to the rat antisense cDNA (463-831 nt). Another human EST, the HL60 3' directed MboI cDNA (391 nt) (accession no. D20756) isolated from the adult female promyelocyte leukemia cell line (HL60) following DMSO-induction of differentiation into granulocytoid cells, has 75% homology (27-209 nt) with the rat antisense cDNA (797-979 nt), and is localized to chromosome 4, the same chromosome assignment as the human FGF-2 gene. The mouse placenta-derived EST, designated 4NbMP13.5 (377 nt) (accession no. AA016863), was found to share 84% homology (42-212 nt) with the rat FGF-2 antisense cDNA (95-264 nt).

Comparison of the full-length rat FGF-2 antisense cDNA with our previously identified human *gfg-1* partial cDNA revealed a surprising high degree of homology (99%) between these two clones. It was then determined that our previously characterized *gfg-1* cDNA, which was believed to be of human sequence and derived from U87-MG glioma cell line, was in fact rat sequence. Further confirmation was provided by comparing the *gfg-1* cDNA sequence with the reverse complement of the

rat FGF-2 sense mRNA sequence, which revealed >98% homology in the region of overlap (138 nt).

**a. Northern Analysis using a Rat FGF-2 Antisense cDNA derived cRNA Probe**

A 630 bp *EcoR1* fragment of the 5' end of the rat FGF-2 antisense cDNA was subcloned into pBluescript and $^{32}$P-labeled cRNA riboprobes were generated by *in vitro* transcription. Using this strand-specific cRNA for northern analysis of poly(A)$^+$ RNA from various stages of developing rat liver detected a 1.5 kb transcript. This transcript displayed the expected developmentally regulated pattern of expression being most abundant in early postnatal (days 3-14) liver (Figure 15), which is in agreement with a similar analysis previously shown by Li et al., (1996a), using the RT-PCR-derived antisense cDNA fragment as a probe.

**b. Identification of the Region of Sequence Overlap with Rat FGF-2 Sense mRNA**

It has been proposed that in *Xenopus laevis* the antisense RNA regulates FGF-2 mRNA by the formation of double-stranded RNA complexes for subsequent RNA editing by DRADA (Kimelman and Kirschner, 1989). However, analysis of the overlap between the sense and antisense transcripts in rat is complicated by the fact that the full length rat FGF-2 mRNA sequence has never been reported and the majority of the 3' UTR is unknown. The longest rat FGF-2 mRNA detected by northern hybridization is approximately 6 kb (Powell et al., 1991), whereas the longest rat FGF-2 cDNA reported to date is 1.3 kb long (Shimisaki et al., 1988). Comparison of our cDNA with the full length (6.7 kb) human FGF-

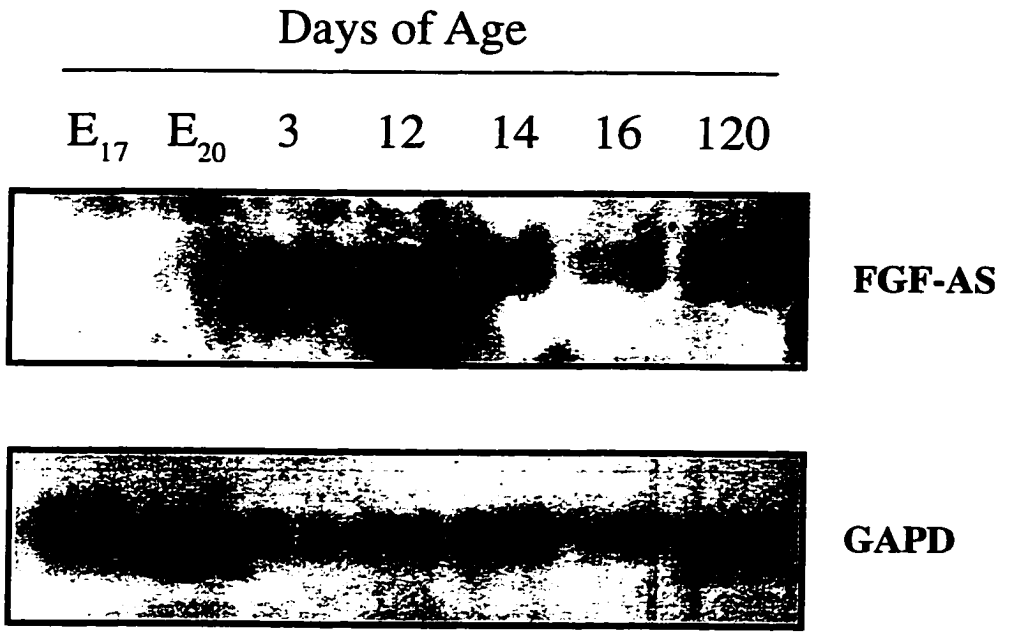



**Figure 15.** Northern analysis of developing rat liver. Poly(A)$^+$ RNA (5 µg/lane) was isolated from rat liver at various stages of embryonic (days 17 and 20) and postnatal development (days 3, 12, 14, 16 and 120). The Northern blot was probed with a $^{32}$P-labeled cRNA derived from the rat FGF-2 antisense cDNA (FGF-AS, top panel) and glyceraldehyde-3-phosphate dehydrogenase (GAPD, bottom panel).

2 cDNA (Prats et al., 1989) demonstrates that the antisense transcript contains sequences complementary to 2 widely separated regions of the FGF-2 sense RNA; one 60 bp region (corresponding to bases 6157- 6216 of the FGF-2 cDNA) includes the most distal polyadenylation signal motif, while the other (corresponding to bases 1362-1766 of the FGF-2 cDNA) lies just downstream of the proximal polyadenylation signal motif (Figure 16).

Three rat FGF-2 RNA sequences have been reported which differ in their 3' UTR sequences (Shimasaki et al., 1988; Kurokawa et al., 1988; el-Husseini et al., 1992). The sequence with the shortest 3' UTR (Kurokawa et al., 1988) has no sequence complementarity with the antisense cDNA. A 722 bp partial FGF-2 cDNA from rat brain contains >300 bp of 3' UTR sequence (el-Husseini et al., 1992). The distal 3' UTR of this cDNA has 100% sequence complementarity with the FGF-2 antisense cDNA in a region corresponding to the site of overlap with the human FGF cDNA (Figure 16). The longest rat FGF-2 cDNA, a 1272 bp sequence from PMSG-primed rat ovary (Shimasaki et al., 1988), lacks 307 bp of 3' UTR sequence present in the rat brain cDNA and may represent a minor splice variant or a cloning artifact (el-Husseini et al., 1992). Beyond the site of this apparent deletion the 3' UTR contains an additional 256 nucleotides with 100% complementarity with the antisense cDNA. By homology with the human FGF cDNA, an additional 60 bp region of homology is predicted in the distal 3' UTR of the 6 kb rat FGF transcript. However, even accounting for the missing distal 3' UTR the total extent of complementarity (425 bp) is less than that found in *Xenopus* (900 bp). The *Xenopus* antisense cDNA has a long 3' UTR with sequence complementary to the FGF-2 3' coding region (Kimelman and Kirschner, 1989). We cannot yet rule out

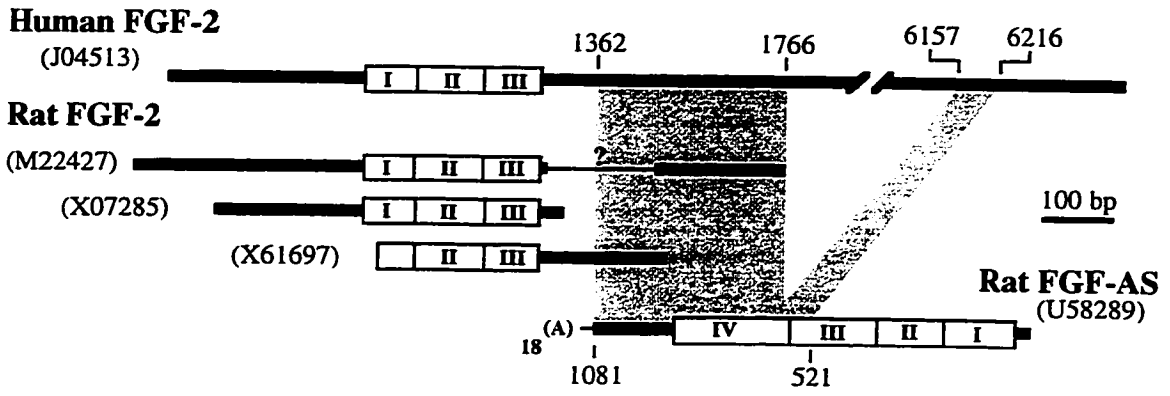



**Figure 16.** The rat FGF-2 antisense transcript is complementary to discrete regions of the 3' UTR of the FGF-2 mRNA. The relationship of the antisense cDNA to the human (top) and rat FGF-2 mRNA sequences is shown. GenBank accession numbers are indicated in parenthesis. Regions of complementarity between sense and antisense cDNAs are indicated by the shaded areas. Complementary sequence in the distal 3' UTR of the 6.7 kb human FGF-2 mRNA (top line) predicts a similar region in the as yet unsequenced 3' UTR of the 6 kb rat FGF-2 mRNA.

the possibility that the rat antisense cDNA described here may be a 3' truncated variant of the rat antisense RNA transcript.

**c. Translation of the FGF-2 Antisense mRNA**

The rat FGF-2 antisense RNA contains a long ORF from 34-975 nts which predicts a 35 kDa translation product containing 313 amino acids. The first ATG (nucleotides 34-36) occurs in a context that matches the essential features for translation initiation codon as defined by Kozak (1986). The deduced amino acid sequence of the rat FGF-2 antisense clone has 69% identity to the predicted 217 amino acid (24 kDa) *Xenopus* protein (Figure 17). However, the ORF in the rat cDNA extends upstream of the putative initiator ATG in the *Xenopus* cDNA, which in the rat sequence is replaced by a CTG (leucine) codon. The rat ORF predicts an $NH_2$-terminal extension of 96 amino acids. This ORF is conserved in the *Xenopus* 5' UTR sequence, suggesting that the original *Xenopus* cDNA sequence may be incomplete.

Initial speculation on the coding capacity of the mammalian antisense mRNA was strengthened by the fact that FGF-2 antisense transcripts were detected in northern gels containing poly(A)$^+$ RNA, therefore the antisense RNA is potentially translatable and may encode a functional protein. *In vitro* translation of the rat and *Xenopus* FGF-2 antisense cDNAs generated (respectively) the predicted 35 kDa and 24 kDa proteins (Figure 18). The corresponding smaller molecular weight band (24 kDa) detected from the *Xenopus* antisense cDNA represents the 5' end truncation in this clone compared to the rat cDNA (see above). Furthermore, the larger product in the *Xenopus* FGF-2 antisense translation (29 kDa) may result from translation initiation upstream of the

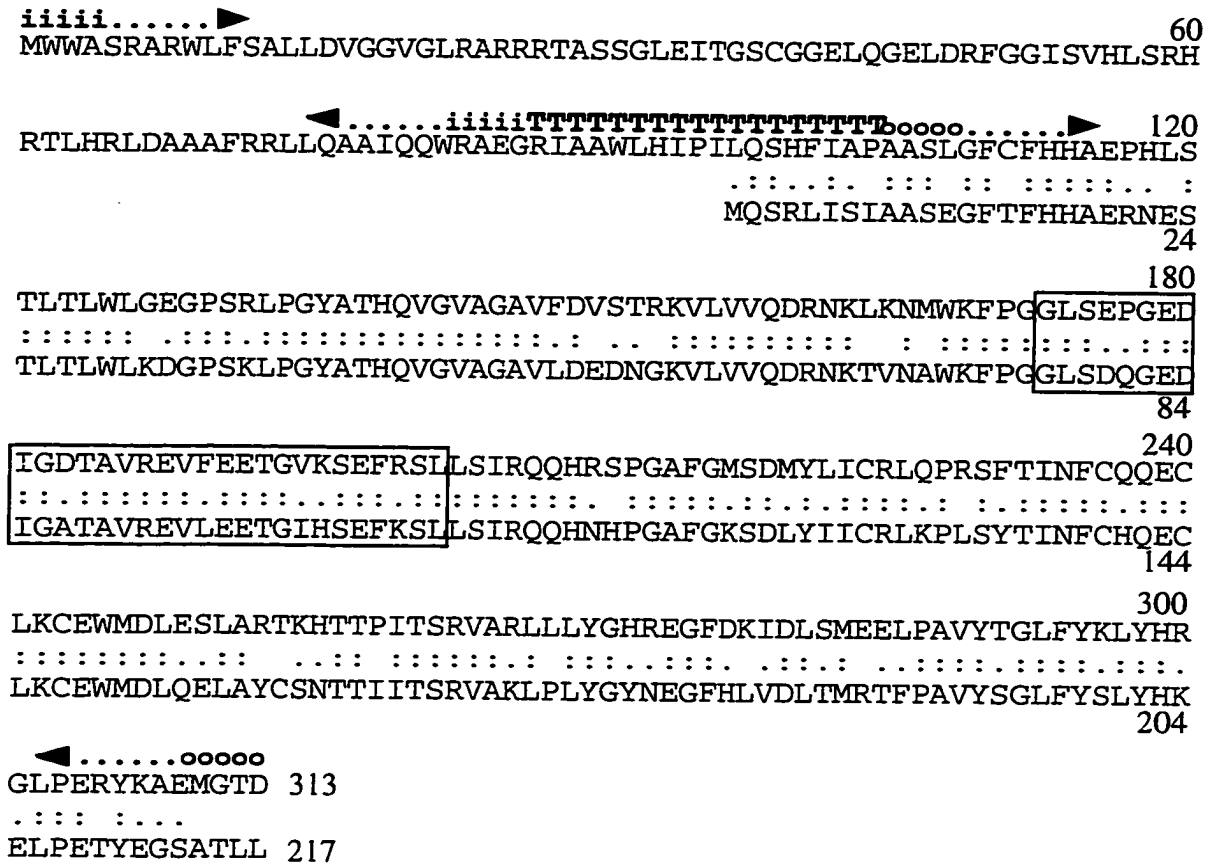



**Figure 17.** Alignment of the deduced amino acid sequence of rat and *Xenopus* FGF-2 antisense cDNA clones. Top and bottom lines correspond to rat and *Xenopus* sequences, respectively. Total overlap is 217 amino acids. Region of overlap is from 97-313 in rat and 1-217 in *Xenopus*. Double dots and single dots represent amino acid identity and similarity, respectively. Percentages of identity and similarity between rat and *Xenopus* antisense amino acid sequences are 69% and 89%, respectively. Representation above the rat antisense sequence corresponds to the topological prediction; i = intracytoplasmic (1-88 a. a.), T = transmembrane (89-107 a. a.), and o = extracytoplasmic (108-313 a. a.). Location of the putative signal sequence is indicated by asterisks. Boxed region corresponds to the conserved MutT domain.

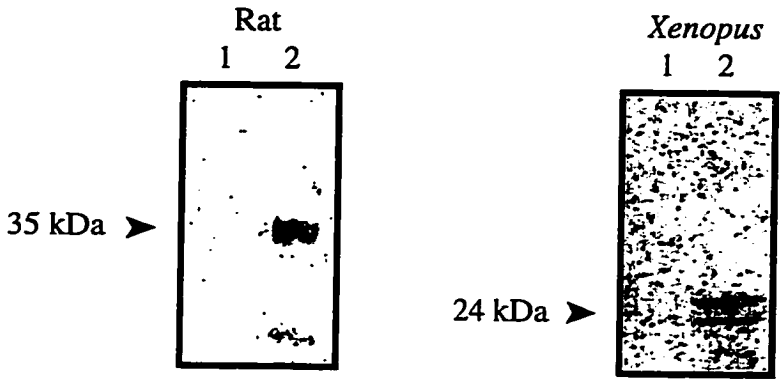



**Figure 18.** Immunoprecipitation of the rat and *Xenopus laevis* FGF-2 antisense proteins. Following *in vitro* transcription/translation of the pcDNA3 vector containing rat antisense cDNA (left panel) and *Xenopus* antisense cDNA inserts in the noncoding (lane 1) and coding (lane 2) orientation. Protein samples were labeled with $^{35}$S-methionine and immunoprecipitated with anti-mutT IgG and protein A-Sepharose. Respective sizes of protein bands are indicated by the arrowhead on the left.

putative initiator ATG codon.

To further characterize the translational product of the rat FGF-2 antisense transcript several bioinformatic programs located at interactive WWW servers were utilized. The SignalP program (Center for Biological Sequence Analysis WWW server, Denmark) predicts a signal peptide cleavage site between residues 21 and 22 in the deduced rat FGF-2 antisense amino acid sequence. Analysis with NetOglyc v1.1 (Center for Biological Sequence Analysis WWW server, Denmark [Hansen et al., 1995]) identified a possible O-glycosylation site at threonine 257 in the FGF-2 antisense protein. PHDTopology (EMBL WWW server, Heidelberg), a program for the prediction of transmembrane helices and topology (Rost, 1996), identified an intra-cytoplasmic amino-terminus segment of 88 residues followed by a short transmembrane region (18 residues) and a large extra-cytoplasmic carboxy-terminus of 207 residues (Figure 17). This models suggests that the FGF-2 antisense protein is a membrane-anchored protein, the bulk of which is inserted into a non-cytoplasmic compartment. This is consistent with our recent finding that the rat FGF-2 antisense protein is localized in the nuclear fraction of liver homogenates (Li et al., unpublished observations).

**d. Nucleotide and Protein Database Searches**

The GenBank protein database (NCBI) was searched for protein sequences sharing homology with the rat FGF-2 antisense putative protein. Using the BLAST algorithm (Altshul et al., 1990), the deduced amino acid sequence was found to share homology with the consensus sequence of the mutT domain of prokaryotic and eukaryotic antimutator (8-oxo-dGTPase) proteins (Figure 19). The mutT domain (first defined by

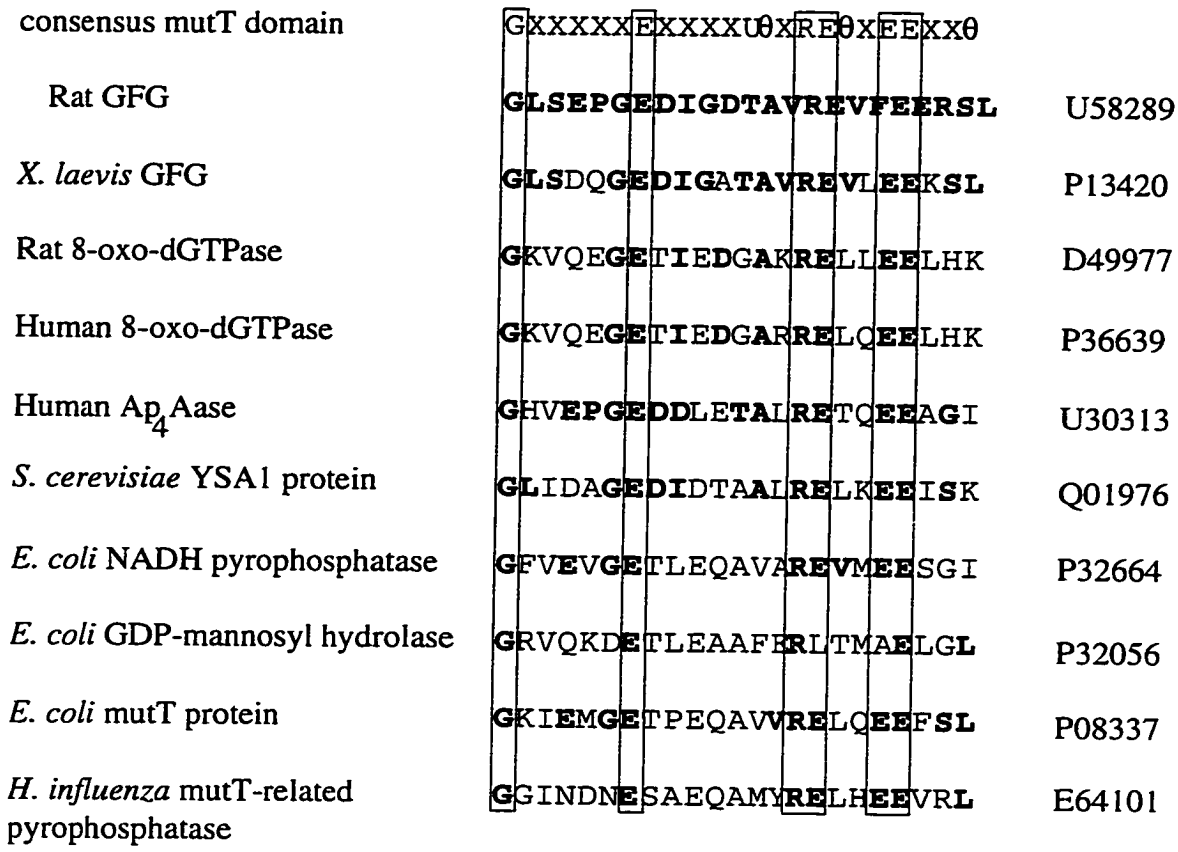



**Figure 19.** Comparison of the mutT domain of the FGF-2 antisense protein with those of other members of the mutT/Nudix family of proteins. In the consensus sequence (top line), U represents a bulky aliphatic amino acid (I, L, or V), θ represents a bulky hydrophobic amino acid, and X may be any amino acid. Bold letters indicate identities with the rat FGF-2 antisense protein sequence. The GenBank accession number for each sequence is shown.

Koonin (1993)) contains the signature sequence $G(X)_5E(X)_4U\theta XRE\theta XEE(X)_2\theta$ (where X= any amino acid, U= a bulky aliphatic residue and θ = a bulky hydrophobic residue). Within the consensus sequence, the rat FGF-2 antisense amino acid sequence has strong similarity to the corresponding domains in proteins from a range of organisms including the *Haemophilus influenzae* MutT-related pyrophosphatase (27% identity), *Escherichia coli* MutT (40% identity), yeast YSA1 hypothetical protein (47% identity), the human and rat 8-oxo-dGTPases (37% identity), human Ap4A hydrolase (57% identity), and the *Xenopus* FGF-2 antisense protein (77% identity).

The MutT family includes *Escherichia coli* mutT (8-oxo-dGTPase) and mammalian homologues; mouse, rat and human 8-oxo-dGTPase (MTH1). The antimutator prototype was originally described in the mutant strain of *E. coli* (*mutT*1), which exhibited spontaneous mutation frequencies ranging from 100- to 10,000-fold higher than normal (Treffers et al., 1954). Unlike typical defective mutator genes causing different DNA derangements like transitions, transversions, and frameshifts, *mutT* specifically causes a single unidirectional A:T to C:G transversion (Yanofsky et al., 1966). This transversion could therefore have arisen from mismatch of A with G, or T with C. Complementation of the *mutT* mutator phenotype permitted cloning of the *mutT*+ gene (Bhatnagar and Bessman, 1988). The defective *mutT*1 allele was shown to result from a IS1 insertion in the *mutT*+ gene, confirming the direct role of *mutT*+ in preventing the high frequency of A:T to C:G transversions. Further studies involving the expression, purification and characterization of the mutT gene product (Bhatnagar et al., 1991), revealed that it is a nucleoside triphosphatase with a preference for dGTP which catalyzes a reaction with

the following stoichiometry: dGTP + $H_2O$ ➡ dGMP + $PP_i$. The specific substrate of this nucleoside-triphosphate pyrophosphatase has been reported to be 8-oxo-dGTP (Maki and Sekiguchi, 1992). The mutagenic nucleotide, 8-oxo-dGTP, is generated by oxidation of free dGTP, and can induce A:T to C:G transversions (Cheng et al., 1992) due to its proclivity to mispair with adenine during replication. MutT proteins "sanitize" the nucleotide pool by hydrolyzing mutagenic 8-oxo-dGTP and thereby suppress the occurrence of spontaneous oxidative mutagenesis (Mo et al., 1992). The MutT consensus domain forms a loop-helix-loop motif not found in other nucleotide-binding sites (Frick and Bessman, 1995).

Recently, the mutT domain has been shown to occur in a larger group of proteins referred to as the family of "nudix" hydrolases (Bessman et al., 1996); these proteins all hydrolyze a nucleoside diphosphate linked to some other moiety, X. However, aside from these two properties of containing the mutT/"nudix" signature sequence and hydrolyzing X-linked nucleotide diphosphates, members of this family appear quite diverse in their substrate preferences, which include nucleoside triphosphates, coenzymes, nucleotide sugars, and dinucleoside polyphosphates.

### iii. FGF-2 Sense and Antisense mRNA Expression in Developing Rat Tissues

Amplification of FGF-2 sense and antisense RNA by RT-PCR demonstrates the tissue-specific distribution and developmental pattern of expression of the sense and antisense transcripts (Figure 20). The FGF-2 antisense mRNA was most abundant, and FGF-2 least abundant, in liver at all developmental stages examined, and the ratio of sense to antisense

**Figure 20.** RT-PCR analysis of FGF-2 sense and antisense mRNA expression in developing rat tissues. **(A)** embryonic (day 17 post-fertilization), **(B)** neonatal (postnatal day 12), and **(C)** adult (day 120). PCR primers for FGF-2 sense mRNA were the same as those used in previous analyses and additional primers for FGF-2 antisense mRNA (As1 and As2) were designed based on 5' end sequence. Glyceraldehyde-3-phosphate dehydrogenase (GAPD) mRNA levels were determined as a loading control. Reactions with no RNA were included for each product as a negative control (not shown). Graphs show the relative expression for FGF-2 sense and antisense following normalization with GAPD; samples with no visible product are denoted by an asterisk.

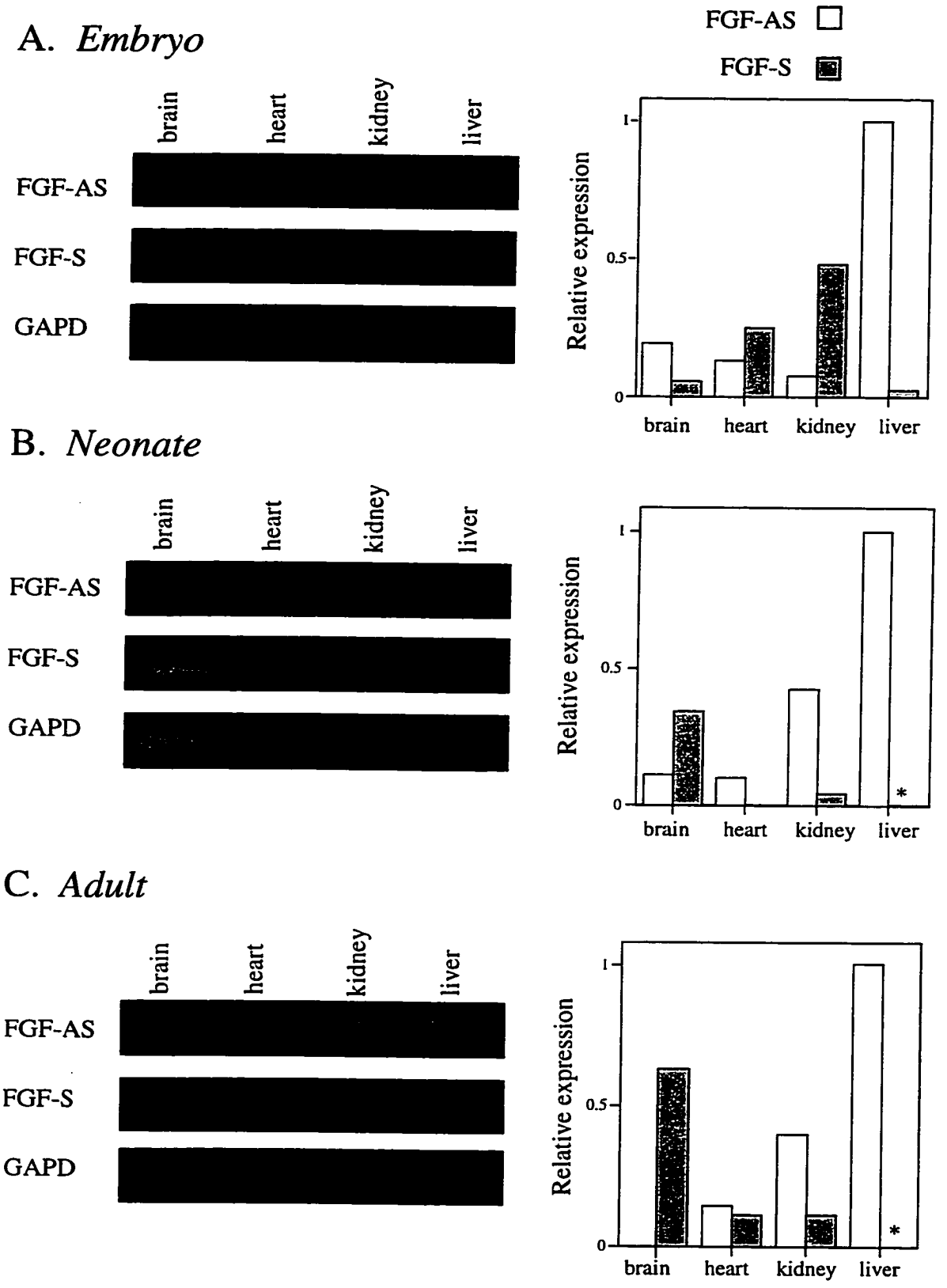

A. Embryo
brain
heart
kidney
liver
FGF-AS
FGF-S
GAPD
FGF-AS
FGF-S
Relative expression
1
0.5
0
brain
heart
kidney
liver
B. Neonate
brain
heart
kidney
liver
FGF-AS
FGF-S
GAPD
Relative expression
1
0.5
0
*
brain
heart
kidney
liver
C. Adult
brain
heart
kidney
liver
FGF-AS
FGF-S
GAPD
Relative expression
1
0.5
0
*
brain
heart
kidney
liver

Figure 20

changed little throughout this period. In heart and kidney, FGF-2 mRNA transcripts were more abundant than FGF-2 antisense transcripts in embryonic samples, but in postnatal samples the situation reversed due to an increase in abundance of FGF-2 antisense transcripts. In sharp contrast, in the developing brain FGF-2 mRNA expression increased throughout development, while FGF-2 antisense decreased in a reciprocal fashion, confirming our earlier observation by northern hybridization using the human 301 bp cRNA probe (Li et al., 1996a). The *gfg-1* PCR product was used as a 3' end derived fragment for PCR analysis, and an additional set of PCR oligonucleotides were designed to amplify a 5' end fragment. It was necessary to design primers for amplifying a product in the 5' end, because the original 3' end PCR fragment (*gfg-1*) was found to overlap the mutT domain typical of various NTPases. Further, a 5' end PCR product would not contain any complementary sequence with the FGF-2 sense mRNA and would give additional specificity for semi-quantitative analysis of the FGF-2 antisense RNA in various expression studies.

**III. Discussion**

We have isolated a rat FGF-2 antisense cDNA from neonatal liver which contains a conserved ORF and displays 67% homology to the reported *Xenopus* antisense sequence. The region of overlap between rat FGF-2 sense and antisense RNAs occurs within the 3' UTR of the sense transcript and the 3' UTR and part of exons III and IV of the antisense transcript. Sequence homology suggest that the structural organization of the FGF-2 sense/antisense transcription unit is highly similar between *Xenopus* and rat indicating a strong evolutionary conservation of the relationship between these two genes.

The generation of two individual RNA species sharing regions of homologous overlap, suggests the possibility of interstrand RNA duplex formation, which could play a role in strand-specific or mutual regulation of expression. In *Xenopus*, the FGF-2 sense and antisense RNAs are assumed to form duplexes in the cytoplasm of immature oocytes because of extensive modification by double-stranded RNA-specific adenosine deaminase (DRADA) in these transcripts following germinal vesicle breakdown (Kimelman and Kirschner, 1989). Given the rapid decline in FGF-2 sense transcript stability at this time, it has been suggested that destabilization of the dsRNA complexes by DRADA modification may target the transcripts for degradation by dsRNA-specific RNases (Kimelman and Kirschner, 1989). Post-transcriptional A-I editing of GluR-B pre-mRNA results in a functionally significant glutamine (C<u>A</u>G) to arginine (C<u>G</u>G) substitution in the glutamate receptor. In contrast, the extensive degree of covalent modification reported in the *Xenopus* FGF-2 sense mRNA suggests that DRADA is not involved in specific editing of the mRNA, but may play a role in the regulation of mRNA stability.

Alternatively, dsRNA complexes formed between FGF-2 sense and antisense may function in the modulation of nucleocytoplasmic transport or pre-mRNA splicing as suggested for the c-*erbA*$\alpha$ and N-*myc* antisense genes.

The presence of overlapping sequences between FGF-2 sense and antisense is primarily in their 3' UTR. Multiple FGF-2 sense transcripts (1.8-7.0 kb in human) arise from differential length of their 3' UTRs. The functional significance of the variable 3' UTR in FGF-2 mRNA may be associated with the presence of AU-rich motifs, which are important in mRNA stability and perinuclear localization. There are 15 copies of the AU-rich motif in the largest human FGF-2 mRNA transcript (7.0 kb), 2 copies in the 3.7 kb species, and none in the 1.2 kb mRNA (Kurokawa et al., 1987; Prats et al., 1989). Although their significance is unknown, the expression of different 3' UTR variants occurs in a cell- or tissue-specific and developmentally-regulated manner (Powell et al., 1991; Kurokawa et al., 1987; Bost and Hjelmeland, 1993; Braunhut et al., 1989). Regulated use of polyadenylation sites has been associated with developmental processes in other systems (Paris and Richter, 1990). In the developing rat brain, short (1.2-1.8 kb) FGF-2 mRNA transcripts are preferentially expressed prenatally, while the major (6 kb) transcript is increasingly expressed after birth, corresponding with increased FGF-2-like bioactivity (Powell et al., 1991). Li et al., (1996a) have demonstrated that the appearance of the 6 kb FGF-2 mRNA is coincident with a decline in the expression of the antisense RNA.

The selective expression of different FGF-2 3' UTR sequences may be a physiological mechanism to determine the extent of RNA interaction with the antisense transcript. By comparing the human FGF-2 sense

transcript with the rat antisense transcript, it is evident that the FGF-2 antisense transcript has sequence complementarity with 2 widely separated regions of the FGF-2 sense mRNA; one overlaps the most distal 3' polyadenylation site of the 7 kb sense transcript, the other overlaps the proximal 3' UTR, just downstream of the first consensus polyadenylation signal. The identification of restricted regions of complementarity in the vicinity of the proximal and distal FGF-2 polyadenylation sites suggests that the FGF-2 antisense RNA may be involved in polyadenylation site selection.

In *Xenopus*, the region of overlap extends to include a portion of the 3' coding region of FGF-2. Although this does not appear to be the case in rat or human, in chicken the FGF-2 antisense transcript overlaps with FGF-2 mRNA in the 3' UTR and all of exon 3 (Borja et al., 1993). The function associated with hybridization of the sense and antisense transcripts may therefore be to direct differential spicing of FGF-2 mRNA.

Although the FGF-2 sense and antisense mRNA transcripts share substantial complementarity for dsRNA complex formation *in vivo*, these two transcripts may not interact. The CYP/TN-X gene locus generates the steroid 21 hydroxylase (sense) and tenascin-X (antisense) mRNA transcripts. However, despite the presence of nearly 300 nt of perfect complementary sequence at their 3' ends, this sense-antisense pair were demonstrated to not form *in vivo* dsRNA duplexes (Speek and Miller, 1995). It was further shown in this case that sequence-specific binding of nuclear proteins prevented annealing between the complementary strands. Given the variety of proteins which facilitate or suppress RNA annealing, it is possible that hybridization of FGF-2 sense and antisense

RNAs may be regulated on a tissue-specific basis by nuclear and/or cytoplasmic RNA binding proteins.

We have recently reported that the onset of expression of the 6 kb FGF-2 transcript in the developing rat brain coincides with a corresponding decline in FGF-2 antisense expression measured by northern hybridization with the antisense-specific cRNA probe (Li et al., 1996a). The present RT-PCR data comply with this observation, and further demonstrate that the overall level of FGF-2 mRNA in brain (regardless of transcript size) is inversely related to the level of FGF-2 antisense mRNA in that tissue. Our expression analyses are also consistent with a recent RNase protection study which reported an inverse relationship between the level of FGF sense and antisense RNA abundance in the developing rat CNS (Grothe and Meisinger, 1995).

Northern and RNase protection analyses revealed significant levels of FGF-2 antisense RNA during chicken embryogenesis (stage 16 to 24) (Borja et al., 1993). Interestingly, the expression of FGF-2 sense and antisense mRNA diverge in differentiated cells (Savage and Fallon, 1995). Most obvious is the mesonephros, in which FGF-2 sense mRNA was readily detected and antisense mRNA absent. Further, the presence or absence of the antisense mRNA correlated with the level of FGF-2 protein detected in cartilage, ectoderm, mesenchyme and mesonephros. For example, in the mesonepheros, FGF-2 mRNA and protein were detected at the highest levels in the absence of antisense mRNA. Conversely, in adult chicken organs such as liver, the level of expression of antisense mRNA is notably higher than sense mRNA.

We have demonstrated that FGF-2 antisense expression is very low in the human glioma cell line, U87-MG (Murphy and Knee, 1994) which

expresses an unusually stable FGF-2 sense RNA transcript (Murphy et al., 1990). Preliminary experiments in our lab indicate that transfection of human (U87-MG) or rat (C6) glioma cells with an expression vector containing the 301 bp *gfg-1* cDNA inhibits glioma cell growth in monolayer culture and renders the cells FGF-responsive for proliferation. The mechanism of this inhibition remains to be determined, but the observation suggests that the 3'UTR of the FGF-2 mRNA is susceptible to antisense targeting. Since the antisense transcript is complementary to regions of the 3' untranslated region of the FGF-2 mRNA it is conceivable that formation of double-stranded RNA helices in these regions may in some way regulate polyadenylation site usage and/or mRNA half-life.

Sequence analysis of the *Xenopus laevis* and rat FGF-2 antisense cDNA clones reveal a conserved ORF, however the function of its translational product remains unknown. Our recent observation of the mutT motif within the rat full-length FGF-2 antisense sequence demonstrated that this protein belongs to the mutT/nudix family. The prototypic member of this family of nucleotide pyrophosphatases is the *E. coli* MutT protein whose consensus domain shares 40% identity with that of the rat FGF-2 antisense protein. *E. coli* MutT is an antimutator protein which functions in suppressing the occurrence of spontaneous oxidative mutagenesis by removal of mutagenic 8-oxo-dGTP from the nucleotide pool. The enzymatic activity associated with this protein involves the conversion of 8-oxo-dGTP to 8-oxo-dGMP via the reaction $NTP^{4-} + H_2O \rightarrow NMP^{2-} + PP_i^{4-} + 2H^+$ (Weber et al., 1992). Human and rat MutT homologs (MTH1) are also 8-oxo-dGTPases, and are predominantly mitochondrial and cytosolic (Kang et al., 1995), consistent with their proposed role in

sanitizing the nucleotide pools located in these compartments (Mo et al., 1992).

The mutagenic nucleotide, 8-oxo-7, 8-dihydro-2'-deoxyguanosine 5'-triphosphate (8-oxo-dGTP) and its DNA analog, 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxo-dG), are formed by oxidative damage and appear to play critical roles in carcinogenesis and aging (Ames and Gold, 1991; Ames et al., 1993). Since 8-oxo-dGTP is able to base-pair with both cytosine and adenine, the misincorporation of this mutagenic substrate from the free nucleotide pool during DNA synthesis can result in A:T to C:G transversions (Cheng et al., 1992). Inactivation of the mutT gene in *E coli* resulted in a dramatic specific increase in the frequency of A:T to C:G transversions 100 to 10000 times the level of wild-type (Yanofsky et al., 1966). The DNA analog 8-oxo-dG, can mismatch with adenosine during DNA replication, resulting in the formation of G:C to T:A transversions (Shibutani et al., 1991; Moriya, 1991). Other structurally unrelated antimutator proteins include mutM which removes 8-oxo-dG residues from DNA, and mutY which specifically suppresses G:C to T:A transversions. Due to the presence of antimutator proteins, the frequency of mutations caused by endogenous reactive oxygen species remains low in normal growing cells (Schapper et al., 1986; Mo et al., 1992). The measurement of 8-oxo-dG in cellular DNA from mouse liver or *Salmonella typhimurium* revealed approximately 0.6-1.4 nucleotides per $10^5$ dG (Kasai et al., 1986).

Increasing evidence suggests that 8-oxo-dG is the major product of base modification formed by reaction with singlet oxygen. Singlet oxygen ($^1O_2$) is an excited state of molecular oxygen and is one of the major oxidative species generated during biological and chemical processes such as

photosensitization (Piette, 1991), the decomposition of dioxetanes (Epe et al., 1992), dismutation of superoxide radical (Khan and Kasha, 1994), and the cleavage of hydroperoxides by cytochrome P450 monooxygenase (Cadenas and Sies, 1982).

The exposure of cells to hydrogen peroxide results in a variety of cytotoxic effects, such as the rapid attenuation of intracellular ATP, loss of intracellular NAD, activation of ADP-ribose polymerase, and DNA damage, all of which occur prior to loss of plasma membrane integrity (Abe et al., 1995). The conversion of hydrogen peroxide to reactive oxygen species (ROS), such as the hydroxyl radical, is believed to require transition metal ions (Blakely et al., 1990), which explains the DNA damage effect of hydrogen peroxide due to the trace amounts of metal ions bound to or near DNA. $H_2O_2$ exposure of *S. typhimurium* or human amniotic WISH cells resulted in a dose-dependent increase from 2- to 6-fold in the proportion of 8-oxo-dG in cellular DNA (Kasai et al., 1986; Abe et al., 1995). Since it is likely that the extent of 8-oxo-dG modification is proportional to other DNA modifications, the measurement of the 8-oxo-dG in DNA is useful for obtaining an indication of the extent of oxygen radical-induced damage (Kasai et al., 1991).

The mutT consensus domain was initially identified in the *E. coli* MutT and *S. pneumoniae* MutX antimutator proteins (Bullions et al., 1994; Mejean et al., 1994). Computer searches established that this conserved domain defines a large MutT/nudix family of enzymes present in other organisms ranging from viruses to human (Mejean et al., 1994; Koonin, 1993). It has since become apparent that this domain is not restricted to nucleotide triphosphatases and antimutator activity (Bessman et al., 1996).

Additional members of the MutT/nudix family favor different nucleotide compounds such as sugar-nucleotide substrates, including the *E. coli* and *S. cerevisiae* proteins which exhibit GDP-mannosyl and ADP-ribosyl hydrolyase activity, respectively (refer to Figure 20, Chapter 6) (Bessman et al., 1996). Since other MutT/nudix members include enzymes such as *S. cerevisiae* NADH pyrophosphatase, which catalyzes the cleavage of NADH to NMNH and AMP, it is possible that the consensus motif has been conserved throughout evolution and modified for function in various metabolic reactions requiring the cleavage of a nucleotide pyrophosphate bond.

An interesting subgroup of the mutT/nudix family include the human Ap4Aase tetraphosphatase, and related *E. coli* proteins with Ap3Aase or Ap4Aase activity (Bessman et al., 1996). Ap4A (diadenosine 5', 5'''-$P^1$, $P^4$ tetraphosphate) and related adenylylated nucleotides have been implicated in the initiation of DNA replication, as cell-signaling molecules in response to stress or heat shock, as neurotransmitters, and effectors of the cardiovascular system (reviewed in Baxi and Vishwanatha, 1995). The normal cellular role of $Ap_nAase$ is the removal of the corresponding dinucleotide or its conversion to related intermediates.

The elevated levels of Ap4A observed in *E. coli* and *Salmonella*, in response to cellular stress conditions, led investigators to term Ap4A as an "alarmone" (Lee et al., 1983a, b). A similar role of $Ap_nA$ nucleotides in higher vertebrates is also evident. For example, DNA damage in normal human fibroblasts results in alterations in levels of $Ap_nA$ nucleotides. In addition, a role for these nucleotides as secondary messengers was suggested following microinjection of *Xenopus laevis* oocytes with Ap4A nucleotides, which resulted in the initiation of DNA synthesis, implying

that these nucleotides had a direct role in signaling the onset of cellular proliferation (Zourgui et al., 1984).

In brain, Ap4A and Ap5A can be found stored in synaptosomes (Pintor et al., 1992a), however Ap4A and other $Ap_nA$ nucleotides are also found extracellularly in blood, following their release from thrombocytes and adrenal chromaffin cells (Baxi and Vishwanatha, 1995). Release of Ap4A accompanies catecholamines, suggesting the significance of these nucleotides in the neural response (Castillo et al., 1992). In fact, it became apparent during the study of a possible neurotransmitter role for $Ap_nA$ that chromaffin cells are a rich source of these nucleotides (Rodriguez del Castillo et al., 1988; Pintor et al., 1992b). With respect to chromaffin cells, we have observed high levels of FGF-2 antisense mRNA and protein in rat adrenal tissue (Li et al., 1996a, Knee et al., 1997). Further, RT-PCR analysis reveals that the rat PC12 pheochromocytoma cells produce readily detectable levels of FGF-2 antisense mRNA (Knee et al., unpublished observations). Ectohydrolases released into the blood from endothelial and smooth muscle cells (Oligvie et al., 1989; Pearson et al., 1980) cleave dinucleotide polyphosphates into ATP, ADP, or AMP, and finally to adenosine, which may play a role in the regulation of those cells having receptors for these $Ap_nA$ intermediates. For example, the prothrombotic effect of Ap3A is due to the presence of Ap3A hydrolase in plasma, which generates ADP and AMP, whereby the effect is mediated by ADP (Luthje et al., 1985; 1988).

Nucleotide and dinucleotide responses are generated by interaction with purinoreceptors which mediate the transmembrane signal. Purinoreceptors have been categorized into P1 and P2 classes (Hoyle, 1990), and subclassified into $P_{2X}$, $P_{2Y}$, $P_{2Z}$, and $P_{2T}$, depending on the type

ligand with which they interact. Ap4A is suggested to be a $P_{2Y}$-receptor agonist since it inhibits chromaffin cell transport of adenosine, which is known to bind $P_{2Y}$-type receptors (Sen et al., 1993). The inhibition of ADP-induced platelet aggregation by Ap4A, was shown to result from the binding of Ap4A to $P_{2T}$ receptors which also bind ADP (Baxi and Vishwanatha, 1995). The interaction of Ap4A with this receptor was further shown to induce the opening of $Ca^{2+}$ ion channels through the activation phospholipase-inositol pathway (Pfeilschifter, 1990). Evidence has shown that Ap4A receptors are present in the brain, liver, heart, kidney and spleen (Hilderman et al., 1991). However, from the current knowledge of Ap4A signaling it has been concluded that the receptors for Ap4A are probably different from the $P_{2X}$ or $P_{2Y}$ subtypes, and whether intact Ap4A molecules exert their effects directly or through degradative products remains to be determined.

In order to gain insight to the functional role of the FGF-2 antisense protein a series of experiments were conducted to examine its mRNA expression pattern under various culture conditions which involved the manipulation of the levels of 8-oxo-dGTP or $Ap_nA$ nucleotides in different cell lines. Expression of FGF-2 antisense mRNA has been examined in culture under treatments of singlet oxygen, free radicals, and Ap4A, however, no significant changes in mRNA levels were observed under these experimental conditions.

The phenotype of cell lines with dominant-negative mutations in the mutT domain may give some insight into the physiological role of the FGF-2 antisense protein.

# CHAPTER 7: EXPRESSION OF FGF-2 ANTISENSE IN MOUSE LIVER CELLS

## I. Introduction

We have shown that FGF-2 antisense is abundantly expressed in mammalian liver. Considering the complexity of liver, it was of interest to determine the cellular source of FGF-2 antisense in this tissue.

### i. Liver tissue

The liver primordium consists of an endodermal component, giving rise to hepatoblasts which further differentiate into hepatocytes, and a mesodermal component from which the sinusoidal-lining and connective tissue cells originate (Shiojiri et al., 1991). Liver parenchyma cells or hepatocytes are a heterogeneous population of cells which, depending on their location in the liver, contribute differently to the various metabolic processes such as gluconeogenesis, glycogenolysis, and lipogenesis.

Hepatic sinusoids of the liver lobules carry blood throughout the tissue and are separated from the hepatocytes by a thin layer of endothelium interspersed with Kuppfer (reticuloendothelial) cells which are resident macrophages (Desmet, 1994). Kupffer cells comprise 15% of the total number of liver cells and account for 80-90% of the total population of fixed tissue macrophages in the body. Also among the mixture of cell types comprising the liver are a small number of fibroblasts which provide a tenuous supporting framework of connective tissue.

Nonparenchymal cells, such as sinusoidal endothelial and Kuppfer cells, produce paracrine factors which are known to modulate hepatocyte metabolism (Brass and Vetter, 1994). Kuppfer cells produce eicosanoids, cytokines, and nitric oxide in response to macrophage stimuli (Decker,

1990), and hepatic endothelial cells may also contribute to the wide spectrum of bioactive molecules (Kuiper et al., 1988; Feder et al., 1993). Prostaglandin $E_2$ ($PGE_2$) and nitric oxide (NO) treatment of cultured rat hepatocytes was shown to modulate glycogenolysis and its response to glucagon (Okumaura et al., 1988; Brass and Garrity, 1990; Brass and Vetter, 1993), while IL-6 treatment resulted in the induction of lipogenesis (Brass and Vetter, 1994). Kuppfer cells and endothelial cells also generate significant reactive oxygen species (ROS) when properly stimulated by various ligands, such as endotoxin and bacteria (Bhatnagar et al., 1981; Birmelin et al., 1986; Shiratori et al., 1986).

The isolation of hepatocytes in primary cultures has been extensively utilized as an attractive approach to the investigation of the mechanisms associated with liver function and growth. Primary adult hepatocytes in culture have a restricted capacity to proliferate and with time undergo dedifferentiation (Singh and Veltri, 1991). This process of dedifferentiation is signified by reduced cytochrome P450 protein, decreases in mitochondrial DNA and intracellular NAD content, and deterioration of differentiated cellular morphology (Singh and Veltri, 1991; Nemoto and Sakurai, 1994). Hepatocytes from mouse or rat display a low capacity for DNA synthesis in primary culture, however with the exogenous addition of EGF, TGF-$\alpha$ and HGF, show a 5- to 10-fold increase in DNA synthesis (McGowan, 1986).

**ii. Development**

Liver hepatocytes have been observed to exhibit a developmental-specific change in nucleation and polyploidy. In fetal and early neonate rat liver, hepatocytes are predominately mononuclear diploid (~70%),

however, during the third week of postnatal development the level of polyploidy begins to rise dramatically (Sasse, 1986). By week four of postnatal development, the parenchyma is composed of 50% binuclear diploid, 25% mononuclear tetraploid, and only 20% mononuclear diploid cells. In the adult liver, almost 70% of hepatocytes are mononuclear tetraploid, whereas mononuclear diploid cells are less than 5%.

Mammalian liver development is also characterized by a stage-specific accumulation of clusters of enzymes (Greengard, 1971). In rat, the fetal stage is characterized by the emergence of urea cycle enzymes (carbamoylphosphate synthetase, arginase, etc) so that at birth the animal is able to cope with nitrogenous waste products. Also present in the fetal stage are enzymes required for glycogen synthesis (UDPG glycogen glucosyltransferase, UDPG pyrophosphorylase, and phosphosglucomutase), which allow extensive deposition of glycogen during the last few days of gestation. This store of glycogen is rapidly depleted by 24 hr after parturition, as evidenced by hypoglycemia in neonate mammals, including human. The ability to synthesize glucose from glycolytic products and amino acids develops at the beginning of the neonate stage. Phosphoenolpyruvate carboxylase, which catalyzes the rate-limiting step in gluconeogenesis, is a predominant member of the neonatal enzyme cluster. Also, enzymes required for the deamination of amino acids, such as L-serine dehydratase and asparaginase, etc, emerge neonatally. The neonatal cluster also includes UDP glucuronyltransferase which is necessary for the disposal of bilirubin. In the late suckling stage, enzymes such as glucokinase, pyruvate kinase and aminotransferases begin to appear, which are required for adaptation to the changing diet.

Large amounts of oxygen are consumed by the liver; hepatic function results in the production of about 90 nmol of ROS per min per gram of rat liver (Inoue, 1994). Generation of ROS under normal physiological conditions in rat liver by the mitochondria, microsomal, peroxisomal, and cytoplasmic compartments is 15, 45, 35, and 5%, respectively (Chance et al., 1979). The majority of molecular oxygen used in mitochondria is converted to $H_2O$, however a small amount is converted to superoxide radical ($O^-_2$) which is further converted to hydrogen peroxide and related metabolites. The catalysis of reactions by microsomal cytochrome P450 also generates superoxide radicals, and is postulated to be the major mechanism for generation of superoxide radicals (Inoue, 1994). A large proportion of ROS rapidly react with a variety of cellular factors and thereby interfere with their functions.

**iii. Regeneration**

Liver regeneration occurs following surgical resection of tissue or loss of cells due to toxic or viral injury. The most dramatic example of liver regeneration is that of regrowth after partial hepatectomy. Hepatocytes throughout the lobule are highly but not terminally differentiated cells, since they retain their capacity to proliferate but do not under normal conditions (Fausto and Webber, 1994). However, in situations where hepatocyte proliferation is impaired by nutritional and toxic effects of chemicals (Evarts et al., 1987), regeneration can occur via the proliferation and differentiation of oval cells, which are the progeny of liver stem cells (Hu et al., 1996). Oval cells are liver epithelial cells which are smaller than hepatocytes and have not been observed in normal liver, but however become apparent during the use of carcinogens or after extensive liver

damage (Fausto, 1990). The mechanism of liver regeneration is divided into two stages; the priming stage (G0/G1 transition) which involves the posttranslational modification of NF-kB-like transcriptional factors, that provides the hepatocyte with the ability to respond to or produce growth factors, and the progression phase (G1/S transition) which leads to DNA synthesis. The rate of cell proliferation of regeneration at its peak is comparable to that of embryonic cells, and continues at a diminished rate until the original complement of liver tissue is restored (Bucher, 1987).

**iv. Role of FGF in the liver**

The contribution of FGFs to liver growth and regeneration is not yet fully understood. Investigation of FGFR expression in adult rat liver has shown that FGFR-1 is highly expressed and is uniquely localized in the oval cells while absent in hepatocytes, whereas FGFR-2 is detectable in hepatocytes, albeit low levels (Hu et al., 1996). During liver regeneration, FGFR-2 increased while FGFR-1 remained undetectable in hepatocytes, and in the developing liver both FGFR-1 and FGFR-2 were expressed at high levels. Studies to elucidate the role of FGF in the liver have found that FGF-1 is a potent mitogen for cultured rat hepatocytes (Tanahashi et al., 1995) and is among the major growth factors that contribute to liver regeneration (Hu et al., 1996). In addition to its effect on liver regeneration and hepatic differentiation via the stem cell compartment, FGF-1 is also involved in liver development (Marsden et al., 1992). FGF-2 is not believed to play a role in liver regeneration or development, and is undetectable in all developmental stages in rat by northern analysis (Li et al., 1996a). However, according to Baruch et al., (1995), FGF-2 has a hepatotropic capacity in the regeneration of liver parenchymal cells. They

have shown that the injection of FGF-2 into partially hepatectomized rats resulted in significant FGF-2 uptake by hepatocytes and DNA synthesis as measured by labeled-thymidine incorporation.

**v. FGF-2 antisense in the liver**

We have shown that the FGF-2 antisense mRNA is abundantly expressed in neonatal rat liver and antibodies raised against the deduced protein sequence detect abundant expression of the antisense protein in western blots of adult liver extracts (Li et al., 1996a, b; Knee et al., 1997). The FGF-2 antisense protein encodes a novel 35 kDa protein which is a member of the newly described family of MutT/nudix hydrolyases. This diverse family of "housecleaning" enzymes includes the antimutator 8-oxo-dGTPases which "sanitize" the nucleotide pool by hydrolyzing mutagenic 8-oxo-dGTP and thereby suppress the occurrence of spontaneous oxidative mutagenesis (Mo et al., 1992). Subcellular fractionation of adult rat liver revealed that FGF-2 antisense protein is localized predominantly in the nucleus, and to a much lesser extent in the mitochondrial and cytosolic fraction (Li et al., unpublished observations), providing further support for a possible role in maintaining the fidelity of DNA replication.

Given the nature of the putative function of the FGF-2 antisense protein in oxidative damage and nucleotide metabolism, we were prompted to take a closer look at its expression in liver tissue. FGF-2 antisense mRNA and protein were analyzed in whole mouse liver, isolated hepatocytes, Kuppfer cells, and the mouse Hepa1 tumor cell line.

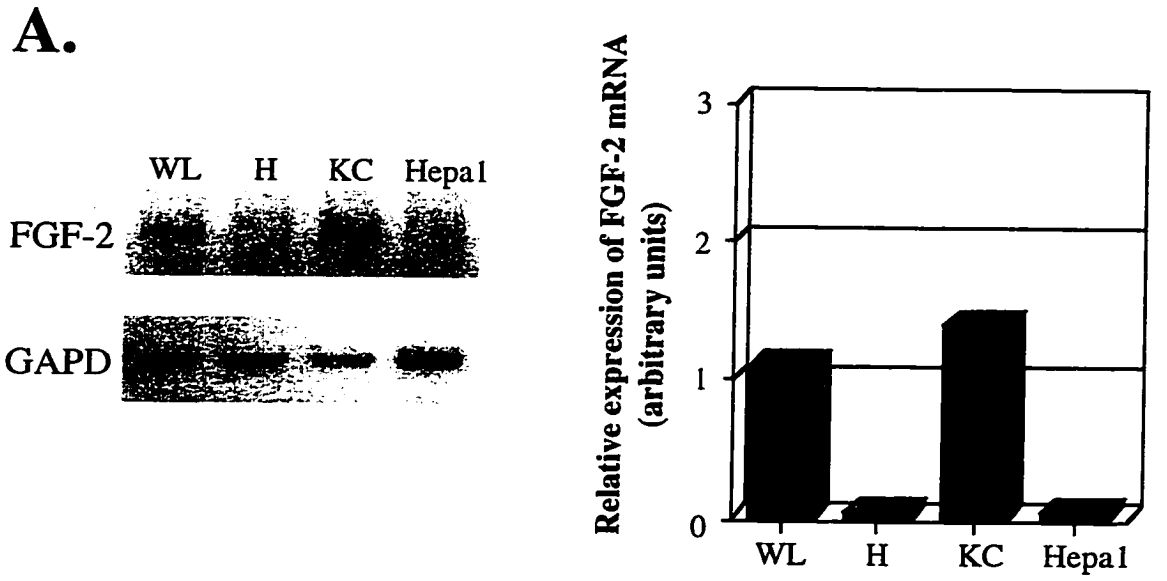
**II. Results**

The preparation of primary cultures of mouse liver cells involved a process of differential centrifugation as described in Materials and Methods. This process yielded a parenchymal fraction consisting of predominantly hepatocytes which is essentially free of Kuppfer cells (~1%), and a nonparenchymal fraction which is enriched for Kuppfer cells, but also contains some other cells, including <4% hepatocytes. The purity of each fraction was then assessed by size and appearance. (The preparation of liver cell fractions was done with the assistance of Dr. Ken Renton and Karen Bedard).

FGF-2 sense and antisense mRNA expression in parenchymal and nonparenchymal cell fractions of mouse liver tissue was determined by semi-quantitative RT-PCR. As shown in Figure 21A, amplification of FGF-2 sense mRNA (352 bp fragment) revealed that this transcript is expressed in whole liver and the nonparenchymal (Kuppfer cell) fraction, but not in hepatocytes. Sequence-specific primers targeted to the 5' and 3' end of the FGF-2 antisense mRNA amplified the predicted 379 and 301 bp PCR fragments in all liver-derived cells (Figure 21B). With respect to the abundance of the 3' end-derived PCR fragment, the relative level of FGF-2 antisense mRNA among the different samples was: whole liver > hepatocytes > Kuppfer cells > Hepa1 tumor cells. Although RT-PCR analysis using the 5' end derived primers amplified the predicted product, there were discrepancies in the abundance of this product and the 3' end product among the different cell types (see discussion).

Antibodies raised against synthetic peptides derived from the MutT domain of the rat FGF-2 antisense protein were used for Western analysis of FGF-2 antisense in mouse liver cells. The 35 kDa FGF-2 antisense

**Figure 21.** RT-PCR detection of FGF-2 sense and antisense mRNA in mouse liver cells. **(A)** RT-PCR analysis of FGF-2 (sense) and glyceraldehyde-3-phosphate dehydrogenase (GAPD) in mouse whole liver (WL), hepatocytes (H), Kuppfer cells (KC), and mouse hepatoma Hepa1 cells. **(B)** RT-PCR analysis using 5' and 3' end-derived PCR fragments of the FGF-2 antisense mRNA for the same samples as in (B). Five microliters of RT (containing one microgram of total RNA) were added to PCR mixes for 40 (FGF-2, FGF-2 antisense) or 20 (GAPD) amplification cycles. Graphs depict relative expression of FGF-2 sense and antisense mRNA in arbitrary units following normalization with GAPD levels as determined by densiometric scanning of agarose gel images.


A.
WL
H
KC
Hepa1
FGF-2
GAPD
Relative expression of FGF-2 mRNA (arbitrary units)
3
2
1
0
WL
H
KC
Hepa1

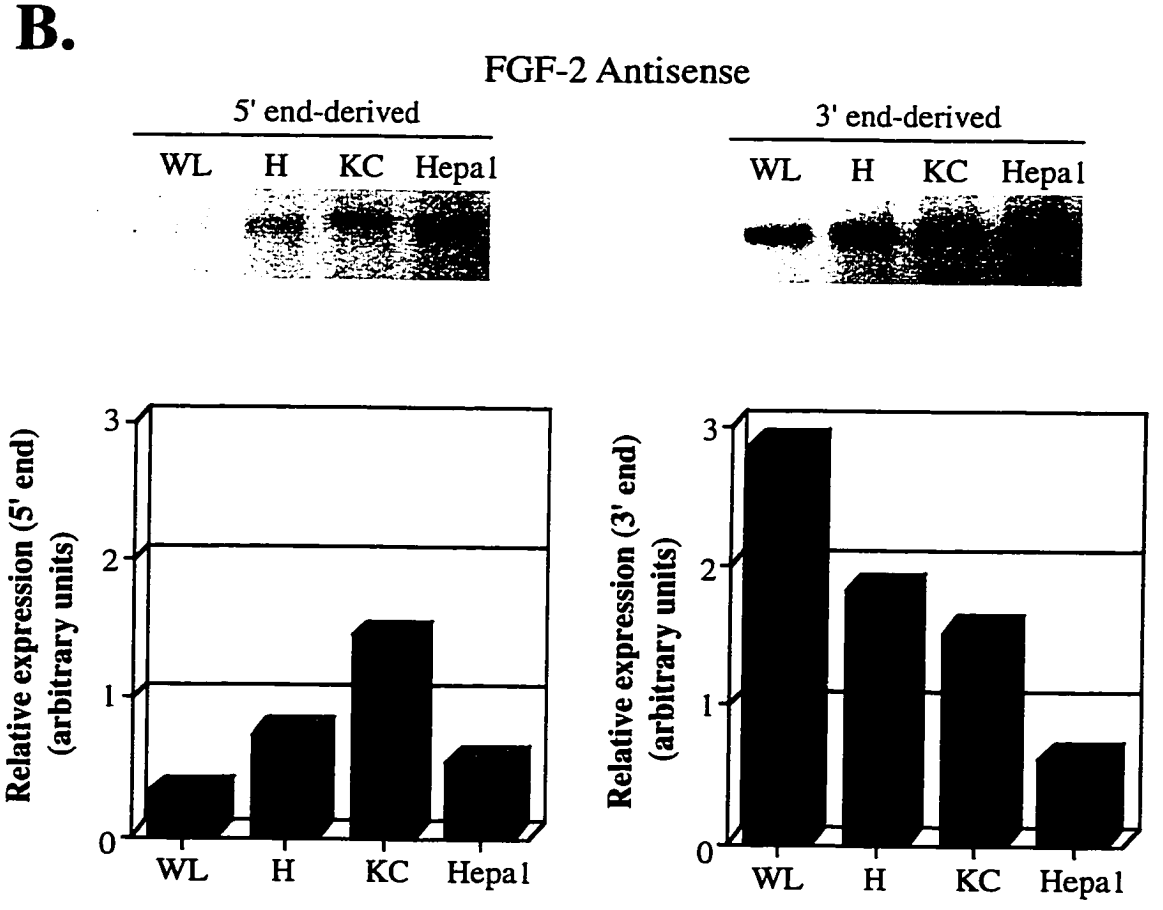

B.
FGF-2 Antisense
5' end-derived
3' end-derived
WL
H
KC
Hepa1
Relative expression (5' end) (arbitrary units)
Relative expression (3' end) (arbitrary units)
3
2
1
0

Figure 21

protein was detected at the highest level of abundance in whole liver, to a lesser extent in isolated hepatocytes (Figure 22), and was undetectable in the nonparenchymal cell fraction and Hepa1 tumor cells. Levels of FGF-2 antisense protein are, however, consistent with those of antisense mRNA as determined by RT-PCR, in which the transcript was most abundant in whole liver and hepatocytes.

Primary adult hepatocytes were harvested from cultures at 5, 16, 24, and 48 hr for total RNA and protein extraction. Control samples (0 hr) were obtained from freshly isolated hepatocytes. Over this period in culture, a pronounced decrease was observed in the level of total RNA (from 0.7 to 0.1 $\mu g/10^6$ cells at 0 and 48 hr, respectively) (Figure 23). The level of total protein also showed a decline (from 1.8 to 0.5 ng/cell at 0 and 48 hr, respectively). RT-PCR and Western analyses of FGF-2 antisense expression in cultured hepatocytes revealed that from 0 to 48 hrs there was a 5- and 4-fold upregulation of its mRNA and protein, respectively (Figure 24).

**III. Discussion**

We provide here the first evidence of FGF-2 antisense expression in mouse and a delineation of its expression within the parenchymal and nonparenchymal cell fractions of mouse liver. FGF-2 antisense mRNA was most abundant in whole liver, which agrees with the high level found in liver tissue of other species including rat, human and chicken. Of the parenchymal and nonparenchymal fractions, FGF-2 antisense mRNA is expressed more abundantly in the parenchymal fractions. Western analysis confirms the association of higher FGF-2 antisense in the parenchymal fraction compared to the nonparenchymal fraction. Since,

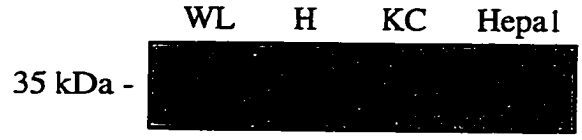



**Figure 22.** Western detection of FGF-2 antisense protein in mouse liver cells. Blot contained 40 micrograms of protein for each of the following samples; whole liver (WL), hepatocytes (H), Kuppfer cells (KC), and the Hepa1 tumor cell line. Immunodetection was performed using the anti-MutT antibody as described in Methods and Materials.

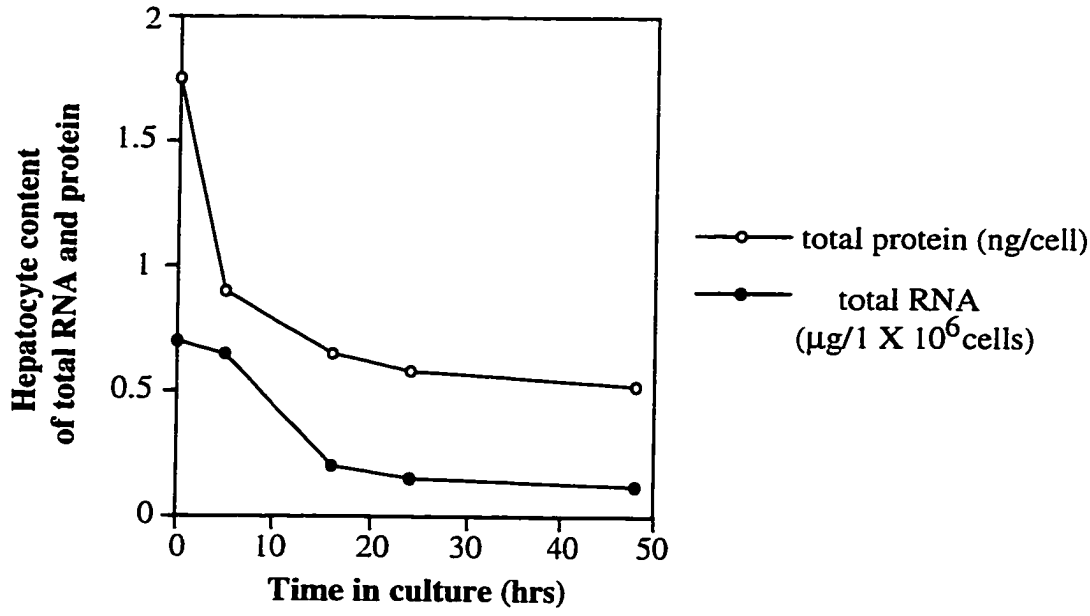
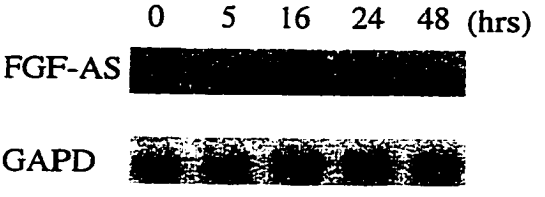
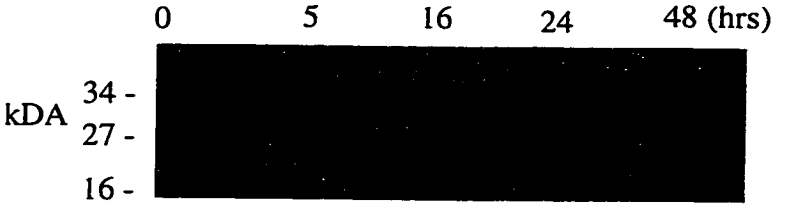



**Figure 23.** Measurement of total RNA and protein content in cultured mouse hepatocytes. Values are expressed as μg/1 X $10^6$ cells (total RNA) or ng/cell (protein).

**Figure 24.** RT-PCR and Western analyses of FGF-2 antisense mRNA and protein in cultured mouse hepatocytes. **(A)** RT-PCR and Western analyses of FGF-2 antisense mRNA and protein expression by mouse hepatocytes in primary culture. RT-PCR analysis of FGF-AS (FGF-2 antisense mRNA; 40 cycles) and GAPD (glyceraldehyde-3-phosphate dehydrogenase; 20 cycles) were performed at the indicated times. Immunblotting using the anti-MutT antibody of hepatocyte lysates isolated from culture at the same time points. Molecular weight markers are indicated on the left. (RT-PCR and Western analyses were performed in duplicate). **(B)** Graph depicts the level of expression of FGF-2 antisense mRNA (after standardizing with GAPD levels) and the corresponding antisense protein levels (arbitrary units).

**A.**


0 5 16 24 48 (hrs)
FGF-AS
GAPD


0 5 16 24 48 (hrs)
kDA
34 -
27 -
16 -

**B.**

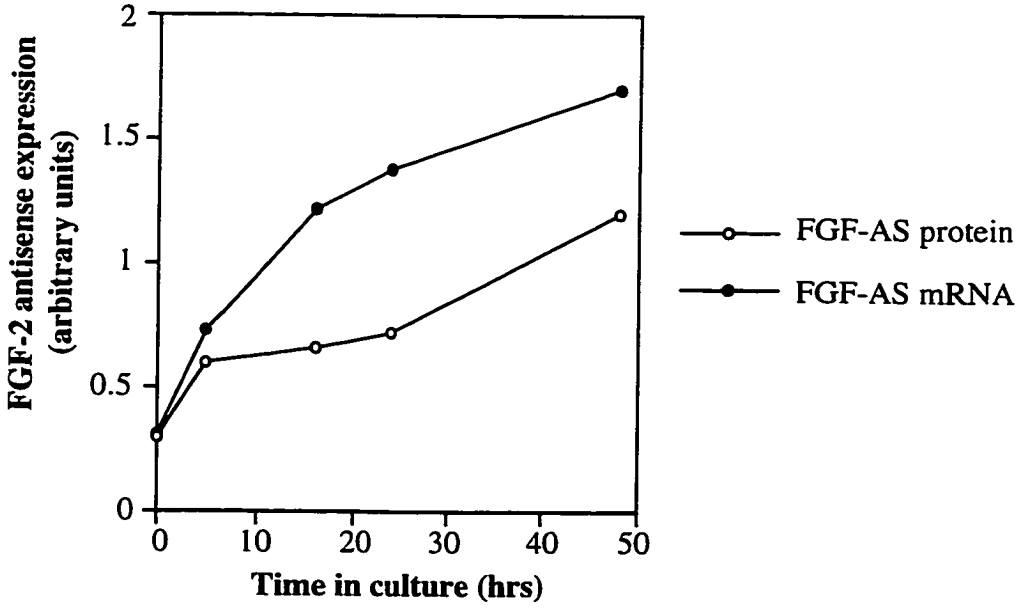

FGF-2 antisense expression (arbitrary units)
2
1.5
1
0.5
0
0 10 20 30 40 50
Time in culture (hrs)
FGF-AS protein
FGF-AS mRNA

Figure 24

hepatocytes comprise >60% of the liver, the majority of FGF-2 antisense mRNA and protein observed in whole liver is likely attributable to hepatocytes. The presence of FGF-2 antisense mRNA in the nonparenchymal cell fraction, comprised predominantly of macrophagic Kuppfer cells, is likely indicative of its expression in cells of high oxidative metabolism. Antisense mRNA was also detected in the mouse hepatoma-derived Hepa1 cell line, but at three-fold lower abundance than that observed in normal hepatocytes.

In the developing rat liver, FGF-2 antisense is low or undetectable at embryonic day 20, promptly induced by postnatal day 3, and remains at a high level into adulthood (day 120) (Li et al., 1996a). This period corresponds to major developmental events in liver; the onset of polyploidy, initiation of specific metabolic enzymes, and immunity adjustments (Greengard, 1971; Sasse, 1986; Inoue, 1994). The level of polyploidy displayed by rat hepatocytes begins to rise dramatically during the second week of postnatal development because of a transition from mononuclear diploid to mononuclear tetraploid cells. In addition, a large increase in the levels of cytochrome P450 enzymatic activity occurs 5 to 6 days postpartum (Vessey, 1982). The catalysis of reactions by microsomal cytochrome P450 is postulated to be the predominant mechanism which generates superoxide radicals (Inoue, 1994), and its cleavage of hydroperoxides also generates ROS such as singlet oxygen (Cadenas and Sies, 1982). Since the independent newborn must also deal with foreign matter, the release of reactive oxygen species such as singlet oxygen from stimulated macrophages (Steinbeck et al., 1993), would also contribute to the degree of oxidative stress. Higher levels of oxidative stress would have such consequences as nucleotide damage leading to mutagenesis.

Increasing evidence suggests that singlet oxygen is the major ROS contributing to the formation of 8-oxo-dGTP, which is the substrate for the *E. coli* MutT antimutator protein and its mammalian homologues. Abundant FGF-2 antisense expression by hepatocytes may therefore be related to the degree of polyploidy, since these cells are predominantly mononuclear tetraploid. This is further supported by our observation that mouse Hepa1 cells, which display an average of 69 chromosomes (heteroploid) (Darlington et al., 1980), express lower levels of FGF-2 antisense compared to normal hepatocytes. The function of FGF-2 antisense in the liver tissue may be related to a role in protecting the increased cellular content of genetic material. The increasing degree of polyploidy coupled with rising oxidative stress in the neonate hepatocyte may therefore underlie the abundance of FGF-2 antisense expression at this time. Consistent with the association of FGF-2 antisense and degree of polyploidy, is the predominantly nuclear localization of antisense protein observed in the subcellular extracts of rat liver tissue (Li et al., unpublished data). A protective role for FGF-2 antisense in "sanitizing" the nucleotide pool during oxidative stress, is further suggested by its conserved MutT domain and proposed antimutator activity.

A discrepancy was observed between levels of abundance of the 5' and 3' end-specific FGF-2 antisense PCR products. This may be due to species-specific differences in the 5' end sequence since these oligonucleotides were derived from rat sequence. However, the dissimilar patterns of expression observed between the 5' and 3' end-derived PCR products raises the possibility of alternative splicing. Consistent with this possibility is the observation of two different antisense transcript species (2.3 and 2.6 kb) detected by northern analysis in chicken embryos (Borja et

al., 1993). The different results obtained using the 5' end-derived primers may be also due to the presence of secondary structure, which may inhibit the annealing of these primers in the 5' end region of the transcript.

Primary cultures of isolated hepatocytes are frequently used as a means to study their differentiation. After plating, hepatocytes begin to undergo a process of dedifferentiation in which these cells revert to the fetal phenotype (Rauckman and Padilla, 1987). This is characterized by decreased expression of adult enzymes such as glutathione S-transferase, and increased production of α1-fetoprotein, fetal-specific pyruvate kinase isozymes, and increased DNA synthesis. These changes conceivably reflect the transition toward a proliferative state. The coincident upregulation of FGF-2 antisense mRNA and protein over the same period, despite decreasing total RNA and protein level, suggests a function possibly related to the nuclear events which occur during the adult-to-fetal hepatocyte transition.

We have also shown that the FGF-2 sense mRNA is expressed in whole liver and the nonparenchymal cell fraction, but at lower levels compared to FGF-2 antisense mRNA. Previously, FGF-2 sense mRNA was observed to be undetectable in neonate and adult rat liver by northern and RT-PCR analysis (Li et al., 1996a; Knee et al., 1997). In the present study, PCR samples contained 5-fold more cDNA, which gave increased sensitivity and permitted FGF-2 mRNA detection in the adult liver tissue. FGF-2 mRNA expression in Kuppfer cells has not previously been reported. However, although the nonparenchymal cell fraction contained predominantly Kuppfer cells, we cannot rule out the possibility that FGF-2 mRNA detection in this fraction may have resulted from the presence of contaminating fibroblasts and epithelial cells.

# CHAPTER 8: EXPRESSION OF FGF-2 ANTISENSE RNA AND PROTEIN IN NORMAL AND CANCEROUS HUMAN TISSUES

## I. Introduction

In a further effort to establish the functional role of FGF-2 antisense, we performed an expression analysis of its mRNA and protein in human cancer cell lines. Levels of FGF-2 sense mRNA were also determined. The comparison between levels of sense and antisense RNA in normal and tumorous tissue may expose patterns of expression indicative of a regulatory relationship between these two RNAs, similar to that which has been suggested to occur in *Xenopus oocytes* (Kimelman and Kirschner, 1989).

The importance of FGF-2 in tumor biology is supported by numerous studies which have demonstrated abundant FGF-2 mRNA and protein in different neoplastic cells (Halaban et al., 1988; Murphy et al., 1989; Takahashi et al., 1991), the reversal of transformed phenotypes by antisense oligonucleotides against FGF-2 mRNA (Murphy et al., 1992), and FGF-2 stimulation of angiogenesis *in vivo* (Stan et al., 1995). The potent mitogenicity of FGF-2 and its ubiquitous expression in endodermal-derived cells suggests an importance in the aberrant growth of such cells. Several lung carcinoma cell lines have been shown to express moderate steady-state levels of FGF-2 mRNA (Goldsmith et al., 1991). Downregulation of the steady-state levels of FGF-2 mRNA and protein in colon carcinoma is induced by interferon $\alpha$ or $\beta$, which are known to stimulate the regression of highly vacularized tumors (Singh et al., 1995). Better understanding of the role of FGF-2 in tumor growth is being achieved through the ongoing elucidation of control mechanisms which underlie its regulation. Recently, a novel mechanism in the regulation of

FGF-2 gene expression has been identified whereby its mRNA stability is believed to be controlled by heteroduplex formation with a naturally occurring antisense mRNA transcribed from the same gene.

Antisense RNA expression from the FGF-2 gene locus has been reported in several species including *Xenopus*, human and rat (Kimelman and Kirschner, 1989; Murphy and Knee, 1994). In *Xenopus*, sense and antisense RNAs share a 900 bp region of complementary overlap at their 3' ends (Volk et al., 1989). Double-stranded RNA (dsRNA) hybrids formed between FGF-2 sense and antisense transcripts are believed to be modified by dsRNA-specific adenosine deaminase (DRADA) during oocyte maturation (Kimelman and Kirschner, 1989). We have recently cloned and characterized the rat full-length FGF-2 antisense mRNA (Knee et al., 1997) which, like in *Xenopus*, has complementary overlap in its 3' end with that of the FGF-2 sense mRNA.

Examination of FGF-2 antisense mRNA in human found these transcripts to be of very low abundance in human U87-MG glioma cells (Murphy and Knee, 1994), which express high levels of FGF-2 sense RNA due to a high stability (Murphy et al., 1990). Further, human T47D breast tumor cells, which do not produce detectable amounts of FGF-2 sense mRNA, have abundant levels of FGF-2 antisense mRNA. We therefore proposed that a disruption of the relative expression of FGF-2 sense and antisense transcripts may lead to the overexpression of FGF-2 protein in certain tumors (Murphy and Knee, 1994).

In addition to its putative regulatory role, the FGF-2 antisense mRNA encodes a 35 kDa protein with a conserved MutT catalytic domain found in the MutT/nudix hydrolase family of nucleotide pyrophosphohydrolases (Knee et al., 1997). Other members of this family

have been found to play a role in preventing spontaneous mutations due to oxidative DNA damage. The rat FGF-2 antisense protein has been shown to be translated *in vivo* in a tissue-specific manner (Li et al., 1996b; Li et al., unpublished) and transfection of MutT-deficient *E. coli* with rat FGF-2 antisense cDNA constructs shows that this protein also exhibits antimutator activity. Further, in liver the antisense protein displays a nuclear localization, providing further evidence of its putative role in maintaining the fidelity of DNA replication.

The expression of FGF-2 antisense mRNA and protein in human tumor cells has not been previously examined. We have employed semiquantitative RT-PCR for determination of FGF-2 sense and antisense mRNA levels in different human cancer cell lines. The patterns of FGF-2 sense and antisense mRNA expression provide additional insight to the potential role of the antisense mRNA in the posttranscriptional regulation of FGF-2 expression. Western analysis was performed for examination of the translational activity associated with FGF-2 antisense mRNA. In this study we show that the FGF-2 sense/antisense mRNA pair are differentially expressed in human tumor cells derived from brain, lung, and colon.

## II. Results

### i. Semiquantitative RT-PCR Analysis

Examination of FGF-2 sense and antisense mRNA transcripts in the selected human tumor cell lines reveals a differential pattern of expression of these two mRNA species. With the exception of HT-29 colon carcinoma cells, FGF-2 sense RNA-derived PCR products were detected in all cell lines and most abundantly in the NSCLC cell lines (Figure 25). Previous northern detection of FGF-2 sense mRNA in U87-MG glioma cells is consistent with the present RT-PCR data. The antisense PCR product was detected at comparable levels in the all tumor cell lines, with the exception of U87-MG glioma cells which displayed a significantly lower level of antisense mRNA expression (Figure 25). The U87-MG cells are characterized by a high ratio of sense to antisense (>5 : 1), NSCLC cell lines had ratios corresponding to approximately equal levels of both sense and antisense mRNA (A427, 0.6 : 1 and A549, 1.6 : 1), while colon carcinoma cells lines showed a very low ratio of sense to antisense mRNA (HT-29, <0.1 : 1 and T84, 0.2 : 1). The disruption of FGF-2 sense and antisense mRNA ratios may have implications on the antisense mRNA mechanism to control FGF-2 mRNA stability in certain tumors (see discussion).

### ii. Western Analysis

Antisera against the mutT domain of the FGF-2 antisense protein detects the presence of a 35 kDa immunoreactive band in all tumor cell lines examined and not in normal human tissues (Figure 26A). The relative abundance of FGF-2 antisense protein in the human tumor cell lines is shown in Figure 26B. Comparison of antisense protein levels with

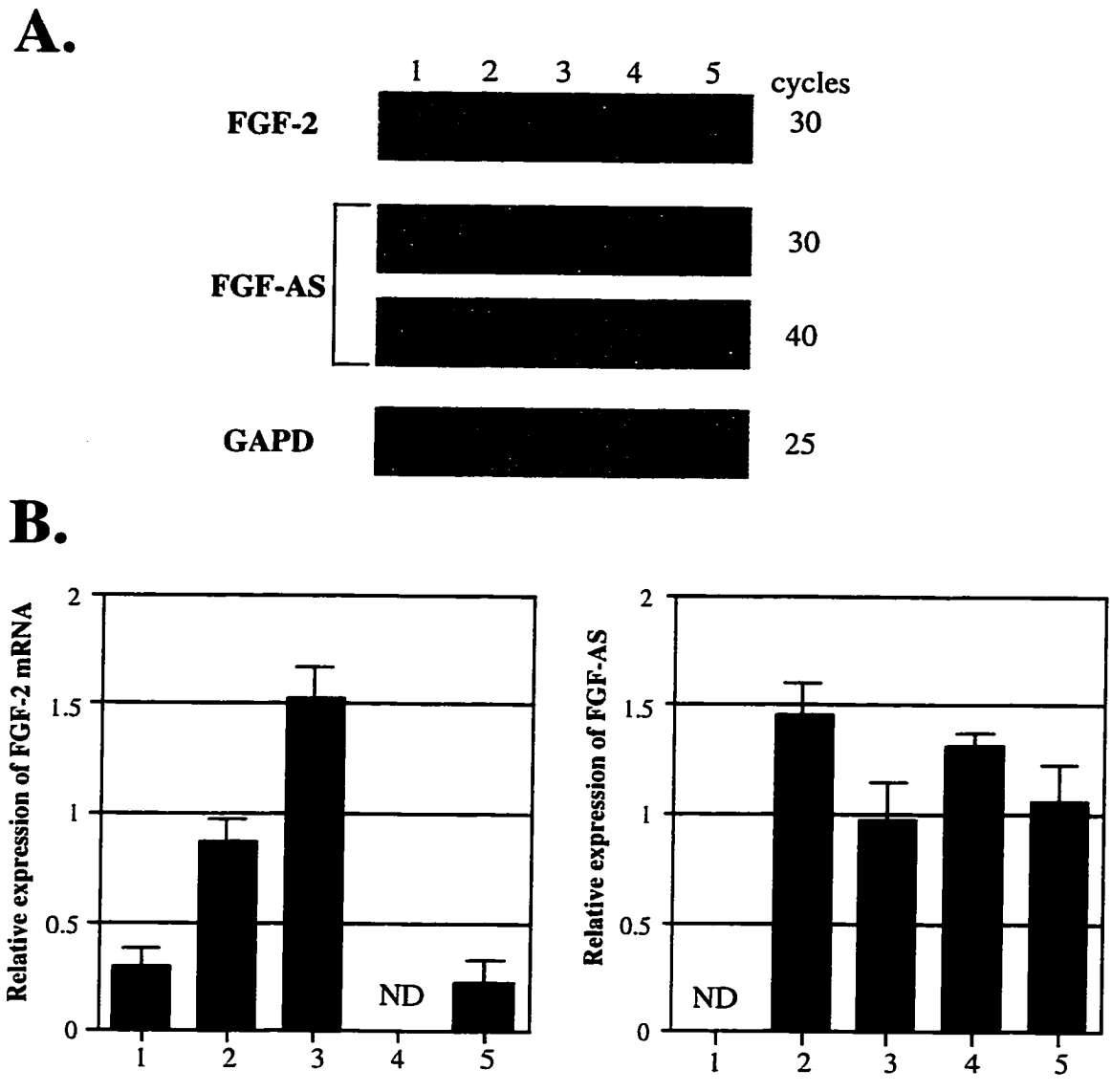
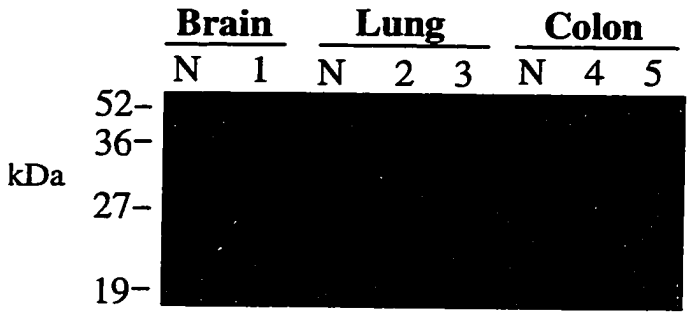



**Figure 25.** RT-PCR analysis of FGF-2 sense and antisense mRNA in human tumor cell lines. **(A)** Agarose gel electrophoresis of RT-PCR samples corresponding to FGF-2 (sense; top panel), FGF-AS (antisense; center panels), and GAPD (glyceraldehyde-3-phosphate dehydrogenase; bottom panel) for cell lines; lane 1, glioma (U87-MG); lane 2 and 3, NSCLC (A427, A549); lane 4 and 5, colon carcinoma (HT-29 and T84). Cycles numbers for PCR amplification of each product are indicated on the left. ND, not detected. **(B)** Relative levels of FGF-2 sense and antisense mRNA in the human tumor cell lines. Agarose gels in (A) were subjected to densitometric analysis and values for FGF-2 sense and antisense products (both at 30 cycles) were normalized with GAPD values to correct for differences in input RNA. Experiments were perfomed in triplicate.

**A.**




**B.**

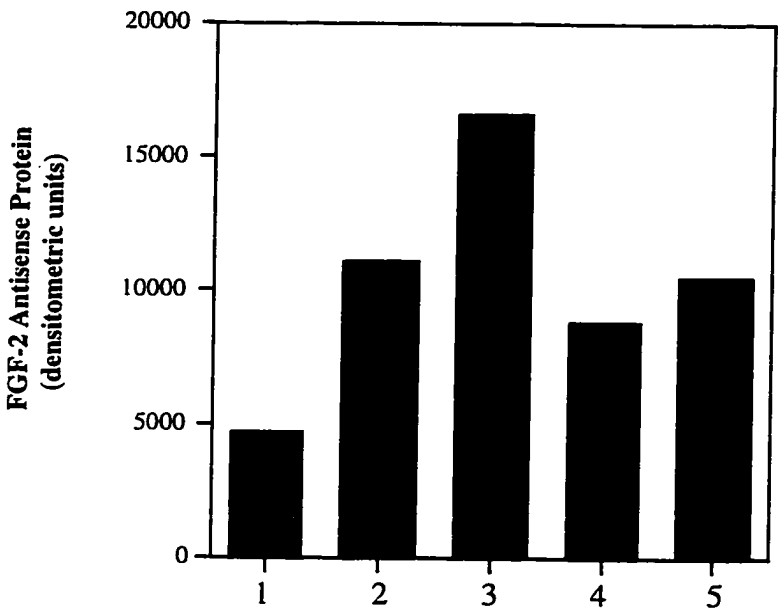



**Figure 26.** Western analysis of FGF-2 antisense protein in human tumor cell lines. **(A)** Immunodetection of FGF-2 antisense protein in cell lysates (50 μg) of the respective normal human tissues (N); and U87-MG, A427, A549, HT-29, and T84 cell lines, corresponding to lanes 1-5, respectively. **(B)** Densitometric scan analysis of the levels of FGF-2 antisense protein in the same human tumor cell lines (1-5).

that of its mRNA as determined by RT-PCR reveals a complementary pattern. Whereas lung- and colon-derived cell lines show readily detectable levels of antisense protein, the U87-MG gliomas cells produce significantly less.

**III. Discussion**

In the present study we show that FGF-2 antisense protein is produced by tumor cell lines derived from different tissues and provide the first report of expression of this protein in human cells. The FGF-2 antisense protein is a novel member of the MutT-like family of NTPases, which includes *E. coli* MutT (8-oxo-dGTPase) and the mammalian homologues; mouse, rat, and human 8-oxo-dGTPases (MTH1). MutT proteins suppress the occurrence of spontaneous oxidative mutagenesis by hydrolyzing 8-oxo-dGTP (Mo et al., 1992). The mutagenic nucleotide, 8-oxo-dGTP, is generated by oxidation of free dGTP, and can induce A:T to C:G as well as G:C to T:A transversions during DNA replication (Maki and Sekiguchi, 1992). Interestingly, hMTH1 mRNA expression is increased in human renal cell carcinoma (RCC) (Okamoto et al., 1996). Further, the increased expression of hMTH1 is directly proportional to the advancing clinical stages of RCC, and therefore may, in part, adjust for the increased oxidative stress which occurs during RCC progression. Northern analysis of FGF-2 antisense mRNA in various normal human tissues including brain, lung and colon, revealed very low to undetectable levels of this mRNA species (Knee et al., 1994), consistent with the lack of detectable levels of FGF-2 antisense protein shown here by western analysis of those same tissues. Although the precise function of the FGF-2 antisense protein is unknown, its expression in all tumor cell lines and absence in

normal tissue, suggests a role in the transformed cell phenotype possibly related to the differentiation status and/or continuous proliferation of these cells.

The importance of FGF-2 in the development and progression of tumor growth is supported by numerous studies which have examined its angiogeneic and mitogenic effects in various cell models. Although two separate groups were unable to demonstrate FGF-2 mRNA expression in either primary or cell line colon carcinomas using northern (Sternfield et al., 1988) and *in situ* hybridization (Li et al., 1994), the FGF-2 protein has been detected in some colon carcinoma cell lines. Immunoblotting of cell lysates from GEO, CBS, FET, MOSER, and HCT 116 colon carcinoma cell lines detects both immunoreactive FGF-2 and FGF receptor in these cell lines (New and Yeoman, 1992), suggesting that FGF-2 may function in an autocrine manner to stimulate the proliferation of these cells. This is further supported by the observation that GEO and LIM 1215 carcinoma cells exhibit proliferative responses in a dose-dependent manner to the exogenous addition of FGF-2 (New and Yeoman, 1992; Whitehead et al., 1990). In contrast, Dignass et al. (1994) found that FGF-2 stimulated only a modest increase in proliferation in IEC-6, Caco-2, and HT-29 colon carcinoma cells.

A unique regulatory mechanism of eukaryotic gene expression is believed to be associated with the posttranscriptional control of FGF-2, whereby its mRNA stability is modulated by dsRNA hybrid formation with an endogenous antisense mRNA transcript (Kimelman and Kirschner, 1989). Previous northern analysis (Murphy and Knee, 1994) is supported by our present RT-PCR data which reveal that FGF-2 antisense expression is very low in human U87-MG glioma cells which express an

unusually stable FGF-2 mRNA. The putative function of the antisense transcript in the regulation of FGF-2 sense mRNA stability is supported by other expression studies which have identified a reciprocal relationship between the levels of FGF-2 sense and antisense mRNAs in specific tissues. Northern analysis and RT-PCR demonstrate that the level of FGF-2 mRNA in rat brain is inversely related to the level of FGF-2 antisense mRNA in that tissue (Li et al., 1996a; Knee et al., 1997). RNase protection analysis also shows a high ratio of FGF-2 sense to antisense mRNA in the developing rat CNS (Grothe and Meisinger, 1995). Expression patterns of FGF-2 sense and antisense mRNA in adult chicken organs, also lends support to the putative regulatory function of the antisense transcript. Borja et al., (1993) consistently found a high ratio of antisense to sense mRNA in tissues such as liver, kidney, and heart. Our present demonstration of the inverse levels of FGF-2 sense and antisense mRNAs in both HT-29 and T84 cells, suggest that the antisense mRNA regulation of FGF-2 sense mRNA stability in particular colon carcinomas can not be excluded.

## CHAPTER 9: SUMMARY

FGF-2 antisense was first identified in *Xenopus laevis* as a 1.5 kb mRNA transcribed in the opposite direction from the 3' end of the FGF-2 gene locus (Kimelman and Kirschner, 1989). In immature oocytes, the antisense transcript has been suggested to form dsRNA complexes with the sense transcript as a posttranscriptional regulatory mechanism for targeting the degradation of FGF-2 mRNA. In addition, the antisense RNA contains an ORF which encodes a hypothetical 24 kDa protein of unknown function. The goal of this thesis was to characterize the mammalian FGF-2 antisense mRNA with the intention to gain understanding of its translational potential and putative RNA regulation of FGF-2 expression.

In most normal tissues FGF-2 mRNA levels are generally below the limits of Northern detection yet readily detectable in various tumor-derived cell lines. The human U87-MG malignant glioblastoma cell line is characterized by a high level of FGF-2 mRNA that correlates with high concentrations of FGF-2 (Sato et al., 1989). The elevated levels of FGF-2 mRNA in U87-MG glioma were demonstrated to be attributable to an increased stability of the transcripts in these cells (Murphy et al., 1990), however, the mechanism underlying this stability has not been elucidated. The observation that an endogenous antisense RNA may regulate FGF-2 mRNA stability in *Xenopus* oocytes (Kimelman and Kirschner, 1989) prompted us to select the U87-MG glioma cell line as a model to investigate the mammalian antisense mRNA and its putative role in the regulation of FGF-2 gene expression.

By performing a sequence comparison between the human FGF-2 sense and *Xenopus* antisense RNA we identified the presence of two strongly

conserved regions from which PCR oligonucleotides were derived. RT-PCR amplification of U87-MG glioma RNA generated an antisense cDNA fragment of the predicted size whose identity was then confirmed by sequence analysis. The detection of this fragment revealed that the mammalian FGF-2 antisense mRNA is expressed and structurally homologous to the *Xenopus* transcript.

The PCR-derived clone was then used for cDNA library screening, however, our initial efforts to isolate a full-length clone from the human brain and liver cDNA libraries were unsuccessful. It was later determined by Northern analysis of rat tissues that FGF-2 antisense mRNA exhibits a tissue-dependent and developmentally regulated pattern of expression. This informed us that the selected cDNA libraries did not contain levels of antisense favorable for screening.

Our construction of a cDNA library from neonatal liver, however, finally enabled us to isolate the full-length cDNA. Sequence analysis revealed that the rat FGF-2 antisense RNA is a 1.1 kb polyadenylated transcript and contains a conserved ORF which specifies a 35 kDa protein. The nature of overlap between the sense and antisense transcripts is such that the 3' UTR of the sense RNA is complementary to exon III and IV and the 3' UTR of the antisense RNA.

The restriction of the region of overlap to the 3' UTR of FGF-2 mRNA is suggestive of regulatory implications with respect to the function of heteroduplexes formed between sense and antisense transcripts. Multiple FGF-2 transcripts arise by differential length of the 3' UTR, yet the significance of this is not completely understood. In eukaryotic mRNA, the 3' UTR contains all of the signals required for mRNA localization, most of those required for the control of polyadenylation, and many of the

signals which regulate mRNA stability (Jackson, 1993). In addition, the 3' UTR of some eukaryotic mRNAs contain controlling signals for translation initiation, particularly in those mRNAs found in germline cells (Jackson, 1993).

The patterns of sense and antisense expression in developing rat brain are such that the largest FGF-2 transcript (6 kb) is increasingly expressed after birth, while at this time there is a corresponding decline in the expression of FGF-2 antisense mRNA (Li et al., 1996a). We have found that the complementary overlap between FGF-2 sense and antisense includes two widely separated regions, near the proximal and distal polyadenylation signal in the 3' UTR. Taken together these observations suggest that the function of antisense RNA may involve hybrid formation for temporal selection of the FGF-2 RNA polyadenylation site. Since the 6 kb FGF-2 transcript corresponds to the presence of FGF-2 bioactivity (Powell et al., 1991), the formation of heteroduplexes with antisense transcripts may target the degradation of this transcript as a mechanism to suppress the synthesis of FGF-2 protein during neonatal development.

Interestingly, the antisense transcript was abundantly expressed by adult rat tissues which displayed low levels of FGF-2 sense transcripts, as well as in human T47D breast tumor cells (Murphy and Knee, 1994), which express undetectable levels of FGF-2 (Li and Shipley, 1991). In contrast, the expression of the antisense transcript appears to be lowest in human U87-MG glioma cells which express an unusually stable transcript. The inverse levels of sense and antisense mRNA levels is suggestive of a negative regulation of sense mRNA stability. Further to this role, abundant FGF-2 protein in chicken tissues such as limb bud ectoderm, undifferentiated mesenchyme, and the mesonephros, has been noted

specifically in the absence of the antisense counterpart (Borja et al., 1993). The inverse levels of expression between other sense and antisense partners such as the Hoxa11 and PCNA/YC transcriptional units (Hsieh-Li et al., 1995), suggests that the targeting of sense RNA stability may be a common mechanism of antisense-mediated regulation.

DsRNA complexes formed between FGF-2 sense and antisense transcripts may also be targeted for covalent modification by mammalian RNA-specific adenosine deaminases. This family includes the dsRNA-specific adenosine deaminase (DRADA), and the type 1 and 2 dsRNA-specific editase (RED1 and RED2) (Melcher et al., 1996). DRADA has been shown to edit the R/G site of glutamate receptor subunit B (GluR-B) pre-mRNA, whereas RED1 edits both the GluR-B Q/R and R/G sites. As well as their differences in substrate specificity, the demonstration that RED2 is exclusively expressed in rat brain suggest that members of this family also exhibit a tissue-specific expression. It is therefore conceivable that the developmental appearance of FGF-2 sense or antisense mRNA may permit the formation of duplexes on a tissue-specific basis. Subsequent modification by adenosine deaminase may alter FGF-2 or its antisense gene-encoded protein by selective changes in their mRNA. It has also been reported that deaminated transcripts are as stable as nonmodified transcripts (Bass, 1992), therefore it may be unlikely that destabilization of heteroduplexes by DRADA modification promotes the degradation of sense: antisense hybrids.

Alternatively, the relationship between FGF-2 sense and antisense RNAs may be similar to the CYP/TN-X sense/antisense pair which, despite the presence of nearly 300 nt of complementary sequence at their 3' ends, do not form dsRNA complexes *in vivo* (Speek and Miller, 1995). In

this case, the presence of sequence-specific nuclear proteins prevent annealing of the complementary strands. It has also been shown that in general, the extent of RNA duplex formation is dependent on the concentration of heterogeneous ribonucleoprotein (hnRNP) A1 (Portman and Dreyfuss, 1994). As such, lower levels of hnRNP would result in discontiguous basepairing within RNA duplex thereby increasing its sensitivity to RNase degradation (Speek and Miller, 1995). Given the variety of proteins which promote or obstruct RNA annealing, it is possible that hybridization of FGF-2 sense and antisense RNAs may be regulated in a tissue-specific manner by nuclear and/or cytoplasmic RNA binding proteins. As well, the presence of secondary structure may also dictate the interaction between sense and antisense transcripts.

Considering that the level of FGF-2 antisense mRNA and protein are low or undetectable in the brain tissue of both human and rat, our observation of low levels of FGF-2 antisense RNA and protein in U87-MG glioma cells may therefore, represent a tissue-specific expression of the antisense mRNA, instead of an abnormally low level postulated to result in the overexpression of FGF-2 mRNA. However, the *in vivo* function of FGF-2 sense and antisense RNA interaction in terms of its regulatory function, and whether such interaction involves pre-mRNA or mRNA, and strand-specific or mutual regulation, has yet to be determined. Heteroduplex formation between the FGF-2 sense and antisense transcripts could be directly assessed by a double RNase protection assay. The demonstration of dsRNA formation would be a discerning prerequisite for the regulatory capacity of FGF-2 antisense mRNA.

Antisense transcription has been also observed in another member of the FGF family. The FGF-6 antisense transcript was found specifically

expressed in the testes of sexually mature mice, but not in prepubertal mice (Coulier et al., 1991). The conserved genomic organization of the FGF family supports the possibility that other members may also have antisense counterparts. Given the spatial and temporal expression of FGF in development, formation of heteroduplexes with antisense RNA may become a prominent component in their regulation.

Database searches indicate that the predicted amino acid sequence of the rat FGF-2 antisense cDNA contains a conserved domain found in the MutT/nudix family of nucleotide pyrophosphohydrolases. This family includes the prototypic member *E. coli* MutT, and its mammalian homologues, rat and human 8-oxo-dGTPases (MTH). *E. coli* MutT and the MTHs are involved in suppressing the occurrence of spontaneous oxidative mutagenesis by hydrolyzing the mutagenic DNA substrate, 8-oxo-dGTP. *In vitro* translation of the rat antisense clone detects the appropriately sized protein product and Western analysis reveals that the antisense protein is produced *in vivo*. We have recently found that transfection of the rat FGF-2 antisense cDNA can partially complement MutT-deficient *E. coli* (Li et al, unpublished observations), indicating that the FGF-2 antisense MutT domain is functionally conserved.

Although we have established that the FGF-2 antisense protein belongs to MutT/nudix family and is expressed in mammal, we have yet to determine the exact function of this protein. Considering the broad substrate specificity of this family, it is difficult to effectively describe the function of the antisense protein without additional studies. Nonetheless, our studies have made considerable progress in the characterization of the FGF-2 antisense mRNA and protein, not the least of which is the analysis of its expression pattern.

We have observed an association of FGF-2 antisense mRNA and protein expression with those tissues that exhibit high rates of oxidative metabolism, including liver, kidney, adrenals, and testis. This is most obvious in liver which displays a dramatic rise in the level of FGF-2 antisense mRNA during the neonatal stage. The onset of FGF-2 antisense mRNA expression in the early postnatal liver suggests an importance for its translational product during this period. The neonatal liver undergoes several dramatic cellular and functional changes during this same period including a rise in the degree of polyploidy in parenchymal cells and increased activity of neonatal-specific metabolic enzymes (Greengard, 1971; Sasse, 1986). If the antisense protein is involved in the fidelity of chromosomal DNA, the oxidative stress from increased metabolism may require additional antisense protein to protect the accumulating levels of genetic material from the threat of endogenous oxygen radicals. Similarly, high expression of the antisense protein in testis may be related to the presence of rapidly dividing germ cells in this tissue. The accelerated rates of DNA replication, together with presence of added reactive oxygen species arising from the increased oxygen consumption, would require a higher FGF-2 antisense expression in these cells in order to maintain the fidelity of the genome. Interestingly, testis also expresses high levels of two other DNA repair genes involved in excision (Weeda et al., 1991) and glycosylation (Engelward et al., 1993). Expression of the rat 8-oxo-dGTPase (rMTH1) also shows a predominance in testis, as well as heart, kidney and spleen (Cai et al., 1995). Consistent with the possible relationship between antisense protein and the oxidative metabolism of rapidly proliferating cells, we have found abundant levels of FGF-2 antisense protein in several

human cancer cell lines and an absence of this protein in the corresponding normal tissues.

Given the tissue-specific expression and putative role of antisense protein in protecting DNA from endogenous oxidative radicals it is conceivable that FGF-2 and its antisense protein may play complementary roles dependent on the cell's proliferative status. FGF-2 is observed predominantly during embryonic organogenesis, neural patterning and growth, but is absent in the early postnatal stage in most tissues. Therefore, FGF-2 is predominant during differentiation in the embryo, whereas antisense is predominant during the onset of independent tissue functioning at birth. Further to this relationship, the activation of signal transduction pathways by dsRNA formation between the sense and antisense transcripts may serve as a feedback mechanism to control the expression of their respective proteins. DsRNA complexes have been shown to induce dsRNA-dependent protein kinases (DDPK) such as p68 which activates, among several factors, the eIF2 initiation factor (Nellen and Lichtenstein, 1993). Wei et al., (1997) have shown that the expression of several mismatch repair (MMR) genes is decreased in a variety of glioblastomas. By analogy, the low level of antisense mRNA in U87-MG may be associated with pathological changes inherent to glioma cells.

Although we have not yet fully examined the effect of FGF-2 on the expression of antisense protein, the demonstration that a mitogen and DNA repair protein are translated from same genetic locus poses an intriguing relationship. FGF-1 has been found to stimulate the cell-cycle dependent expression of G/T mismatch-binding protein (GTBP) mRNA in NIH 3T3 cells (Donohue et al., 1996). The demonstrated coexpression of FGF-2 and its antisense protein suggests a relationship whereby FGF-2,

which functions primarily in the stimulation of gene expression, is complemented by its antisense protein whose putative function may involve protecting the fidelity of the genome. The relationship between the FGF-2 sense and antisense genes may therefore be similar to the c-erbA/Rev-erbA sense and antisense genes, in which the antisense RNA regulates the post-transcriptional expression of its counterpart sense gene, and also encodes a functionally related protein. A possible interpretation of the tissue-specific expression of FGF-2 antisense mRNA, may be that in certain tissues the antisense transcript mediates the posttranscription regulation of the FGF-2 sense transcript, while in others it provides a template to encode its translational product.

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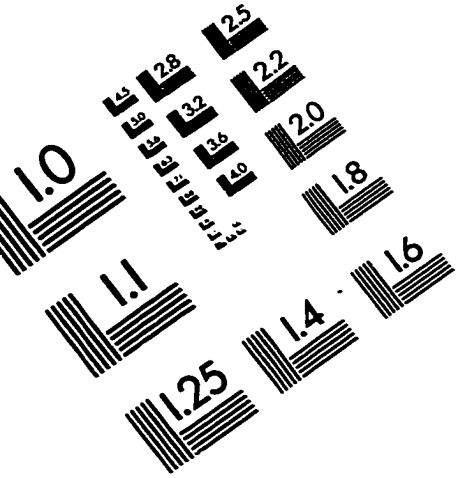
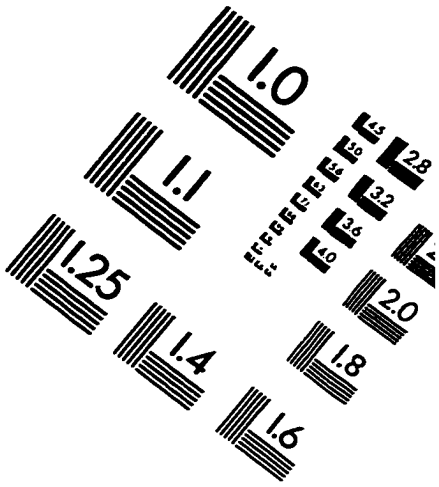
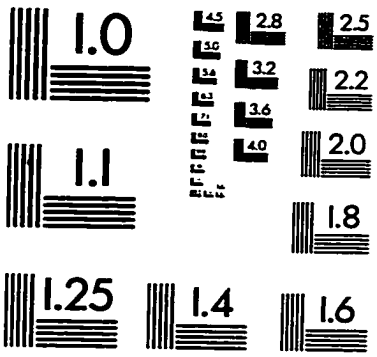
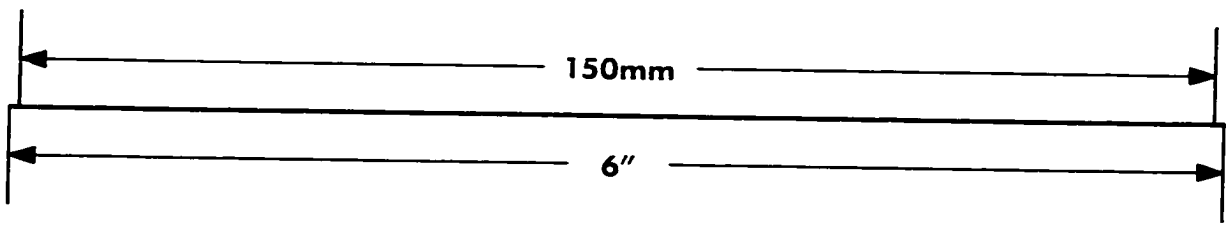
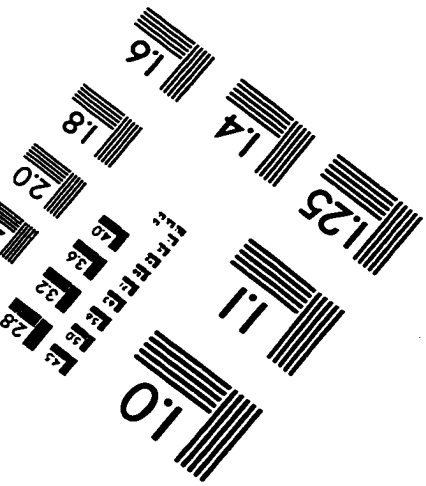
# IMAGE EVALUATION TEST TARGET (QA-3)

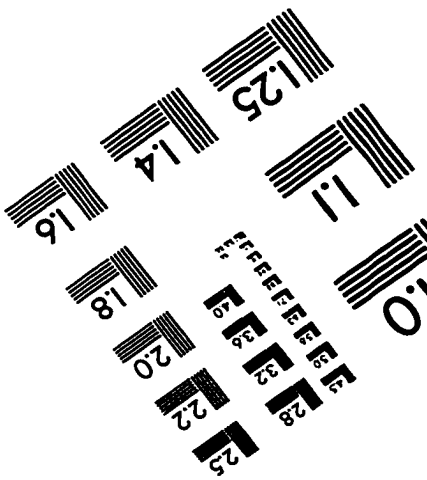
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