The Fn14 Immediate-Early Response Gene Is Induced During Liver Regeneration and Highly Expressed in Both Human and Murine Hepatocellular Carcinomas

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Polypeptide growth factors stimulate mammalian cell proliferation by binding to specific cell surface receptors. This interaction triggers numerous biochemical responses including the activation of protein phosphorylation cascades and the enhanced expression of specific genes. We have identified several fibroblast growth factor (FGF)-inducible genes in murine NIH 3T3 cells and recently reported that one of them, the FGF-inducible 14 (Fn14) immediate-early response gene, is predicted to encode a novel, cell surface-localized type Ia transmembrane protein. Here, we report that the human Fn14 homolog is located on chromosome 16p13.3 and encodes a 129-amino acid protein with ~82% sequence identity to the murine protein. The human Fn14 gene, like the murine Fn14 gene, is expressed at elevated levels after FGF, calf serum or phorbol ester treatment of fibroblasts *in vitro* and is expressed at relatively high levels in heart and kidney *in vivo*. We also report that the human Fn14 gene is expressed at relatively low levels in normal liver tissue but at high levels in liver cancer cell lines and in hepatocellular carcinoma specimens. Furthermore, the murine Fn14 gene is rapidly induced during liver regeneration *in vivo* and is expressed at high levels in the hepatocellular carcinoma nodules that develop in the *c-myc*/transforming growth factor- α -driven and the hepatitis B virus X protein-driven transgenic mouse models of hepatocarcinogenesis. These results indicate that Fn14 may play a role in hepatocyte growth control and liver neoplasia. (*Am J Pathol* 2000, 156:1253–1261)

Polypeptide mitogens such as fibroblast growth factor (FGF)-1 and platelet-derived growth factor-BB stimulate cell cycle progression by binding to specific receptor tyrosine kinases and thereby activating intracellular signal transduction pathways.¹ The activation of cytoplasmic signaling molecules promotes changes in gene expression that are critical for the cellular growth response. Numerous growth factor- and/or serum-inducible genes have been identified and classified into one of three groups: immediate-early, delayed-early, or late response genes.² Immediate-early response genes are rapidly and transiently expressed following mitogenic stimulation of quiescent cells and their transcriptional activation does not require *de novo* protein synthesis. Delayed-early response genes are first expressed a few hours later, in the early to middle portions of the G1 phase, and transcript levels often remain elevated for the remainder of the cell cycle. Late response genes are generally expressed only during the S phase of the cell cycle. Both delayed-early and late response genes require *de novo* protein synthesis for their transcriptional activation. Growth factor-inducible genes encode many types of proteins, including transcription factors, cell cycle regulators, extracellular matrix proteins and metabolic enzymes.^{2–4}

Several years ago our laboratory used a differential display approach to isolate cDNA fragments representing FGF-1-inducible genes in murine NIH 3T3 fibroblasts.^{5,6} One of the immediate-early response genes presently under investigation, the FGF-inducible 14 (Fn14) gene, is located on mouse chromosome 17 and is predicted to encode a 129-amino acid (aa) type Ia transmembrane protein with no significant sequence similarity to any known protein.⁷ Furthermore, we have shown that Fn14 is localized on the plasma membrane and that constitutive Fn14 expression in transfected NIH 3T3 fibroblasts decreases cellular adhesion to extracellular matrix proteins and inhibits growth and migration *in vitro*.⁷

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In this paper, we report that the human Fn14 gene encodes a protein with ~82% amino acid sequence identity to the murine Fn14 protein. This gene is located on chromosome 16 and, like its murine homolog, it is activated following growth factor, serum or phorbol ester treatment of quiescent fibroblasts. Additionally, we show that the human Fn14 gene is expressed at relatively high levels in hepatocellular carcinoma (HCC) specimens. We also report that the Fn14 gene is rapidly induced during liver regeneration in the mouse and activated in two different transgenic mouse models of hepatocarcinogenesis.

Materials and Methods

Cell Culture

Murine NIH 3T3 fibroblasts were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown as described.⁷ Human M426 embryonic lung fibroblasts (kind gift of Dr. J. Rubin, National Institutes of Health) were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mmol/L glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (Mediatech). They were incubated in DMEM/Ham's F-12 medium (50/50 mix) supplemented with 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenious acid (Collaborative Biomedical Products, Bedford, MA) for 48 hours to induce cellular quiescence. The cells were then either left untreated or treated for various time periods with one of the following: 10 ng/ml human recombinant FGF-1 (kind gift of Dr. W. Burgess, Holland Laboratory, Rockville, MD) in combination with 5 units/ml heparin (Upjohn, Kalamazoo, MI), 10% FBS or 30 ng/ml phorbol myristate acetate (PMA; Sigma, St. Louis, MO). The human liver cell lines were obtained from either the ATCC, the Qidong Liver Cancer Institute, or Dr. C. Harris (National Institutes of Health) and grown according to the provider's instructions.

Human Fn14 cDNA Sequence Analysis

Homologous sequences to the murine Fn14 cDNA nucleotide sequence were identified using the National Center for Biotechnology Information BLAST program to search the GenBank human expressed sequence tag (EST) database. Several EST clones with a high degree of sequence identity were found. Two clones were obtained from the IMAGE Consortium through Lawrence Livermore National Laboratory and one of these (GenBank accession no. T57612) was sequenced in its entirety. Sequencing was done either automatically using an Applied Biosystems model 373A DNA sequencer and a Dye Terminator Cycle Sequencing kit (Perkin Elmer, Foster City, CA) or manually using a Sequenase 2.0 kit (U.S. Biochemical) and [α -³⁵S]dATP (1000 Ci/mmol, Amersham, Cleveland, OH). The predicted human Fn14 protein sequence was analyzed using several programs

(SignalP, ScanProsite, PSORT II, TMPred, Piscataway, NJ) accessed through the ExPASy Molecular Biology Server. The nucleotide and deduced amino acid sequence reported in this paper has been deposited in the GenBank database under accession no. AF191148.

RNA Isolation and Northern Blot Hybridization

Total RNA was isolated from NIH 3T3 and M426 cells using RNA Stat-60 (Tel-Test, Friendswood, TX) according to the manufacturer's instructions. Poly(A)⁺ RNA was isolated from human liver cell lines, regenerating mouse liver and liver tissue harvested from *c-myc*/transforming growth factor (TGF)- α double transgenic mice^{8,9} using the guanidium/cesium chloride method and oligo d(T)-cellulose chromatography as described.¹⁰ RNA samples (10 µg of total RNA or 2 µg of poly(A)⁺ RNA) were denatured and subjected to electrophoresis in 1.2% agarose gels containing 2.2 mol/L formaldehyde. The gels were stained with ethidium bromide to verify that each lane contained similar amounts of undegraded rRNA. RNA was transferred onto Zetabind nylon membranes (Cuno Inc, Meriden, CT) by electroblotting and cross-linked to the membrane by UV light irradiation using a Stratalinker (Stratagene, La Jolla, CA). Several Northern blots were purchased from commercial sources. A blot containing 2 µg of poly(A)⁺ RNA isolated from various human tissues was obtained from Clontech, Palo Alto, CA. A blot containing 20 µg of total RNA isolated from several different human tumors including a HCC specimen (catalog no. D3100-01) and a blot containing 20 µg of total RNA isolated from three HCC specimens and one cholangiocellular carcinoma specimen (catalog no. D5080-01) were purchased from Invitrogen (Carlsbad, CA). Membrane prehybridization, hybridization, and washing conditions were as described.⁷ The cDNA hybridization probes were: (a) human Fn14, ~1.0-kb *EcoRI/XhoI* fragment of pBluescript/hFn14; (b) mouse Fn14, ~1.0-kb *EcoRI/XhoI* fragment of pBluescript/mFn14; (c) mouse α -actin, ~1.1-kb *EcoRI* fragment of pVAA (kind gift of Dr. G. Liao, Genetic Therapy Inc.); and (d) rat albumin, ~1.0-kb *PstI* fragment of pRSA13 (kind gift of Dr. T. Sargent, National Institutes of Health). The probes were radiolabeled with [α -³²P]dCTP as described.⁷

Chromosomal Mapping by Fluorescence in Situ Hybridization (FISH)

Normal human metaphase spreads were prepared according to the method of Fan et al.¹¹ Human lymphocytes were cultured for 72 hours at 37°C in RPMI 1640 medium containing phytohemagglutinin and 10% FBS. Cultures were synchronized by treatment with 5-bromodeoxyuridine (0.18 mg/ml, Sigma) for 16 hours, followed by release from the block by incubation in fresh medium containing thymidine (2.5 µg/ml) for 6 hours. Metaphase cells were harvested and chromosome spreads were prepared according to standard procedures. FISH and detection of immunofluorescence were performed essentially as described previously.¹² Briefly, pBluescript/

hFn14 plasmid DNA (1 μ g) was biotinylated in a nick translation reaction containing 10 μ mol/L biotin-16-dUTP (Boehringer Mannheim, Indianapolis, IN) and 2 units DNA polymerase I/DNase I (Gibco BRL, Rockville, MD). Slides were treated with RNase (100 μ g/ml in 2 $\times$ standard saline citrate, SSC) for 1 hour at 37°C, rinsed in 2 $\times$ SSC, dehydrated in a cold ethanol series, and hybridized overnight at 37°C. They were then washed twice in 50% formamide in 2 $\times$ SSC at 43°C for 10 minutes, twice in 2 $\times$ SSC at 37°C for 4 minutes, and once in 4 $\times$ SSC/0.05% Tween 20 at room temperature for 5 minutes. Slides were removed from the buffer, and then the hybridized probe was detected with fluorescein-labeled avidin (Oncor, Gaithersburg, MD). Signals were amplified by adding a layer of anti-avidin antibody (Oncor), followed by a second layer of fluorescein-labeled avidin according to the manufacturer's instructions. The chromosome preparations were stained with diamidino-2-phenylindole (DAPI) and observed using a Zeiss Axiophot fluorescence microscope. Digitized images were captured with a cooled CCD camera connected to a computer work station. Images of DAPI staining and fluorescein signals were merged using Oncor Image software, version 1.6.

Partial Hepatectomy

C57Bl6 $\times$ CBA FI hybrid mice, 7 weeks old, were subjected to a standard 70% partial hepatectomy (PH) as described.¹⁰ They were then sacrificed after various time periods in groups of three, and remnant livers were harvested and pooled for RNA isolation as described above.

In Situ Hybridization

Serial sections of liver tissue from hepatitis B virus X protein (HBx) transgenic mice were the kind gift of Dr. G. Jay (OriGene Technologies). *In situ* hybridization analysis using sense and antisense murine Fn14 riboprobes was performed as described previously.⁷

Results

Human Fn14 cDNA Sequence Analysis

First, we obtained and sequenced a human Fn14 cDNA clone. The BLAST program was used to search the human EST database with the murine Fn14 cDNA nucleotide sequence and several cDNAs were identified. A clone from a human placenta cDNA library was obtained from the IMAGE Consortium and both strands of the ~990-bp insert were sequenced. The DNA sequence, which contained a 29-nucleotide (nt) 5'-untranslated region, a 387-nt open-reading frame, and a 570-nt 3'-untranslated region with a polyadenylation signal and poly(A) tract, had ~80% overall sequence identity to the murine Fn14 cDNA sequence. The open-reading frame encoded a 129-aa protein with a molecular mass of 13,911 daltons and an isoelectric point of 9.37. This protein, like its murine homolog,⁷ is predicted to contain a 27-aa signal peptide sequence, a 53-aa extracellular

Figure 1. Comparison of the mouse and human Fn14 deduced amino acid sequences. Identical residues are boxed and the numbers to the right refer to the last amino acids on the lines. The **solid line** indicates the predicted signal peptide sequence and the **dotted line** indicates the predicted transmembrane domain.

domain, a 21-aa membrane-anchoring domain, and a 28-aa cytoplasmic domain (Figure 1). Human Fn14 has ~82% amino acid sequence identity to murine Fn14 if the signal peptide sequences are included in the analysis. The mature 102-aa human and murine Fn14 proteins have ~90% amino acid sequence identity.

Chromosomal Location of the Fn14 Gene

We next determined the chromosomal position of the human Fn14 gene. The Fn14 locus was mapped to chromosome 16p13.3 by FISH. In an analysis of 23 metaphase spreads, 34% (49/145) of all fluorescence signals hybridized to chromosome 16p. All chromosome-specific signals were localized to 16p13.3 (Figure 2). At least one signal specific for chromosome 16 was observed in 21 of the 23 metaphase spreads examined. The distribution of chromosome 16 signals was as follows: one chromatid (1 cell), two chromatids (14 cells), three chromatids (4 cells), four chromatids (2 cells).

Regulation of Fn14 mRNA Expression in M426 Cells

The murine Fn14 gene is a growth factor-inducible immediate-early response gene in fibroblasts.⁷ We investigated whether the human Fn14 gene was regulated in a similar manner using human M426 lung fibroblasts. First, we performed Northern blot hybridization analysis using RNA isolated from M426 cells and murine NIH 3T3 fibroblasts and found that the human and murine Fn14 genes each encoded a single ~1.2-kb transcript (Figure 3A). Then, we assessed Fn14 mRNA expression levels after the addition of FGF-1, FBS or PMA to serum-starved M426 cells. We found that each of these agents could increase Fn14 gene expression with maximal Fn14 mRNA levels present after either 4 hours (FGF-1 treatment) or 8 hours (FBS or PMA treatment) of cellular stimulation (Figure 3, B–D).

Fn14 mRNA Expression in Human Tissues

The tissue distribution of Fn14 mRNA was evaluated by Northern blot hybridization analysis using RNA isolated from eight different human tissues. Fn14 mRNA was expressed at the highest level in heart, placenta, and kidney and at an intermediate level in lung, skeletal muscle, and pancreas (Figure 4). Fn14 mRNA expression was

Figure 2. Chromosomal localization of the human Fn14 gene by FISH. Two partial human metaphase spreads demonstrating specific hybridization signals at chromosome 16p13.3 are shown. **Inset in right panel** shows specific hybridization to individual chromosome 16 homologues from other metaphase spreads. The photographs represent computer-generated, merged images of fluorescein signals (arrows) and DAPI-stained chromosomes.

relatively low in brain and liver tissue. Rehybridization of the Northern blot to an actin cDNA probe which hybridizes to both the ~2.1-kb β -actin and ~1.7-kb α -actin transcripts demonstrated that intact mRNA was present in all of the gel lanes.

Fn14 mRNA Expression in Human Liver Cell Lines

The liver RNA Northern blot data indicate that the human Fn14 gene is expressed at relatively low levels in differentiated hepatocytes, the major cell type found in this tissue.¹³ We assayed Fn14 mRNA levels in hepatocyte cell lines derived from normal liver (Chang), hepatoblastoma (HB) tissue (HepG2, Huh-6), or HCC tissue (HLE, Hep40, 7703, HLF, PLC/PRF/5, Sk-Hep-1, Huh-1, Focus)^{14–18} to investigate whether the Fn14 gene was activated during hepatocyte immortalization/transformation. Northern blot hybridization analysis indicated that Fn14 mRNA was expressed at relatively high levels in the Chang, HLF, PLC/PRF/5, and Focus cell lines, at intermediate levels in the 7703, Sk-Hep-1, Huh-1, and Huh-6 cell lines, and at low levels in the HLE and HepG2 cell lines (Figure 5). Fn14 mRNA expression was not detected in the Hep40 cells at this autoradiogram exposure.

Fn14 mRNA Expression in Human HCC

We next determined whether Fn14 gene expression was up-regulated in primary human HCC specimens. Northern blots containing equivalent amounts of RNA isolated from either HCC tissue or adjacent noncancerous liver tissue from the same individual were obtained and hybridization analysis was performed. Fn14 gene expression was detected in both HCC tissue and adjacent un-

involved liver tissue samples at this autoradiogram exposure; however, Fn14 mRNA levels were significantly elevated in three of the four HCC samples examined (Figure 6).

Fn14 mRNA Expression in Regenerating Mouse Liver and in Mouse HCC

Because it appeared that human Fn14 gene overexpression correlated with hepatocyte transformation *in vitro* and *in vivo*, we analyzed Fn14 gene expression in mouse models of hepatocyte growth and neoplasia. First, we determined whether the Fn14 gene was expressed during liver regeneration after 70% PH. Northern blot hybridization analysis was performed using RNA isolated from regenerating livers harvested at various times after the PH procedure. Fn14 mRNA expression was relatively low in liver tissue before PH; however, a significant increase in Fn14 mRNA levels was first apparent at 4 hours after PH (Figure 7). Then, the level of Fn14 mRNA expression decreased, increased again to a maximal level at 42 hours, and decreased again to baseline levels by 72 hours after surgery. Rehybridization of the Northern blot to an albumin cDNA probe demonstrated that similar amounts of poly(A)⁺ RNA were present in each gel lane.

Second, we investigated whether Fn14 gene expression was up-regulated in the HCC nodules that develop in two different transgenic mouse models of hepatocarcinogenesis. In the first model, coexpression of the *c-myc* and *TGF- α* transgenes in liver tissue promotes hepatocyte proliferation and eventually HCC formation between 4 and 8 months of age.^{8,9} For this analysis, RNA was isolated from either HCC or adjacent grossly normal liver tissue harvested from three 34-week-old transgenic animals and Northern blot hybridization was performed. El-

Figure 3. Regulation of Fn14 mRNA expression in human M426 cells. **A:** RNA was isolated from human M426 fibroblasts and murine NIH3T3 fibroblasts and equivalent amounts of each sample were analyzed by Northern blot hybridization. The positions of 28S and 18S rRNA are noted on the left. In the bottom section of this panel and the subsequent panels, a photograph of that portion of the RNA gel containing the 18S rRNA band is shown to demonstrate that similar amounts of RNA were present in each gel lane. **B–D:** Serum-starved M426 cells were either left untreated or treated with FGF-1 (**B**), FBS (**C**), or PMA (**D**) for the indicated time periods. RNA was isolated and equivalent amounts of each sample were analyzed by Northern blot hybridization.

evated levels of Fn14 mRNA were detected in all of the HCC specimens examined (Figure 8). Rehybridization of the Northern blot to an albumin cDNA probe demonstrated that similar amounts of poly(A)⁺ RNA were present in each gel lane. In the second model of hepatocarcinogenesis, expression of the hepatitis B virus (HBV) HBx protein in transgenic mice promotes HCC formation between 8 and 12 months of age.^{19,20} For this analysis, the extent of Fn14 mRNA expression in HCC tissue and in adjacent nontumoral tissue was evaluated by *in situ* hybridization analysis of liver specimens harvested from transgenic animals. A high level of Fn14 mRNA expression was detected in the HCC nodules of several animals, and a representative result from a 13-month-old male transgenic mouse is shown (Figure 9).

Figure 4. Fn14 mRNA expression in various human tissues. A Northern blot containing equivalent amounts of poly(A)⁺ RNA isolated from eight human tissues was obtained and hybridization analysis was performed using the two cDNA probes indicated. RNA size markers (in kb) are shown on the left.

Discussion

The addition of either serum or individual polypeptide growth factors to quiescent mammalian fibroblasts promotes the transcriptional activation of numerous genes encoding proteins with diverse functions.^{2–4} Many of these proteins are required for energy generation, organelle and membrane biogenesis, or nucleotide and DNA synthesis; others are known to be directly involved in the control of cell cycle progression and the physiology of wound repair.^{2–4} The genes encoding cell cycle regulatory proteins are of particular interest because their mutation, rearrangement, amplification, and/or overexpression may play a role in cellular transformation and tumorigenesis. We have been studying FGF-1-inducible genes in murine NIH 3T3 fibroblasts and recently reported the identification and characterization of Fn14, an immediate-early response gene encoding a relatively small, plasma membrane-anchored protein.⁷ Here, we present the initial characterization of the human Fn14 gene and provide experimental evidence that Fn14 gene activation is associated with liver regeneration and hepatocarcinogenesis.

We obtained and sequenced a human Fn14 cDNA clone and then used this clone as a probe for FISH and Northern blot hybridization experiments. The human Fn14 gene is predicted to encode a 129-aa protein with ~82% overall amino acid sequence identity to murine Fn14. The majority of the amino acid sequence differences between human and murine Fn14 are found in the predicted signal

Figure 5. Fn14 mRNA expression in human liver cell lines. RNA was isolated from the indicated liver cell lines and equivalent amounts of each sample were analyzed by Northern blot hybridization. The positions of 28S and 18S rRNA are noted on the left. In the **bottom panel**, a photograph of the RNA gel is shown to demonstrate that similar amounts of RNA were present in each gel lane.

peptide and transmembrane regions. Indeed, there are only four amino acid differences in the 53-aa extracellular domain and one amino acid difference in the 28-aa cytoplasmic domain. This indicates that the mature, 102-aa Fn14 protein sequence is highly conserved. The human Fn14 gene maps to chromosome 16p13.3. This result is consistent with the known synteny between this region of human chromosome 16 and the T-locus region of mouse chromosome 17, where the murine Fn14 gene is located.⁷ In addition, this map location is consistent with our finding that there is 100% nucleotide sequence identity between the human Fn14 cDNA sequence and human chromosome 16p13.3 genomic DNA sequence (GenBank accession no. AC004643). The human Fn14 gene encodes a single ~1.2-kb mRNA that is transiently up-regulated in FGF-1-, FBS-, or PMA-treated M426 fibroblasts; thus, the human and murine Fn14 genes encode a transcript of similar size and are regulated in a similar manner *in vitro*. These homologs also have a similar tissue-specific expression pattern *in vivo*; in both human and murine tissues, Fn14 mRNA expression is relatively low in brain and liver but relatively high in heart and kidney.

Figure 6. Fn14 mRNA expression in human HCC specimens. Northern blots containing equivalent amounts of total RNA isolated from HCC tumor tissue (T) and adjacent nontumoral liver tissue (N) from four individuals were obtained and hybridization analysis was performed. The RNA samples from individual no. 1 were on one blot, whereas the other samples were on a second blot, and the hybridization results were combined into this panel. The positions of 28S and 18S rRNA are noted on the left. In the bottom panel, a photograph of the RNA gel is shown to demonstrate that similar amounts of RNA were present in each gel lane.

Our observation that Fn14 gene expression was relatively low in normal human liver tissue, which contains primarily hepatocytes, led us to investigate whether the Fn14 gene was expressed in human hepatocyte cell lines or HCC specimens. HCC is one of the most common malignancies worldwide, with the highest incidence rates found in southeast Asia and sub-Saharan Africa.^{21–23} Several risk factors for HCC development have been identified, but chronic HBV and hepatitis C virus (HCV) infection are considered the two most important etiological agents.^{21–25} First, we examined Fn14 mRNA expression levels in cell lines derived from either normal liver, HB, or HCC tissue. Five of the eight HCC cell lines we examined contain integrated HBV sequences within their genome (Hep40, 7703, PLC/PRF/5, Huh-1, Focus).^{14–18} Fn14 mRNA expression was detected in ten of the eleven cell lines examined. The relative level of Fn14 mRNA expression in these lines did not correlate with the tissue origin of the cell line nor the presence of the HBV genome in cellular DNA; however, in general, there appeared to be higher levels of Fn14 gene expression in the poorly differentiated, HCC-derived cell lines (eg, PLC/PRF/5 and

Figure 7. Regulation of Fn14 mRNA expression during liver regeneration in mice. RNA was isolated from regenerating mouse liver harvested at various times after partial hepatectomy, and equivalent amounts were used for Northern blot hybridization analysis using the two cDNA probes indicated. The positions of 28S and 18S rRNA are noted on the left.

Focus^{15,26,27}). Second, we compared Fn14 mRNA expression levels in HCC tissue and adjacent nontumorous liver tissue isolated from four individuals and found Fn14 overexpression in three of the HCC specimens. This result indicates that Fn14 gene activation may be associated with liver tumorigenesis; however, we will have to obtain and analyze additional human HCC specimens to confirm this association. Several other genes have been reported to be expressed preferentially in human HCC,

Figure 8. Fn14 mRNA expression in HCC specimens from c-myc/TGF- α double transgenic mice. RNA was isolated from HCC tumor tissue (T) and adjacent nontumorous liver tissue (N) from three transgenic animals and equivalent amounts of each sample were analyzed by Northern blot hybridization using the two cDNA probes indicated. The positions of 28S and 18S rRNA are noted on the left.

Figure 9. Fn14 mRNA expression in HCC nodules from HBx transgenic mice. Serial sections of liver harvested from a transgenic animal were used for *in situ* hybridization analysis using Fn14 antisense (A) or sense (B) riboprobes. These two dark-field photographs, which reveal the hybridization signal grains in white, were taken at the same exposure level. A bright-field view showing another serial section stained with hematoxylin and eosin is shown in C. The HCC tumor nodule (T) and the adjacent nontumorous region of the liver (N) are indicated.

including various proto-oncogenes,²⁸ cyclin D1,²⁹ HIP,³⁰ and MXR7.³¹

The possibility that Fn14 could play a role in hepatocyte growth control and the pathogenesis of HCC was further explored using mouse models of liver regeneration and hepatocarcinogenesis. The adult rodent liver is normally a quiescent organ; however, after 70% PH there is compensatory hyperplasia of the parenchymal hepatocytes, and the residual lobes will grow until they attain the size of the original liver, which usually occurs by 1 to 2 weeks.^{32–34} Indeed, liver regeneration represents an excellent *in vivo* model of synchronous cell division; in the mouse, the first wave of hepatocyte DNA synthesis occurs at ~36 hours after PH.^{10,35,36} Previous gene expression studies, primarily using the rat PH model, have demonstrated that numerous growth factor-inducible genes, including proto-oncogenes and genes encoding cell cycle regulators, are activated during liver regeneration *in vivo*.^{10,32,34–37} We found that Fn14 mRNA expression was

low in normal mouse liver, in agreement with our previous report,⁷ but after 70% PH, the level of Fn14 mRNA rapidly increased, with a high level of expression detected at 4 hours after surgery. Fn14 expression then decreased, increased again with a peak at 42 hours, and then returned to basal levels at 72 hours after PH. These results indicate that Fn14 gene expression is first up-regulated in the early phase of liver regeneration, when quiescent hepatocytes enter the G1 phase of the cell cycle, and then there is a sustained high level of expression during the major growth period of the liver.

Transgenic mice and rats have been used by several groups to assess the role of specific oncoproteins, growth factors, or HBV-encoded polypeptides in liver neoplasia.^{38,39} We assayed Fn14 mRNA levels in two mouse models of HCC. In *c-myc*/TGF- α double transgenic mice, constitutive coexpression of the *c-myc* transcription factor and the TGF- α polypeptide mitogen in mouse liver promotes enhanced hepatocyte proliferation, extensive DNA damage, numerous chromosomal aberrations, and the development of HCC lesions in 100% of the male animals by 8 months of age.^{8,9,40–42} In HBx transgenic mice, expression of the HBV-encoded X antigen, a multifunctional, growth-regulatory protein thought to be the critical mediator of HBV pathogenesis,²⁴ promotes the formation of HCC lesions in ~90% of the male animals by 8 to 12 months of age.^{19,20,43} In both of these transgenic mouse models we found that the Fn14 gene was expressed at relatively high levels in HCC nodules.

In summary, we have found that the Fn14 immediate-early response gene is activated during murine liver regeneration and, in addition, relatively high levels of Fn14 gene expression are found in murine and human HCC tumors. It should be noted that another polypeptide growth factor-regulated, immediate-early response gene identified in our laboratory, named Fnk,⁴⁴ is also transiently induced during liver regeneration but not overexpressed in HCCs (unpublished results). Furthermore, Fn14 gene activation is not associated with all cancerous tissues; specifically, we could not detect Fn14 mRNA up-regulation in human breast, ovary, or kidney tumor specimens (unpublished results). Taken together, these results indicate that Fn14 gene activation may have an important and specific role in liver cancer. The biological significance of Fn14 mRNA induction during liver regeneration and hepatocarcinogenesis is presently unknown. We have shown that constitutive expression of the Fn14 protein in NIH 3T3 fibroblasts decreases cellular proliferation *in vitro*.⁷ This result implies that Fn14 is not a positive regulator of cell cycle progression in this particular cell line, but of course it may have different effects on other cell types. It has recently been reported that many of the genes that are activated in serum-stimulated fibroblasts encode proteins implicated in the physiology of wound healing;³ thus, Fn14 expression in regenerating liver may be required for some aspect of tissue repair. In regard to the role of Fn14 in liver tumor biology, we have also shown that constitutive Fn14 expression decreases cellular adhesion to extracellular matrix molecules.⁷ Therefore, it is possible that a high level of Fn14 expression in HCC may promote cell detachment from the primary

tumor, thus contributing to intra- or extrahepatic metastasis. Additional experimentation is required to elucidate the biological function of the Fn14 protein in hepatocytes and other cell types.

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