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### Research Article

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# Overexpression of Human Aspartyl(Asparaginyl) $\beta$ -Hydroxylase in Hepatocellular Carcinoma and Cholangiocarcinoma

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## Abstract

To characterize genes that become upregulated with malignant transformation of human hepatocytes, a library of monoclonal antibodies was produced against the FOCUS hepatocellular carcinoma cell line. Antibody FB-50 reacted with an antigen that was highly expressed in 4 of 10 primary hepatocellular carcinomas, in all 20 cholangiocarcinomas we studied, and in a variety of transformed cell lines. This antigen was also highly expressed in neoplastic epithelial cells of breast and colon carcinomas in contrast to its low level of expression in normal hepatocytes and in non-neoplastic epithelial cells. Among the normal adult tissues studied, high levels were observed only in proliferating trophoblastic cells of the placenta and in adrenal glands. A 636-bp partial cDNA, isolated from a  $\gamma$ GT11 expression library generated with HepG2 human hepatoblastoma cells, and a complete cDNA, generated by reverse transcriptase-PCR, identified the antigen as the human form of aspartyl(asparaginyl) $\beta$ -hydroxylase. This enzyme catalyzes post-translational hydroxylation of  $\beta$  carbons of specific aspartyl and asparaginyl residues in EGF-like domains of certain proteins. Analyses of extracts prepared from several human tumor cell lines compared to their normal tissue counterparts indicate that the increase in hydroxylase,  $\sim 10$ -fold, is controlled at the level of transcription and the protein is expressed in an enzymatically active form. In similar analyses, comparing hepatocellular carcinomas to adjacent uninvolved liver from five patients, enzymatic activity was much higher in the tumor tissue from the four patients whose immunoblots revealed increased hydroxylase protein in the malignant tissue. EGF repeats in the extracellular domain of Notch or its homologs contain the consensus sequence for

hydroxylation. Deletion mutants lacking this domain are gain-of-function mutants, suggesting that the domain modulates signal transduction by the cytoplasmic domain. While the function imparted by  $\beta$  hydroxylation is unknown, our studies raise the possibility that  $\beta$  hydroxylation is regulated in proteins like the mammalian Notch homologs, whose cytoplasmic domains have been shown to be oncogenic. (*J. Clin. Invest.* 1996. 98:1313–1323.) Key words: transformation • oncogene • EGF domain •  $\beta$  hydroxylation • hepatoma

## Introduction

Hepatocellular carcinoma (HCC)<sup>1</sup> is one of the most prevalent tumors in the world today, occurring with especially high frequency in sub-Saharan Africa and the Far East. Despite this, there is actually very little information available regarding the antigenic properties of human hepatoma cells (1). Such information could facilitate the development of improved methods for liver tumor detection and could help elucidate the mechanisms underlying malignant transformation of hepatocytes. To study cell surface changes associated with malignant transformation of these tumors, several libraries of mAbs (2–4) have been produced for the FOCUS human hepatocellular carcinoma cell line (5). One of these mAbs, FB-50, has allowed us to characterize an antigen that is highly expressed in some primary HCCs and a variety of transformed cell lines, including HCC-derived cell lines. In addition, the antigen is highly expressed in all cholangiocarcinomas we have studied. We describe the cellular expression of this antigen in transformed hepatic and bile duct epithelial cells, establish its molecular identity as the human aspartyl(asparaginyl) $\beta$ -hydroxylase (HAAH) enzyme by cDNA cloning and demonstrate that it is expressed in an enzymatically active form.

The bovine form of this enzyme recently has been purified to homogeneity, cloned, and expressed (6–8). It is an  $\alpha$ -ketoglutarate-dependent dioxygenase (9–11) that stereospecifically catalyzes the posttranslational hydroxylation of the  $\beta$  carbon of specific aspartyl and asparaginyl residues in one or more EGF-like domains of certain proteins, if the EGF-like domains possess the consensus sequence (12) required by the enzyme. The possible relationship to the malignant phenotype of regulated aspartyl(asparaginyl) $\beta$ -hydroxylation in EGF-like domains of proteins like the mammalian Notch homologs, known to be involved in cell differentiation and whose cytoplasmic domains have been shown to be oncogenic (13–14a) is discussed.

Laurent Lavaissiere and Steve Jia contributed equally to this work and should be considered co-first authors.

Portions of this work were presented at the Annual Meeting of the American Association for the Study of Liver Diseases, 15 November 1994, and have been published in abstract form (1994. *Hepatology*. 20:275a).

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1. *Abbreviations used in this paper:* HAAH, human aspartyl(asparaginyl) $\beta$ -hydroxylase; HCC, hepatocellular carcinoma; ORF, open reading frame.

## Methods

The following reagents and kits were obtained commercially: all protease inhibitors, catalase, bovine serum albumin, digoxigenin dUTP, antidigoxigenin fluorescent isothiocyanate isomer I, and propidium iodide (Sigma Chemical Co., St. Louis, MO);  $\alpha$ -keto[1- $^{14}$ C]-glutaric acid (51.8 mCi/mmol), and Solvalbe (DuPont-NEN, Boston, MA); ferrous ammonium sulfate (Fisher Scientific Co., Fairlawn, NJ);  $\alpha$ -keto-glutarate (Aldrich Chemical Co., Milwaukee, WI).

**Cell lines and human tissues.** Most cell lines employed were obtained from the American Type Culture Collection (Rockville, MD) and maintained on Dulbecco's Modified Eagle's medium (M.A. Bioproducts, Walkersville, MD) supplemented with 10% fetal calf serum heat inactivated at 56°C, 10  $\mu$ M nonessential amino acids, 1,000 IU/ml of penicillin, and 100  $\mu$ g/ml of streptomycin. The human hepatoma FOCUS cell line was developed in the Wands' laboratory and has been characterized previously (5). Samples of normal human tissues, HCCs paired with adjacent uninvolved liver, and human breast and colon carcinomas were obtained by surgical excision or postmortem examination. These specimens were snap-frozen in liquid nitrogen and stored at -80°C. 20 surgical or autopsy cholangiocarcinoma specimens fixed in 2% paraformaldehyde and paraffin embedded were also studied.

**Production of the mAbs.** The FOCUS human hepatoma cell line was used for immunization. An early passage of this cell line from the original primary HCC tumor was used. Primary immunizations of female Balb/c mice were accomplished intraperitoneally with  $4 \times 10^6$  intact cells/ml that had been removed from tissue culture plates by EDTA/versene buffer and resuspended in 50% Freund's complete adjuvant. After 6–10 wk, the secondary immunizations were performed by an intravenous injection of  $4 \times 10^6$  FOCUS cells in 200  $\mu$ l PBS. 3 d later, splenocytes from immunized mice were fused with the NS1 myeloma cell line and the resulting hybridomas were maintained and selected in hypoxanthine-aminopterin-thymine-containing medium.

**Identification, purification, and specificity testing of mAbs.** Antibody-producing hybridoma cells with initial immunoreactivity to FOCUS cells were subsequently screened by binding activity to a panel of human tumor cell lines. Positive hybridomas were identified, and then cloned twice by limiting dilution. Subsequent specificity testing of cloned hybridomas was performed against various cell lines by an indirect binding radioimmunoassay using  $^{125}$ I-labeled sheep F(ab')<sub>2</sub> anti-mouse immunoglobulins (specific activity: 8–12  $\mu$ Ci/ $\mu$ g protein; DuPont-NEN) as described (1). Ascites fluids were prepared by intraperitoneal injection of hybridoma cells into Balb/c mice primed with 2,6,10,14-tetramethylpentadecane (Pristane; Aldrich Chemical Co.). mAb from double-cloned cell lines of the IgG subclass were purified from the ascites-fluid by using a Sepharose 4B protein-A affinity column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The protein concentration was determined by the method of Lowry (15). Purified mAbs were radioiodinated with  $^{125}$ I using the Iodogen method (Pierce Chemical Co., Rockford, IL) (16), to a specific activity of 5–15  $\mu$ Ci/ $\mu$ g protein.

**Immunoperoxidase staining.** Paraformaldehyde fixed (2%), paraffin-embedded postmortem and surgical specimens of primary HCC and cholangiocarcinomas, together with adjacent uninvolved liver were studied. In addition, sections of breast and colon carcinomas, normal human liver, and other normal human tissues were also fixed and paraffin embedded. The sections (8- $\mu$ m thick) were deparaffinized in xylenes, rehydrated in graded alcohol solution, and equilibrated in PBS. After an overnight incubation at 4°C with FB-50 mAb (5 or 10  $\mu$ g/ml), the sections were immunostained by the avidin-biotin horseradish peroxidase complex method, according to the manufacturer's protocol (Vector Laboratories, Inc., Burlingame, CA), and with 3,3'-diaminobenzidine (0.5 mg/ml in 0.03% hydrogen peroxide) as the chromagen. Nonspecific binding was defined as binding of a  $^{125}$ I-labeled nonrelevant mAb (antitetanus toxoid B2TT) used under the same conditions.

**Tumor cell binding assay.** Focus cells ( $10^5$ ), removed from plates with EDTA/versene buffer, were resuspended in 100  $\mu$ l of PBS/20% fetal calf serum and incubated at room temperature for 1 h with  $^{125}$ I-FB-50 mAb ( $10^5$  cpm). The cells were then washed three times and bound radioactivity was counted in a gamma well counter.

**Enzymatic activity assay.** Aspartyl(asparaginyl) $\beta$ -hydroxylase activity was assayed as described previously (6, 7, 10) using as the substrate the first EGF-like domain of bovine protein S with an asparagine replacing the aspartic acid at position 18. Incubations were carried out at 37°C for 30 min in a final volume of 40  $\mu$ l containing 48  $\mu$ g of crude cell extract protein and 75  $\mu$ M EGF substrate.

**cDNA library screening and DNA sequencing.** A  $\gamma$ GT11 expression vector library constructed from the human hepatoblastoma cell line HepG2 (4) was immunoscreened with  $^{125}$ I-FB-50 mAb. About  $10^6$  independent recombinant plaque-forming units were screened on blotted filters with  $^{125}$ I-FB-50 mAb as described (4), and one clone with persistent immunoreactivity was plaque purified. The partial cDNA clone was subcloned into the pT7 blue TA cloning vector (Novagen, Inc., Madison, WI) after PCR amplification using  $\gamma$ GT11 screening amplicon primers (Clontech, Palo Alto, CA), and then transformed into *Escherichia coli*. Miniprep plasmid DNA was prepared from transformed bacteria, and one clone-containing insert was sequenced. DNA sequencing was performed along both strands using the dideoxynucleotide chain termination method (Sequenase version 2.0 T7 DNA polymerase; United States Biochemical Corp., Cleveland, OH) and  $\alpha$ - $^{35}$ S-labeled dATP (DuPont-NEN). The DNA sequence was assembled using the Mac Vector Software version 4.1 and analyzed using a Sequence Analysis Software of the Genetics Computer Group version 7.3 as implemented on a MicroVAX II computer (17).

**Identification of the complete HAAH cDNA.** Total cellular RNA was extracted from the FOCUS cell line with a commercially prepared phenol-guanidine isothiocyanate reagent according to the directions of the manufacturer (Ultraspec RNA isolation system; Biotecx Laboratories, Houston, TX). RNA was reverse transcribed using RNase H-free reverse transcriptase (Superscript II; Life Technologies, Inc., Grand Island, NY) and random hexamer primers. Two specific oligonucleotide primers, selected from the HAAH open reading frame sequence and modified to include 5'KpnI-BamHI and 3'XhoI-EcoRI

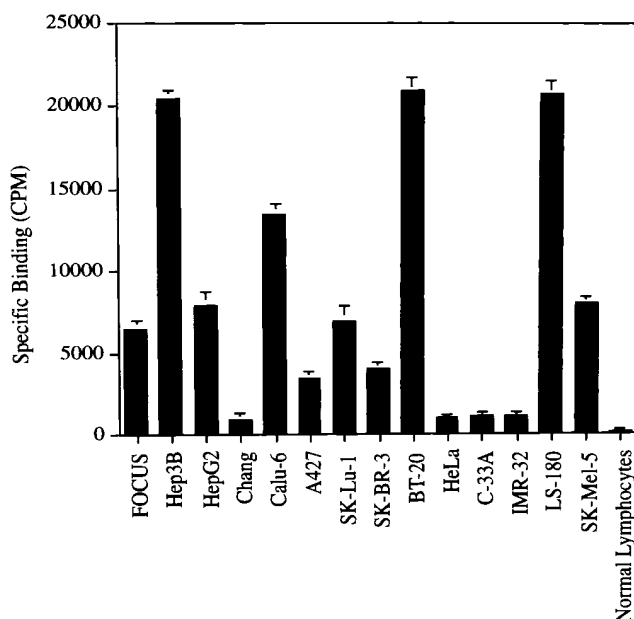


Figure 1. Immunoreactivity of  $^{125}$ I-FB-50 mAb with intact human tumor cell lines as determined by a direct binding assay (see Methods).

**Table I. Expression of Proteins in Human Carcinomas and in Normal Human Tissue that React with the FB-50 mAb as Revealed by Immunoperoxidase Staining**

Tissue type	Number positive/ number studied	Comments
<b>Tumor tissue</b>		
Hepatocellular carcinoma	4/10*	Heterogeneous staining among tumor cells within and between tumors.
Cholangiocarcinoma	20/20	Diffuse homogeneous staining of all transformed bile duct cells in all tumors studied.
Breast carcinoma	4/4	Heterogeneous staining among tumor cells.
Colon carcinoma	6/10	Heterogenous staining among tumor cells within and between tumors.
<b>Proliferating tissue</b>		
Term placenta	4/4	Diffusive homogeneous staining of the placental trophoblastic cells of the chorionic villi.
<b>Normal tissue</b>		
Normal liver	9/9	Diffuse weak staining of all hepatocytes.
Normal bile ducts	0/4	No staining.
Adrenal glands	2/2	Intense staining of cells in the medulla and a subpopulation of cells in the zona fasciculata and reticularis.
Heart	Negative	
Kidney	Negative	
Thyroid	Negative	
Small intestine	Negative	
Large intestine	Negative	
Muscle	Negative	
Spleen	Negative	
Lung	Negative	

\*All 10 HCCs were reactive with the FB-50 mAb. However, while the level of immunoreactivity in four of the tumors was clearly greater than in normal liver, in the other six HCCs examined, diffuse weak staining similar to that seen in normal liver was observed.

restriction enzyme sites, were synthesized in an Applied Biosystem DNA synthesizer: 5'-GGTACCAGGATCCA-77 ATGGCCAGCGTAAGAAT-3' and 5'-CTCGAGGAATTC-2418 CACCCAAGGAGATGGAACC-3'. 30 PCR cycles were carried out for 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C, followed by a final 10-min extension at 72°C. The resultant 2,374-bp DNA amplification product was purified from agarose gel, subcloned into the pT7 blue TA cloning vector, and sequenced.

**Expression of bacterial recombinant fusion protein and production of rabbit polyclonal antibodies.** The bacterial expression vector pTrcHis B (Invitrogen Corp., San Diego, CA) was used to subclone the partial FB-50 cDNA into the BamH1 and EcoR1 sites. This vector was chosen because it encodes a 5'G-His sequence, which can be used to isolate the resulting fusion protein by chelate chromatography. The open reading frame was confirmed by sequencing both strands at the 5' end. Recombinant fusion protein induction in transformed *E. coli* was achieved by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside during the log growth phase. The fusion protein was purified using ProBond resin (Invitrogen Corp.). Two rabbits were immunized by intramuscular injections of 0.5 mg purified recombinant fusion protein emulsified with Freund's complete adjuvant. Booster injections were performed 2 and 4 wk later with ~ 0.25 mg purified fusion protein emulsified with Freund's incomplete adjuvant. Sera were obtained 7 d after the final injection.

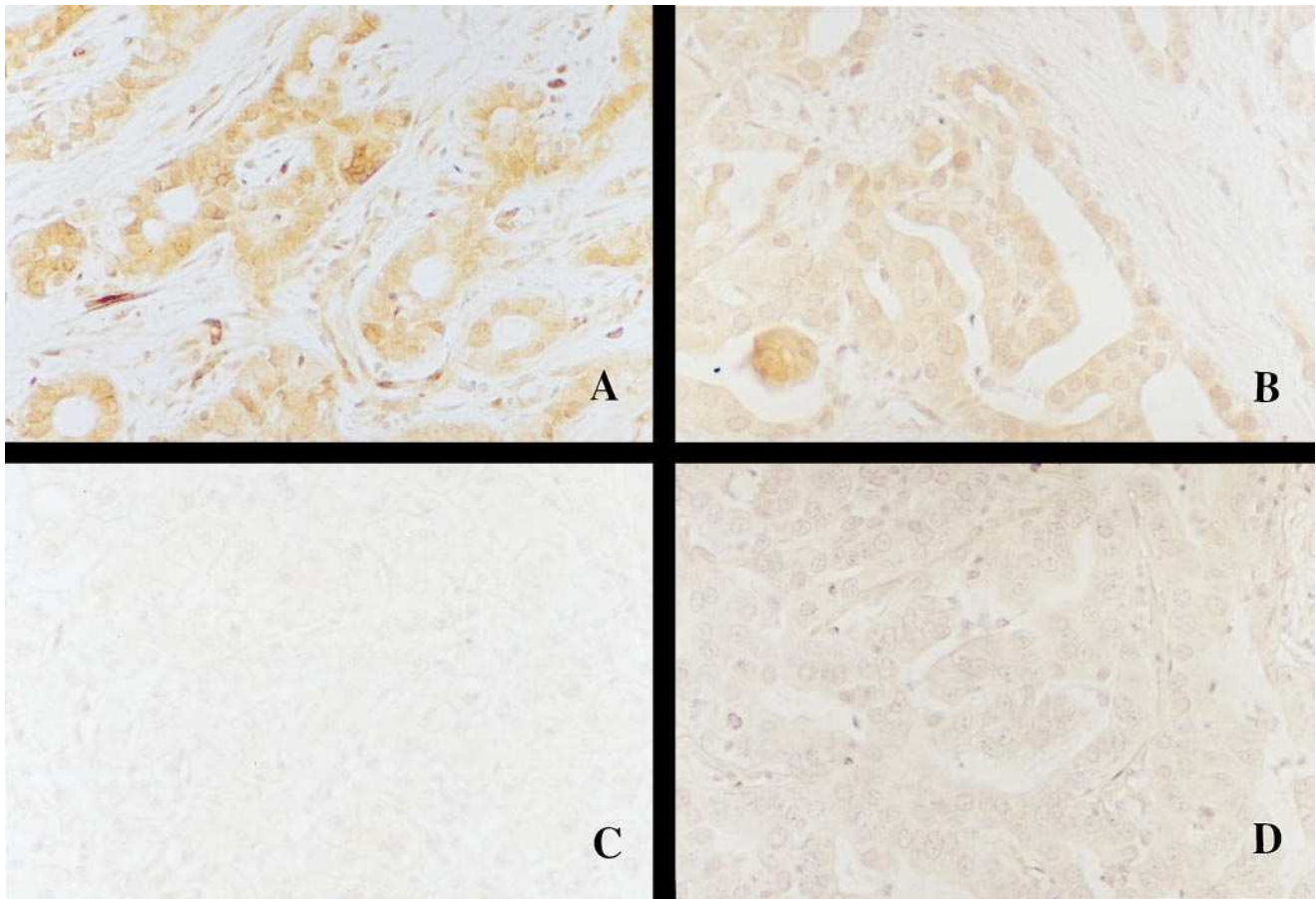
**Northern blot analysis.** Samples of 15 μg total cellular RNA were electrophoretically fractionated in 1% denaturing agarose gels

and transferred to nylon membranes (Hybond N+; Amersham Corp., Arlington Heights, IL). After prehybridization in Rapid Hybrid buffer (Amersham Corp.), the blots were hybridized for 3 h at 65°C with the partial FB-50 cDNA probe labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (DuPont-NEN) using the Megaprime labeling method (Amersham Corp.). Blots were washed at 65°C in 0.1% SSC and 0.5% SDS for 30 min and subjected to film autoradiography at -80°C. To assess RNA integrity and the relative loading in each lane, the blots were stripped and reprobbed with [ $\gamma$ -<sup>32</sup>P]αATP labeled synthetic 30 mers corresponding to 18S ribosomal RNA in probe excess.

**Subchromosomal localization of the HAAH gene.** Fluorescent in situ hybridization analysis was performed as described by Pinkel et al. (18) using a human hydroxylase genomic clone as the probe (Genome Systems, Inc., St. Louis, MO).

## Results

**Distribution of antigen for the FB-50 mAb.** The FB-50 mAb was initially selected because of its high level of binding to the FOCUS cell line used as the immunogen. Antigen distribution was assessed by determining the binding of <sup>125</sup>I-FB-50 mAb to a variety of intact HCC and other transformed cell lines. As shown in Fig. 1, <sup>125</sup>I-FB-50 mAb exhibited high level binding to the three human HCC lines (Hep 3B, Hep G2, FOCUS), to



**Figure 2.** Representative examples of immunoperoxidase staining of two cholangiocarcinoma tissues (*A* and *B*). There was strong homogeneous cytoplasmic staining of transformed bile ducts in all 20 cholangiocarcinoma specimens studied, while the normal liver and bile ducts (*C*) have little immunoreactivity. Cholangiocarcinoma stained with a nonrelevant mAb (*D*) served as a negative control.

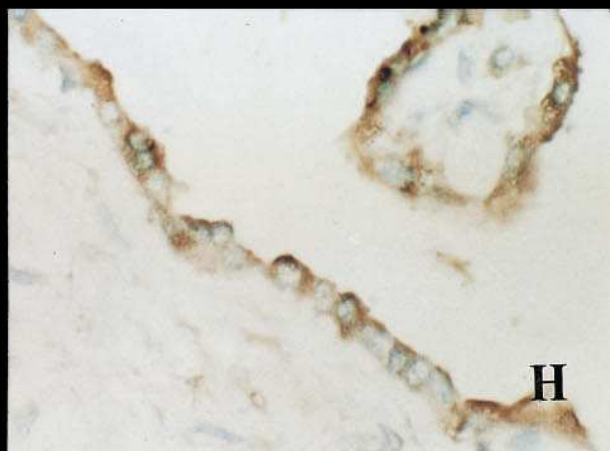
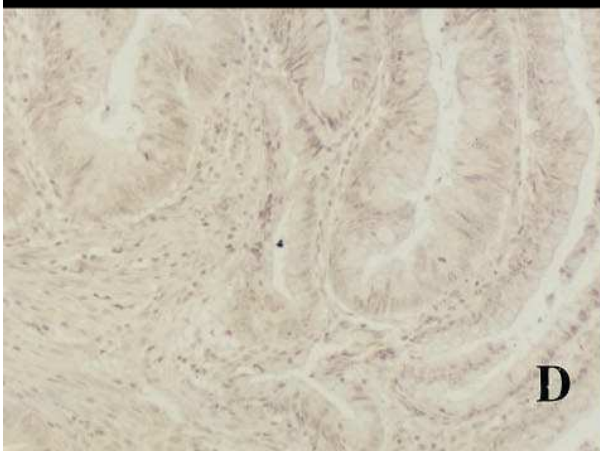
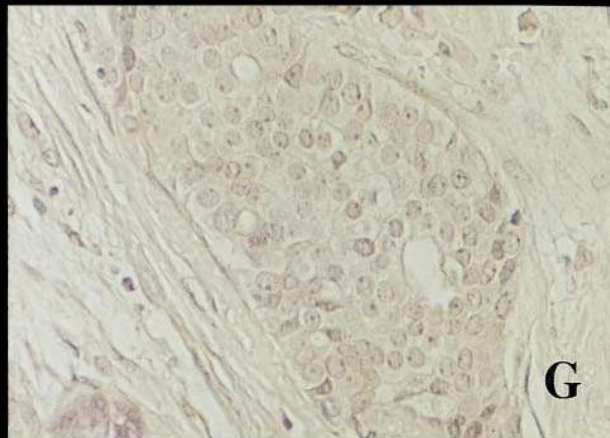
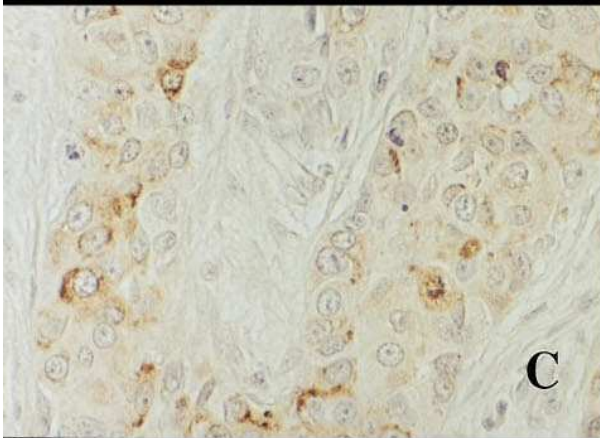
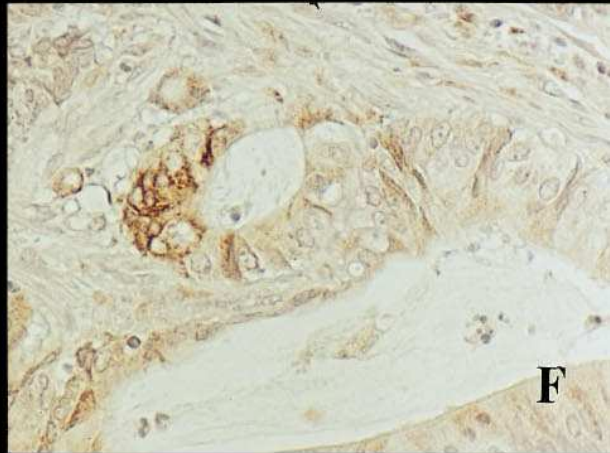
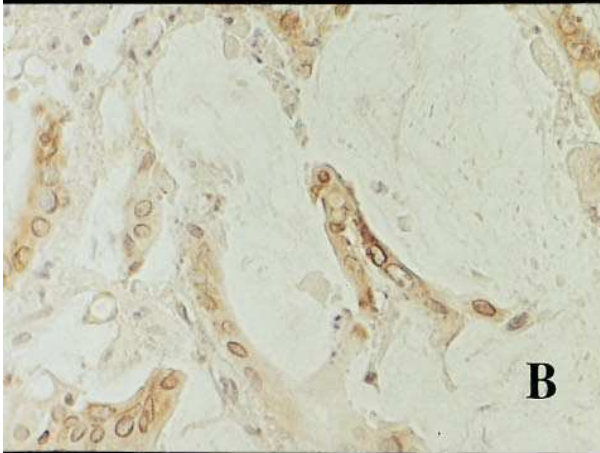
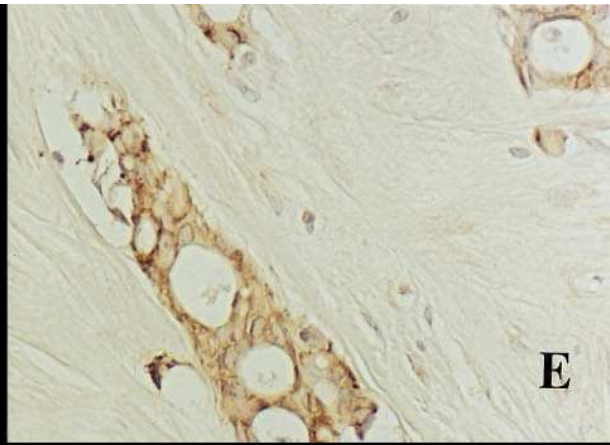
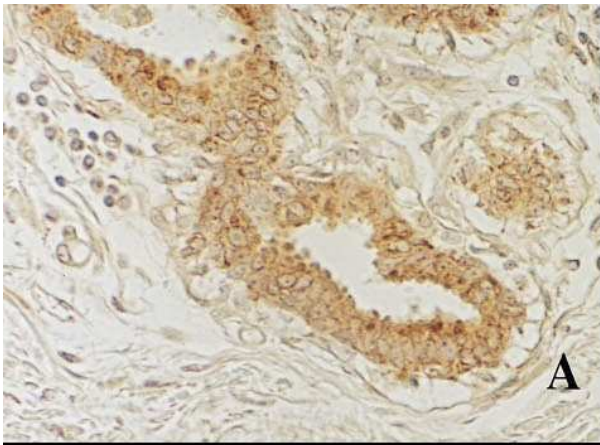
human adenocarcinoma cell lines derived from lung (Calu 6, SK-lu-1), breast (BT 20), and colon (LS180), and to a melanoma cell line (SK-Mel-5). Lower degrees of binding were observed with Chang, Hela, cervical carcinoma (C-33A), and neuroblastoma (IMR-32) cell lines as well as to another lung (A427) and another breast (SK-BR-3) cell line. In contrast, normal human lymphocytes were unreactive.

The cellular localization of antigen in human tissues was confirmed by immunohistochemical staining studies (Table I). Normal hepatocytes exhibited diffuse and very low level expression. Four HCCs exhibited high levels of FB-50 immunoreactivity, while the remaining six had the staining pattern seen in normal liver. Distribution of antigen was heterogeneous among the cells in the tumors with high level immunoreactivity. By contrast, all 20 cholangiocarcinomas examined exhibited intense and uniform immunoreactivity (Table I, Fig. 2). In addition, discrete FB-50 immunoreactivity was localized in the perinuclear cytoplasm of neoplastic glandular epithelial cells in 4 of 4 breast and 6 of 10 colon carcinomas studied (Table I, Fig. 3). Mucin-producing and well differentiated neoplastic foci within the specimens exhibited the most striking FB-50 immunoreactivity. In contrast, nests, cords, and diffusely infiltrative neoplastic cells exhibited low level or absent FB-50 immunoreactivity. The high level FB-50 expression was a feature of both primary and metastatic lesions. As observed with respect to the cholangiocarcinomas, FB-50 immunoreactivity

was not detected in normal, nonneoplastic epithelial cells and stroma. All specimens exhibited immunoreactivity for glyceraldehyde-3-phosphate dehydrogenase (positive control) and absent immunolabeling with the primary antibody omitted. Of a number of normal tissues examined, in addition to liver, only the adrenal glands displayed immunoreactivity. Finally, we examined antigen expression in proliferating term placental trophoblasts. As shown in Table I and Fig. 3, there was intense staining in the syncytiotrophoblastic cells of the chorionic villi.

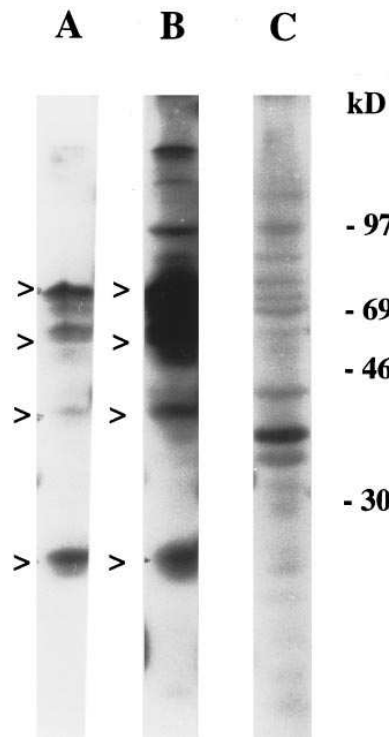
*Molecular cloning of the antigen: its identification as HAAH.* A 636-bp cDNA clone was obtained after screening of a Hep G2  $\gamma$ GT11 library with  $^{125}$ I-FB-50 (mAb). The Hep G2-derived cDNA had a single major open reading frame (ORF) capable of encoding for a 212 amino acid protein, as illustrated by the underlined sequences in Fig. 4. The ORF had neither a methionine start codon nor a termination codon, but was in phase with the bacteriophage-derived *lac Z* gene read-

**Figure 3.** Representative examples of immunoreactivity. FB-50 immunoreactivity in colon (*A–D*) and breast (*E–G*) carcinoma, and in normal placenta (*H*). Intense FB-50 immunoreactivity in well differentiated glandular secretory neoplastic epithelial cells in both colon (*A* and *B*) and breast (*E*) carcinomas. With loss of glandular architecture, FB-50 immunoreactivity was diminished (*C*, *F*, and *G*). No immunoreactivity was observed with the primary antibody omitted (*D*).



1 CGGACCGTGCAATGCCCCAGCGTAAGAAATGCCAAGAGCAGCGGCAACAGCAGCAGC  
1 M A Q R K N A K S S G N S S S  
57 AGCGGCTCCGGCAGCGGTAGCAGAGTGGGGCAGCAGCAGCCCCGGGGGAG  
16 S G S G S G S T S A G S S S S P G A R R  
113 AGAGACAAAGCATGGAGGACACAAGAAATGGGAGGAAGGCGGACTCTCGGAACATT  
35 E T K H G G H K N G R K G G L S G T  
169 CATCTTCACGTGGTTTATGGTGATTCGATTCGTTGGCGCTCTGGACATCTGTAGCT  
53 S [ F F T W F M V I A L L G V W T S V A  
225 GTCGTTTGGTTTATCTTGTGACTATGAGGAAGTTCTAGGAAACATAGGAATCTA  
72 V V W F ] D L V D Y E E V L G K L G I Y  
281 TGATGCTGATGGTATGGAGATTTTATGATGGATGATGCCAAAGTTTATTAGGAC  
91 D A D G D G D F D V D D A K V L L G  
337 TTAAAGAGAGATCTACTTCAGAGCCAGCAGTCCCGCCAGAGAGGCTGAGCCACAC  
109 L K E R S T S E P A V P P E E A E P H  
393 ACTGAGCCCCAGGAGGAGTCTCTCTGAGGCGAGACCCAGAAATATCGAAGATGA  
128 T E P E E Q V P V E A E P Q N I E D E  
449 AGCAAAAGAACAAATTCAGTCCCTCTCCATGAAAATGGTACACGAGAACATGTTG  
146 A K E Q I O S L L H E M V H A E H V  
505 AGGGAGAGACTTGCAACAGAGATGGACCCACAGGAGAACCAACAGAGGAT  
164 E G E D L Q O E D G P T G E P Q Q E D  
561 GATGAGTTCTTATGGCGACTGATGTAGATGATAGATTGAGACCTTGGACCTGA  
184 D E F L M A T D V D D R F E T L E P E  
617 AGTATCTCATGAAGAAACCGAGCATAGTTACCCGCTGGAAGAGACAGTTTCACAG  
203 V S H E E T E H S Y H V E E T V S Q  
673 ACTGTAATCAGGATATGGAAGAGATGATCTCTGAGCAGGAAAAATCCAGATCCAGT  
221 D C N Q D M E E M M S E Q E N P D S S  
729 GAACCACTAGTGAAGATGAAGATTCACCATGATACAGATGATGTACATACCA  
240 E P V V E D E R L H H D T D D V T Y V  
785 AGTCTATGAGGAACAGCAGTATATGAACCTCTAGAAAAATGAAGGATAGAAATGA  
259 V Y E E Q A V Y E P L E N E G I E I  
841 CAGAAGTAAGTCTCCCGCTGAGGATAATCTCTGTAAGATTCACAGGTAAATTGTA  
277 T E V T A P P E D N P V E D S Q V I V  
897 GAAGAAGTAAGCATTTTCTCTGGAAGAACAGCAGGAAAGTACCACCAAGAACAAA  
296 E E V S I F P V E E Q Q E V P P E T N  
953 TAGAAAAACAGATGATCCAGAACAAAGCAAAAGTTAAGAAAAAGAGCCATAAC  
315 R K T D D P E Q K A K V K K K P K  
1009 TTTTAAATAAATTTGATAAGACTATTAAGCTGAATCTGATGCTGCAGAAAACTC  
333 L L N K F D K T I K A E L D A A E K L  
1065 CGTAAAGGGGAAAAATTTGAGGAAGCAGTGAATGCAATTAAGAACTAGTAGCACA  
352 R K R G K I E E A V N A F K E L V R K  
1121 ATACCCTCAGAGTCCAGCAGCAAGATATGGGAAGGCGAGTGTGAGGATGATTTGG  
371 Y P Q S P R A R Y G K A Q C E D D L  
1177 CTGAGAAGAGGAGAAGTAATGAGGTGCTAGCTGGAGCCATCGAGACCTACCAAGAG  
390 A E K R R S N E V L R G A I E T Y Q E  
1233 GTGGCCAGCCTACCTGATGTCTCTGAGAGCTGCTGAAGCTGAGTTTGAAGCGTGG  
408 V A S L P D V P A D L L K L S L K R R  
1289 CTCAGACAGGCAACAAATTTCTAGGTCAATATGAGAGGTTCCCTGCTTACCTGCAGA  
427 S D R Q Q F L G H M R G S L L T L Q  
1345 GATTAGTTCAACTATTTCCCAATGACTCTTCTTAAAAATGACCTTGGCGTGGGA  
446 R L V Q L F P P N D T S L K N D L G V G  
1401 TACCTCTTGAAGAGATTAATGACAAAGCAAGAAATTTATGAAGAGGTGCTGAG  
464 Y L L I G D N D N A K K V Y E E V L S  
1457 TGTGACACCTAATGATGCTTTTCTTAAAGTCCATTATGGCTTCATCTGAGGCAC  
483 V T P N D G F A K V H Y G F I L K A  
1513 AGAACAAATTTGCTGAGAGCATCCCATATTTAAAGGAAGGAATAGAAATCCGAGAT  
502 O N K I A E S I P Y L K E G I E S G D  
1569 CTTGGCAGCTGATGAGGAGATTTTATTTCCACCTGGGGGATGCCATTCAGAGGGT  
520 P G T D D G R F Y F H L G D A M Q R V  
1625 TGGGAACAAAGAGGCATATAAGTGGTATGAGCTTGGGCACAAGAGGAGCACTTTG  
539 G N K E A Y K W Y E L G H K R G H F  
1681 CATCTGTCTGCAACGCTCCTCTCAATGTGAATGGACTGAAAGCACAGCCTTG  
558 A S V W Q D A V N V N G L K A Q P W  
1737 TGCACCCCAAGAAACGGGCTACACAGAGTTAGTAAAGTCTTTAGAAAGAACTG  
576 W T P K E T G Y T E L V K S L E R N W  
1793 GAAGTTAATCCAGATGAAGGCTTGCAGTGTGATGAAGCCAAAGGCTCTCTCC  
595 K L I R D E G L A V M D K A K G L F  
1849 TGCCCTGAGGATGAAGAACTGAGGAGAAAGGGGACTGGAGCCAGTTCAGCGTGTGG  
613 L P E D A E N L R E K G D W S Q F T L W  
1905 CAGCAAGGAAGAAATGAAATGCTGCAAGAGGCTCTTAAAGCTGTACCTT  
632 Q Q G R R N E N A C K G A P K T C T L  
1961 ACTAGAAAAGTTCCCGGAGACACAGGATGCAGAAGAGGACAGATCAAAATATTC  
651 L E K F P T G C R R G Q I K Y S  
2017 TCATGCAACCCCGGAGTCACTGTGGCGCACACAGGGCCCAAACTGCAGGCTC  
669 I M H P G T H V W P H T G P T N Q R L  
2073 CGAATGCACCTTGGGCTTGGTATTCCTCAAGGAAGGCTGCAAGATTTCGATGTGCCAA  
688 E M H L G L Y I E K E G C K I R C A N  
2129 CGAGACCAAGACCTTGGAGAGAGGCAAGGTGCTCATCTTTGATGACTCTTTGAGC  
707 E T R T W E E G K V L I F D D S F E  
2185 ACGAGGTATGGCAGGATGCCTCATCTTCCGGCTGATATTTCATCGTGGATGTGTG  
725 H E V W Q D A S F R L D I F I V D V W  
2241 CATCCGGAATGACACACAGAGAGAGCAGCCTTCCAGCAATTTAGCATGAAT  
744 H P E L T P Q Q R R S L P A I \*\*\*  
2297 TCATGCAAGCTTGGGAACCTCGGAGAGA

**Figure 4.** Nucleotide and deduced amino acid sequences of cDNA encoding HAAH. The amino acid sequence is denoted under the nucleotide sequence in the standard one-letter code. Amino acids are numbered, starting with the first in-frame methionine as 1, the codon of which is indicated in bold. The translation termination codon is designated by \*\*\*, and indicated in bold type. The amino acid residues obtained by immunoscreening and the corresponding FB-50



Immobilon-P (Millipore Corp., Bedford, MA), the membranes were blocked for 1 h at room temperature with PBS, pH 7.5, containing 5% nonfat dry milk, and then probed for 2 h at 20°C with mouse ascites fluid FB-50 diluted to 1:5,000 or 10 µg/ml of purified FB-50 mAb in PBS containing 0.1% Tween 20 and 3% nonfat dry milk. Antibody binding was detected with horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham Corp.) diluted 1:5,000, using the ECL system (Amersham Corp.). C was blotted with preimmune rabbit serum.

ing frame, indicating that the epitope recognized by the FB-50 mAb derived from a linear amino acid sequence. When the FB-50 cDNA and deduced amino acids were compared with the available sequences in the data banks, it was found to be 81% homologous to a region in the enzyme bovine aspartyl(asparaginyl)β-hydroxylase (7) and 99% homologous to the same region of the human form of the enzyme cloned from an osteosarcoma cell line MG-63 (19). The partial FB-50 cDNA clone was shown to lack 480 nucleotides in the amino terminal coding region, as well as 1,450 nucleotides spanning the COOH-terminal coding and 3' untranslated regions. The full length ORF (Fig. 4) was obtained using specific oligonucleotide-based PCR (see Methods).

To further confirm that the FB-50 mAb recognized HAAH and to insure the specificity of the expression cloning technique, the following experiments were performed: first, polyclonal antibodies were produced against a recombinant fusion

partial cDNA are underlined. The amino acids and nucleotides found different from the human sequence cloned from the osteosarcoma cell line MG-63 (19) are indicated in relief. Three putative glycosylation sites (NXT, NXS) are indicated by a double underlining. A putative transmembrane domain is bracketed. A putative basic dipeptide endoplasmic reticulum retention motif (22) is underlined and indicated in bold type. The His-2 motif (8, 23) is underlined with a dashed line.

protein derived from the FB-50 mAb partial cDNA clone. As shown in Fig. 5, the FB-50 mAb and the polyclonal antibodies raised against the recombinant fusion protein recognized the same cellular proteins in FOCUS cell lysates. Rabbit preimmune serum used as a negative control did not recognize FB-50 protein bands. In addition, immunoprecipitation of a FOCUS cell lysate with both antibodies followed by a Western immunoblot analysis with the reciprocal antibodies also revealed the same protein bands (data not shown). The multiple bands detected (Fig. 5) reflect the proteolysis of the extended, noncatalytic NH<sub>2</sub>-terminal region of the hydroxylase that has been described for the bovine enzyme (6).

**Characteristics of the HAAH cDNA.** The complete ORF (Fig. 4) contains 2,277 bp that encode for a predicted protein having 759 amino acids and a molecular weight of 85 kD. Nearly complete identity (99%) was found between this sequence and that reported by Koriath et al. (19), but there were differences at residues 565 (Tyr to Ile), 575 (Trp-Trp-Thr changed to Cys-Gly), 585 (Asp to Gln) and 709 (Arg to Lys). A nucleotide difference was also found at position 161 (TCG to TCA) that does not modify the corresponding Ser residue. It is noteworthy that the amino acids predicted at residues 565, 575, 585, and 709 from the cDNA derived from FOCUS cells were also found in the bovine sequence (7), suggesting conservation of these residues between the two species.

Bovine aspartyl(asparaginyl) $\beta$ -hydroxylase is associated with the endoplasmic reticulum (10). Computer analysis of the deduced HAAH sequence revealed one area of high hydrophobicity as judged by the Kyte and Doolittle model (20). This hydrophobic region (also present in the bovine sequence) is located between residues 54 and 75 and is flanked by positively charged amino acid residues at its amino-terminal side and negatively charged residues at its carboxyl-terminal side. This

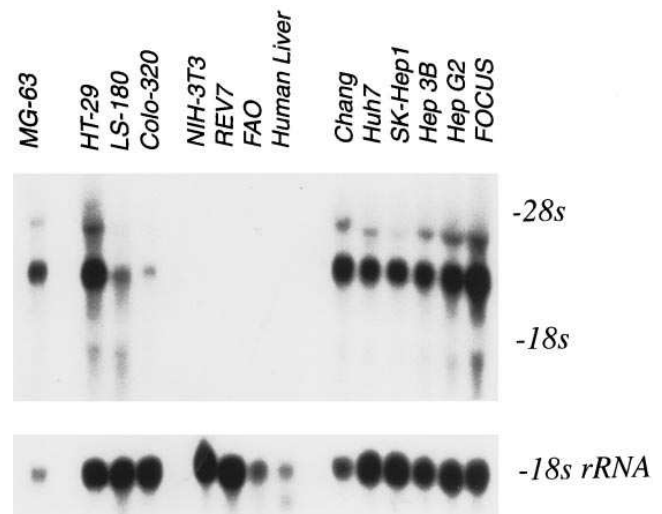


Figure 6. Northern blot analysis of HAAH gene expression in human tumor cell lines. An 18S rRNA oligonucleotide has been used as a control for assessment of RNA loading and integrity.

hydrophobic region is the only putative transmembrane domain in the protein; together with the positions of the adjacent basic and acidic amino acids, it very likely functions as the signal anchor of a type II transmembrane protein (21). The predicted orientation of the hydroxylase would have its NH<sub>2</sub> terminus in the cytoplasm and the COOH terminus, containing the catalytic domain, in the lumen of the endoplasmic reticulum (N<sub>cyt</sub>C<sub>exo</sub>). Furthermore, the Arg-Lys at residues 3 and 4 is an NH<sub>2</sub>-terminal basic motif that has been shown to target type II membrane proteins to the endoplasmic reticulum (22).

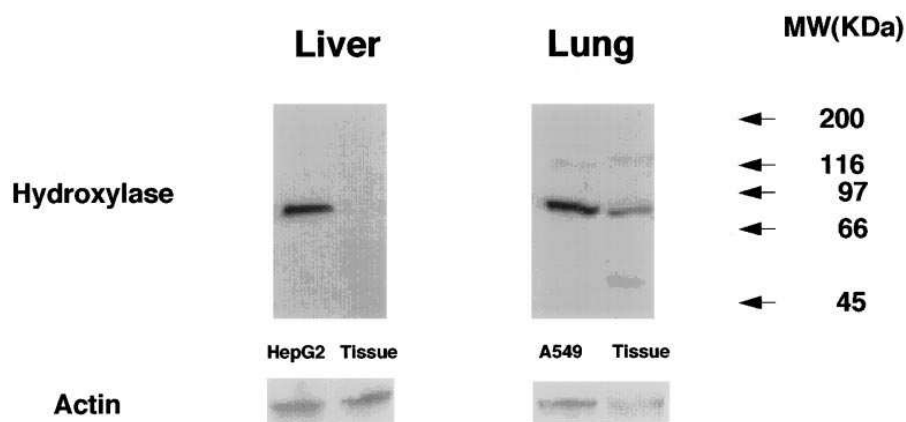


Figure 7. Western immunoblot analysis of HAAH extracted from lung and liver carcinoma cells compared to their normal tissue counterparts. Confluent cell cultures were harvested and washed with PBS. Cells (10<sup>8</sup>) were suspended in 3 ml ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 10 mM DTT, 0.9% NP-40, and the following protease inhibitors: 1  $\mu$ g/ml, aprotinin; 1  $\mu$ g/ml, leupeptin; 1  $\mu$ g/ml, pepstatin; 1 mM, PMSF; 0.01%, soybean trypsin inhibitor). The cells were then lysed on ice by sonication using three 30-s pulses at 1-min intervals at 50% maximum intensity (Sonifier 450; Branson Ultrasonics Corp., Danbury, CT). The lysate was centrifuged at 105,000 g for 60 min. Proteins in the super-

natant (~20 mg/ml) were precipitated by adding 0.7 g solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to 1 ml. After 60 min at 4°C, the suspension was centrifuged and the pellet was dissolved in 0.5 ml lysis buffer to a protein concentration of 30 mg/ml. 1 g of thawed human lung or liver was cut into smaller pieces that were washed with PBS, resuspended in 10 ml ice-cold lysis buffer, and homogenized on ice three times at top speed (Tissumizer; Tekmar Co., Cincinnati, OH) for 10 s at 1-min intervals. The homogenates were subjected to sonication, and then centrifuged as above. Proteins in the supernatant (~40 mg/ml) were precipitated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and dissolved in 1 ml of lysis buffer to a final concentration of 30 mg/ml (as described above). Protein (100  $\mu$ g) from each extract was subjected to SDS-PAGE. After transfer to nitrocellulose, immunoblotting was carried out as follows: the membranes were washed and blocked as described in Fig. 4; blotting was for 18 h at 4°C with rabbit monospecific antisera to the 52 kD bovine hydroxylase (6); an identical membrane was also blotted with a mouse anti-human actin mAb as an internal control; after washing, bound antibodies were detected either by goat anti-mouse IgG antibodies coupled to alkaline phosphatase (Bio Rad Laboratories, Hercules, CA) or by goat anti-rabbit IgG antibodies coupled to horseradish peroxidase using the ECL system. The level of hydroxylase protein in the Hep G2 cells was at least 13-fold greater than its level in normal liver, while in the A549 cells it was approximately sixfold more abundant than in normal lung. Estimates were made by scanning densitometry after normalizing for the internal controls.

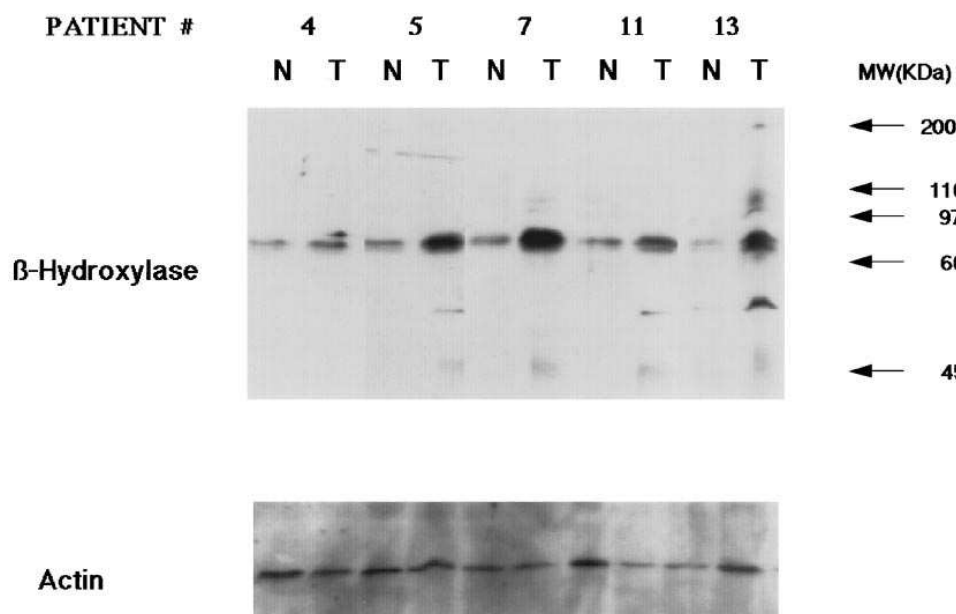


Figure 8. Western immunoblot analysis of HAAH extracted from liver biopsy specimens obtained from five patients with HCC. Protein (100  $\mu$ g) from extracts of each tumor and from its adjacent normal liver parenchyma were fractionated; HAAH was quantitated as described in Fig. 7 except that both bound antihydroxylase and anti-human actin antibodies were detected using the ECL system. The approximate increases of enzyme protein in the tumors compared to their respective normal tissues were: patient 4, 1.5-fold; patient 5, 6-fold; patient 7, 8-fold; patient 11, 4-fold; and patient 13, 15-fold.

Although the bovine enzyme has been shown to be in the endoplasmic reticulum and the cDNA predicts that HAAH would also reside there, FB-50 reacts with intact cells (Fig. 1). This indicates that the epitope recognized by FB-50 is present on the surface of these cells. This situation may be analogous to that of the protein ERGIC-53, which contains an endoplasmic reticulum retrieval signal, but which, when overexpressed, is transported to the cell surface (23).

The overall identity with the bovine enzyme is 68%, but, in regions suggested to be functionally important (7), conservation is considerably higher: for the COOH-terminal catalytic domain, amino acids 310–759 (90%); for the putative transmembrane domain, amino acids 54–75 (100%). In addition, the “His-2 motif” (amino acids 679–697), present in  $\alpha$ -ketoglutarate-dependent dioxygenases (8, 24) and containing a histidine essential for  $\text{Fe}^{2+}$ / $\alpha$ -ketoglutarate binding (8), is identical.

Finally, in a fluorescence in situ hybridization analysis for subchromosomal localization of the gene, all 80 of the metaphase cells examined by in situ hybridization exhibited specific labeling of the proximal long arm of chromosome 8. Measurements of 10 such chromosomes demonstrated that the gene is located 14% of the distance from the centromere to the telomere of chromosome arm 8q, an area corresponding to band 8q12 (data not shown). Band 8q12 is a region known to undergo translocations that are associated with salivary gland pleomorphic adenomas (25, 26), although there is as yet no evidence that these adenomas contain altered levels of HAAH.

Table II. HAAH Activity of Crude Extract from Human Carcinoma Cells and Normal Tissue Counterparts

Enzyme source	Activity* ( $\text{CO}_2$ released, in picomoles)
A549 cell culture	239 $\pm$ 10
Human lung tissue	25 $\pm$ 4
Hep G2 cell culture	133 $\pm$ 16
Human liver tissue	14 $\pm$ 0.1

\*The average value of two assays, each run in duplicate.

**HAAH gene expression in transformed cell lines.** Northern blot analysis of total cellular RNA, using the partial FB-50 cDNA as a probe (Fig. 6), revealed a major transcript of  $\sim$  2.8 kb and a minor transcript of  $\sim$  5 kb in the FOCUS, Hep G2, Hep 3B, and HuH 7 human HCC cell lines, the SK-Hep 1 liver adenocarcinoma cell line, and in transformed Chang cells. Transcripts were also detected in a number of other human tumor cell lines. Consistent with the  $^{125}\text{I}$ -FB-50 mAb binding studies, little, if any, transcript was detected when total cellular RNA from normal human liver was analyzed. As has been reported (19), when polyA<sup>+</sup> RNAs were analyzed, transcripts of roughly equivalent abundance were detected in preparations from normal liver, lung, heart, kidney, pancreas, skeletal muscle, and placenta and, at lower abundance, from brain. These transcripts were found to be elevated  $\sim$  10-fold in several human tumor cell lines relative to their normal tissue counterparts (data not shown). For both the bovine (7) and human (19), (Fig. 4), the size of the ORFs ( $\approx$  2.3 kb) are such that the smaller of the two transcripts are large enough to contain the entire ORF. While the sizes of the larger transcripts are consistent with that of the full length cDNA, the relative contributions of the two transcripts to cellular levels of the enzyme remain to be determined.

**Elevated levels of hydroxylase protein and activity in carcinoma cell lines and in primary HCCs.** When Western blotting

Table III. HAAH Activity of Crude Extracts from Human Hepatocellular Carcinoma Tissues and from Adjacent Uninvolved Liver

Patient number	Enzymatic activity ( $\text{CO}_2$ released, in picomoles)	
	Normal	Tumor
4	17 $\pm$ 13	13 $\pm$ 4
5	< 1	42 $\pm$ 9
7	2.7 $\pm$ 2	105 $\pm$ 1
11	10 $\pm$ 4	39 $\pm$ 1
13	< 1	26 $\pm$ 2

was carried out using a monospecific antiserum (6) directed against the carboxyl-terminal catalytic domain of the bovine hydroxylase (as opposed to the FB-50 mAb, which is directed against an epitope in the amino terminal noncatalytic region of the human enzyme), the levels of hydroxylase protein in Hep G2 cells and A549 cells were greater than in normal liver and lung tissue, respectively (Fig. 7). The molecular weight of the major species in both the carcinoma cells and in normal tissues is  $\sim 72$  kD. With longer exposure (data not shown), the blots of the carcinoma cell preparations reveal multiple molecular weight species (50–120 kD)<sup>2</sup>. Previously, multiple species of hydroxylase (52–90 kD) were identified in bovine liver (6).

When equivalent amounts of total protein from preparations of normal lung or liver or from the carcinoma cell lines were assayed for hydroxylase activity, the activities in the carcinoma cells were substantially higher ( $\sim 10$ -fold) (Table II). The lower levels of enzyme activity in the extracts of normal tissues did not result from the presence of an endogenous inhibitor(s) since, in incubations of extracts to which were added purified bovine hydroxylase, there was no inhibition of the activity of the bovine enzyme (data not shown). Furthermore, the specific activities of the hydroxylase found in the normal lung and liver are quite comparable to those previously reported for bovine liver and mouse L cells (9, 10); thus, the marked increases seen with the carcinoma cell lines represent true increments over what is observed in normal tissue.

When similar analyses were made comparing HCCs to adjacent uninvolved liver from five patients, enzymatic activity was much higher in the tumor tissue from the four patients whose immunoblots revealed increased hydroxylase protein in the malignant tissue (Fig. 8, Table III). Thus, the increase in hydroxylase enzymatic activity extends to malignant tissue.

## Discussion

To characterize cellular genes that are upregulated with malignant transformation of human hepatocytes, a library of mAbs was produced against the FOCUS HCC cell line. The antigen recognized by the mAb FB-50 was particularly abundant. The fact that immunoreactivity was maintained in Western blots under reducing conditions suggested that the epitope was linear. Accordingly, it was possible to clone the antigen, which was identified as HAAH, by directly immunoscreening the FOCUS cDNA library. Our studies indicate that high level expression of HAAH is not restricted to the FOCUS cell line; rather, it is found with a wide variety of tumor cell lines. Furthermore, the enzyme is overexpressed in a significant proportion (40%) of primary HCCs and in all 20 cholangiocarcinomas examined as well as in breast and colon carcinomas. The high levels of HAAH seen in syncytiotrophoblasts demonstrate that this increased expression is not restricted to carcinomas. In this regard, in preliminary studies we have also found increased HAAH expression in mouse fetal tissues (data not

shown). The increased expression may be characteristic of growth in normal tissues.

Recent results from several independent lines of research highlight a need for determining the relevance of the increase of HAAH in transformed cells. A series of reports have documented that deletion mutants, lacking the EGF-repeat rich extracellular portions of *Drosophila* Notch (27–30) or of homologous invertebrate (31, 32) and vertebrate proteins (33–36), which are involved in cell fate determinations and cellular differentiation, are gain-of-function mutants. This suggests that in these transmembrane proteins, the extracellular domain regulates signal transduction by the cytoplasmic domain. Many of the EGF-like repeats in the extracellular domain (more than 20 of the 36 repeats in *Drosophila* and mammalian Notches) contain the putative consensus sequence (12) for aspartyl(asparaginyl) $\beta$ -hydroxylation. The low abundance of these proteins has to date precluded a direct demonstration of  $\beta$ -hydroxyaspartate or -asparagine in them. However, it seems likely that at least some, if not all, of the consensus-containing EGF repeats in Notch homologues are hydroxylated since the enzyme is present in all mammalian (mouse, rat, bovine, human) (6, 9) and invertebrate (*Drosophila*, *Spodoptera*) (37) species in which it has been sought and is widely distributed in adult and fetal tissues (19). It is tempting to hypothesize that the extent and/or pattern of  $\beta$  hydroxylation modulates the regulation of signal transduction by the extracellular domain.

Two of the aforementioned gain-of-function mutations are particularly noteworthy. First, the mouse mammary tumor virus genome, which contains no known oncogene, induces mammary tumors by acting as an insertional mutagen (14), integrating at one or more specific (int) loci. Integration at the int-3 locus causes transcription of a novel truncated 2.3-kb cellular RNA species, the deduced amino acid sequence of which shows significant homology with the intracellular, ankyrin repeats of Notch (38). The untranslated portion of the gene located 5' to the integration site encodes for the 36 EGF-repeat rich extracellular domain of Notch. Transgenic mice, containing a DNA fragment composed of the 3'-end of the mouse mammary tumor virus genome and the flanking cellular int-3 sequences, developed focal and, in some instances, poorly differentiated mammary and salivary adenocarcinomas (14). Second, in some human T lymphoblastic neoplasms, the TAN-1 gene, a human homolog of *Drosophila* Notch that maps to chromosome 9, is broken by a recurrent (7, 9) (q34, q34.3) chromosomal translocation (13). The translocation separates the TAN-1 gene into two nearly equal portions, one consisting of the extracellular domain and the second comprised of the transmembrane and cytoplasmic domains. When a cDNA, composed of this second portion of the gene, was inserted via a retroviral vector into mouse bone marrow cells, which were subsequently transplanted into lethally irradiated syngeneic mice, the mice developed T cell leukemias (14a).

In addition to being an integral part of receptors involved in cell growth and differentiation, EGF domains containing the consensus sequence for hydroxylation may also act as important regulation elements in ligands. The vitamin K-dependent protein encoded by the growth-arrest-specific gene 6 has recently been shown to be a ligand for the oncogenic receptor Axl (39, 40). The growth-arrest-specific gene 6 protein contains four EGF-like domains with the hydroxylation consensus sequence (41) and can be predicted to be hydroxylated. While vitamin K-dependent gamma glutamyl carboxylation may be

2. Western immunoblots of these preparations show different patterns (Fig. 5 vs Fig. 7). This occurs because the multiple molecular weight species derive from proteolysis of the amino terminal portion of the enzyme and because the FB-50 mAb (Fig. 5) and the monospecific antiserum (Fig. 7) recognize different epitopes. Whether the proteolysis is ordered or random is not known and we have not characterized the resulting amino terminal fragments.

required for full stimulation of receptor autophosphorylation and, presumably, signal transduction (39, 40), a prototype receptor tyrosine kinase is the EGF receptor; by analogy, the EGF domains in the growth-arrest-specific gene 6 protein may participate in signal transduction and/or receptor endocytosis and recycling. Here, too, the extent of  $\beta$  hydroxylation could determine the binding affinity of the protein ligands, the extent of autophosphorylation and/or the efficiency of signal transduction by these receptor tyrosine kinases. Receptor endocytosis and recycling could also be affected, as it could for Notch and its homologues.

These experimental systems offer new opportunities to define the function subserved by the  $\beta$  hydroxylation of specific aspartic acid and asparagine residues in proteins. They further provide a starting point for studies designed to determine whether the substantially increased activity of HAAH in carcinomas is merely associative or contributes to the generation and/or maintenance of the malignant phenotype.

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