Growth-inhibitory Activity and Downregulation of the Class II Tumor-suppressor Gene *H-rev107* in Tumor Cell Lines and Experimental Tumors

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Abstract. The *H-rev107* gene is a new class II tumor suppressor, as defined by its reversible downregulation and growth-inhibiting capacity in *HRAS* transformed cell lines. Overexpression of the *H-rev107* cDNA in *HRAS*-transformed ANR4 hepatoma cells or in FE-8 fibroblasts resulted in 75% reduction of colony formation. Cell populations of *H-rev107* transfectants showed an attenuated tumor formation in nude mice. Cells explanted from tumors or maintained in cell culture for an extended period of time no longer exhibited detectable levels of the H-rev107 protein, suggesting strong selection against *H-rev107* expression in vitro and in vivo. Expression of the truncated form of H-rev107 lacking the COOH-terminal membrane associated domain of 25 amino acids, had a weaker inhibitory effect on proliferation in vitro and was unable to attenuate tumor growth in nude mice. The *H-rev107* mRNA is expressed in most adult rat tissues, and immunohistochemical analysis showed expression of the protein in differentiated epithelial cells of stomach, of colon and small intestine, in kidney, bladder, esophagus, and in tracheal and bronchial epithelium. *H-rev107* gene transcription is downregulated in rat cell lines derived from liver, kidney, and pancreatic tumors and also in experimental mammary tumors expressing a *RAS* transgene. In colon carcinoma cell lines only minute amounts of protein were detectable. Thus, downregulation of *H-rev107* expression may occur at the level of mRNA or protein.

RAS gene mutations contribute to a large proportion of human cancers. Point mutations activating one of the three *RAS* genes are detected in 50% of colon carcinomas and adenomas >1 cm in size (Vogelstein et al., 1988), in ~90% of pancreatic tumors, in 30% of lung carcinomas and myeloid leukemias, and in 50% of thyroid tumors (for review see Bos, 1989). In most of these tumors, *RAS* mutations are present in early stages of the neoplastic disease, suggesting a role in the initiation of malignant transformation. The normal RAS protein is activated by GDP/GTP nucleotide exchange factors in response to the binding of different ligands to their cognate receptors. Thereby, RAS couples extracellular stimuli with the activation of genes controlling proliferation, differentiation, and growth arrest (for review see Marshall, 1995). In its active, GTP-bound form, RAS targets the protein kinase, Raf, to the cell membrane, where Raf initiates a protein kinase cascade resulting in transcription factor activation (Leevers et al., 1994; Stokoe et al., 1994). RAS is inactivated by hydrolysis of GTP to GDP. Mutations affecting amino acids 12, 13, or 61 within the RAS protein not only lead to a reduction of its intrinsic GTPase activity but also to its resistance to the action of various GTPase activating proteins that highly enhance the GTP to GDP conversion of normal RAS. These RAS mutants are trapped in the active GTP-bound state and constitutively stimulate downstream kinases. Similar effects can also be achieved by mutations in one of these GAPs, such as *NF1*. Mutations within the coding sequence of the *NF-1* gene disrupt this activity and are thought to contribute to neoplasia associated with neurofibromatosis type I (Cawthon et al., 1990; Viskochil et al., 1990). Recently, considerable progress has been made in the elucidation of the pathways activated by GTP-bound RAS (for review see Marshall, 1995). Several different protein kinases, including Raf-1, PI-3-kinase, PKC-ζ, and MEK kinase, have been identified as downstream effectors of RAS. All of them stimulate distinct protein kinase cascades, resulting in mitogenic stimulation and morphological alterations (Marshall, 1995). These effects are thought to be mediated via distinct transcriptional targets of the protein kinase

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cascades. Two well known genes activated in response to oncogenic RAS-activation are the immediate–early genes *Fos* and *Jun* encoding the heterodimeric AP-1 transcription factor. The AP-1 complex stimulates the transcription of genes encoding tissue remodeling enzymes relevant for the metastatic phenotype such as type IV collagenase and the metalloprotease stromelysin (Scher et al., 1983; Muller et al., 1988; Chauhan et al., 1991). Gene products involved in cellular metabolism, such as glucose transporters or ornithine-decarboxylase, are also found at increased levels in RAS-transformed cells and may contribute to the increased division rate of tumor cells (for review see Aoyama and Klemenz, 1993).

Oncogenic mutations of Ras also lead to the transcriptional downregulation of critical genes. They encode the F-actin capping proteins such as gelsolin (Müllauer et al., 1993), matrix modifying enzymes such as rrg-1 (Contente et al., 1990), and the F-actin bundling proteins tropomyosin (Prasad et al., 1993) and vinculin (Fernandez et al., 1992). In addition, the expression of protein kinase C family isoforms is abrogated after activation of oncogenic *HRAS* (Geiges et al., 1995), and some downregulated genes have been described with as yet unknown function, e.g., the 322 gene (Lin et al., 1995). Several of those genes were shown to be reexpressed in the revertant cell lines derived from *HRAS*-transformed fibroblasts (for review see Schäfer, 1994). Therefore, the reversible functional loss of these gene activities may be causally involved in the process of neoplastic transformation. These observations suggest a specific interference of the proteins encoded by downregulated genes with the oncogenic signaling pathways.

We have established a series of closely related immortal, nontumorigenic 208F rat fibroblasts, *HRAS*-transformed FE-8 cells, and phenotypic revertants for the identification of transcriptional targets suppressed by an activated *HRAS* gene (Schäfer et al., 1988). Using cDNA subtraction and differential hybridization, we analyzed the expression pattern of known transformation-sensitive genes in these cells (Oberhuber et al., 1995) and isolated new sequences, designated *H-rev* genes, associated with the normal and the revertant phenotype (Hajnal et al., 1993, 1994; Kiess et al., 1995). One of these genes, *H-rev107*, a novel, 16-kD protein without known sequence homologies, is expressed in immortal rat fibroblasts, downregulated after *HRAS*-transformation, reexpressed in the revertant clone F9, and also highly expressed in two independent *RAS*-resistant fibroblast lines (Hajnal et al., 1994). This result suggested that the *H-rev107* gene was directly involved in the resistance toward *HRAS*-mediated transformation. In this study, we demonstrate the growth-inhibitory effects of *H-rev107* overexpression on *HRAS*-transformed cells in vitro and in vivo. Furthermore, we show the ubiquitous expression of *H-rev107* in normal tissues and its downregulation in rat tumor cell lines and in experimental tumors.

Material and Methods

Cell Lines and Cell Culture

The *EJ-ras* transformed rat hepatoma cell line ANR4, the rat colon carcinoma cell lines DHD and WB2054, the rat pancreatic tumor cell lines CRI-D2 and CRI-D11, and the *Ki-RAS* transformed rat kidney cell line KNRK were obtained from the European Collection of Animal Cell Cultures (Porton Down, UK). The immortal rat pancreatic cell line AR4-IP and the transformed rat pancreatic cell line AR4-2J were obtained from Dr. O. Hagenbüchle (Lausanne, Switzerland). The immortal rat kidney cell lines, NRK 49F and NRK 52E, were obtained from the American Type Culture Collection (Rockville, MD). 208F (Quade, 1979) and REF52 (Franza et al., 1986) are immortalized, nontumorigenic rat fibroblast cell lines. The FE-8 cell line is an *HRAS*-transformed derivative of 208F (Griegel et al., 1986). Clone F9 is a phenotypic revertant cell line derived from FE-8 (Schäfer et al., 1988). Cells were grown in DME medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml), standard medium. ANR4 cells were grown in Williams E medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). DHD cells were grown in DME/Hams F10 medium (1:1); AR4-2J, AR4-IP, and WB2054 cells were grown in RPMI medium supplemented with the same additives as above.

Northern Blot Analysis

Total cellular RNA was isolated as described (Chomczynski and Sacchi, 1987). 10 μg of RNA was denatured using glyoxal and separated in 1% agarose gels (McMaster and Carmichael, 1977), and transferred to nylon membranes (Genscreen plus, New England Nuclear, Boston, MA). Northern blots were hybridized with a 523-bp *H-rev107* cDNA fragment encoding the 5′ untranslated sequence and part of the coding sequence as a probe. Fragments were ^{32}P labeled by random priming (Feinberg and Vogelstein, 1983), and hybridization was carried out either in 50% formamide, 10% dextran sulfate, 1 M sodium chloride, 5X Denhardt's solution, and 100 μg/ml denatured salmon sperm DNA at 42°C, or in the Quickhyb solution from Stratagene (La Jolla, CA) at 65°C.

H-rev107 Expression Plasmids

H-rev107 cDNA was cloned into expression vectors in which transcription of the inserted DNA is driven by the cytomegalovirus promoter. Plasmids pCMVTK107+ and pCMVTK107− encode *H-rev107* mRNA in sense and in antisense orientation, respectively (Hajnal et al., 1994). Plasmid pCDNA-107+ was constructed by cloning the 900-bp BamHI insert, obtained from the cDNA clone pBS107C, into the BamHI site of pCDNA3 (Invitrogen, San Diego, CA). This fragment contains the complete coding region of *H-rev107*, 33 bp of 5′ untranslated region, and 386 bp of 3′ untranslated region. Plasmid pCDNA-107− drives transcription of antisense *H-rev107* mRNA. The pCDNA107 ΔC vector was constructed by PCR amplification of a 416-bp fragment obtained from pBS107C using the *H-rev107* specific primers 5′ TTGAATTCAT GCCCATACCAGAACCCAAG 3′ and 5′ GGGAGCTCTCGCCACCTTGACGATGTCT 3′, followed by insertion of the PCR product into the EcoRI and XhoI sites of pCDNA3. The termination codon is just downstream of the XhoI site in the vector. This cDNA clone expresses only the first 135 amino acids of the H-rev107 protein. For the production of a tetracycline-inducible *H-rev107* (pUHD-107), a 714-bp BamHI fragment from cDNA clone pBS107B (Hajnal et al., 1994) was cloned into the BamHI site of the plasmid pUHD 10-3 (Gossen & Bujard, 1992).

DNA Transfections and In Vivo Tumorigenicity Assays

Plasmid DNA was transfected into 208F, FE-8, and ANR4 cells by calcium–phosphate precipitation, as described (Wigler et al., 1978). 5 μg of linearized recombinant vector DNA, 1 μg of linearized plasmid pY3 carrying the hygromycin B resistance gene, and 20 μg genomic carrier DNA were used for transfection of 10^6 recipient cells. 48 h after transfection, cells were subjected to selection in standard medium containing 400 μg/ml hygromycin B. When colonies had reached a size of 500–1,000 cells, the cells were trypsinized and pooled. H-rev107 protein expression was analyzed by immunofluorescence using the anti-H-rev107 antibody. To obtain FE-8 cells harboring a tetracyclin-inducible H-rev107, the cells were first transfected with the plasmid pUHD172-1neo and stable clones selected as described above. This plasmid expresses the modified tetracycline repressor which was turned into an activator (Gossen et al., 1995). These clones were analyzed by transient transfection using the plasmid pUHC13-3 containing the luciferase-reporter gene under the control of a tet operator (Gossen and Bujard, 1992). Clones showing a preferable ratio of induction to background luciferase actvity were chosen for further transfection with the pUHD-107 plasmid and clones selected by cotransfection with the pY3 plasmid as described above. 48 h after induction of H-rev107 expres-

sion using 2 μg/ml doxycyclin, protein expression was monitored either by Western blot analysis or by immunofluorescence. For control cells (10-3-3), the pUDH10-3 vector alone was used instead of pUHD-107. For tumorigenicity assays, 10^6 cells were injected subcutanously into either side of ICR nu/nu mice. Tumor growth was controlled daily. After the indicated time the animals were killed, tumors removed, and either frozen in liquid nitrogen or explanted in tissue culture medium after digestion with trypsin/EDTA. Tumor cells were incubated in standard medium containing 50 μg/ml gentamicin.

Preparation of Cell Extracts and Western Blot Analysis

For subcellular fractionation, adherent cells were washed twice in PBS, incubated in hypotonic lysis buffer (10 mM Tris, 0.1 mM dithiothretiol, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 50 μg/ml TLCK, pH 8) for 5–10 min on ice, and lysed using a Dounce homogenizer. Nuclei were obtained after centrifugation at 500 *g* at 4°C for 10 min, washed twice in PBS, and purified via centrifugation through a sucrose gradient (Blobel and Potter, 1966). The 500 *g* supernatant was centrifuged at 100,000 *g* at 4°C for 30 min to obtain the membrane fraction. Cytoplasmic proteins were precipitated from the 100,000 *g* supernatant using methanol/chloroform (Wessel and Függe, 1984). All pellets were resuspended in SDS sample buffer, boiled for 5 min, and stored at −20°C. Protein concentration was determined using amido black staining of protein extracts on nitrocellulose filters (Schaffner and Weissmann, 1973). Equal amounts of protein were separated on 16% polyacrylamide gels by SDS-gel electrophoresis (Laemmli, 1970) and transferred to nitrocellulose membranes (Immobilon P; Millipore Corp., Milford, MA). Membranes were blocked overnight in TBST (10 mM Tris, pH 8, 150 mM NaCl, 0.05% Tween 20) containing 1% BSA, incubated with affinity purified anti-H-rev107 antibody (Hajnal et al., 1994) diluted 1:1,000 in TBST for 4 h. Membranes were washed three times for 5 min in TBST and incubated for 45 min with an anti–rabbit horse-radish peroxidase coupled antibody and processed according to the manufacturer's instructions (ECL-Kit; Amersham Intl., Buckinghamshire, England).

Immunohistochemistry and Immunofluorescence

Indirect immunoperoxidase staining on frozen tissue sections was performed using the Vectastain-kit (Vector Laboratories, Burlingame, CA) according to the protocol of the manufacturer. In blocking experiments, the H-rev107 antiserum was preabsorbed with 8 μg/ml of bacterially expressed H-rev107 protein prior to incubation with the sections. For immunofluorescence analysis, cells were grown on glass coverslips coated with polyornithine (100 μg/ml). After fixation in 3% paraformaldehyde in phosphate-buffered saline for 20 min, cells were permeabilized using 0.5% Triton X-100 in PBS for 2 min. Coverslips were incubated with the H-rev 107 antiserum, diluted 1:100 in PBS/1% BSA, washed in PBS, incubated with a DTAF-conjugated goat anti–rabbit antibody (Dianova GmbH, Hamburg, Germany), washed in PBS, mounted in 5% Mowiol and analyzed with a fluorescence microscope (Zeiss Inc., Oberkochen, Germany). For nuclear staining, cells were incubated with 1 μg/ml Hoechst 33258 fluorescent dye together with the second antibody.

Results

Growth-inhibitory and Tumor-suppressing Activity of the H-rev107 Protein

REF52 cells which overexpress *H-rev107* (Hajnal et al., 1994), are resistant to *RAS* transformation, and forced overexpression of *RAS* blocks their growth and eventually causes cell death (Hirakawa and Ruley, 1988). Interestingly, the adenovirus E1A nuclear protein, which represses the *H-rev107* gene, abolishes the resistance of REF52 cells to *RAS* (Hajnal et al., 1994). These observations suggested that *H-rev107* is responsible for *RAS*-induced growth arrest or cell death. To test the hypothesis that overexpression of *H-rev107* is growth inhibitory for *RAS*-transformed cells but not for normal cells, we have stably transfected different *H-rev107* cDNA expression constructs into immortal, nontumorigenic 208F rat fibroblasts, into the *HRAS* transformed rat fibroblasts FE-8, or into the EJ-*RAS* transformed rat hepatoma cell line ANR4. FE-8 cells do not express endogenous *H-rev107*, while ANR4 cells exhibit very low levels of endogenous *H-rev107* mRNA (Fig. 1 *A*). The plasmid pCDNA107+ directs moderate expression of the full-length *H-rev107* cDNA. An antisense expression vector pCDNA107−, the plasmid pCDNA107 ΔC expressing the truncated *H-rev107*

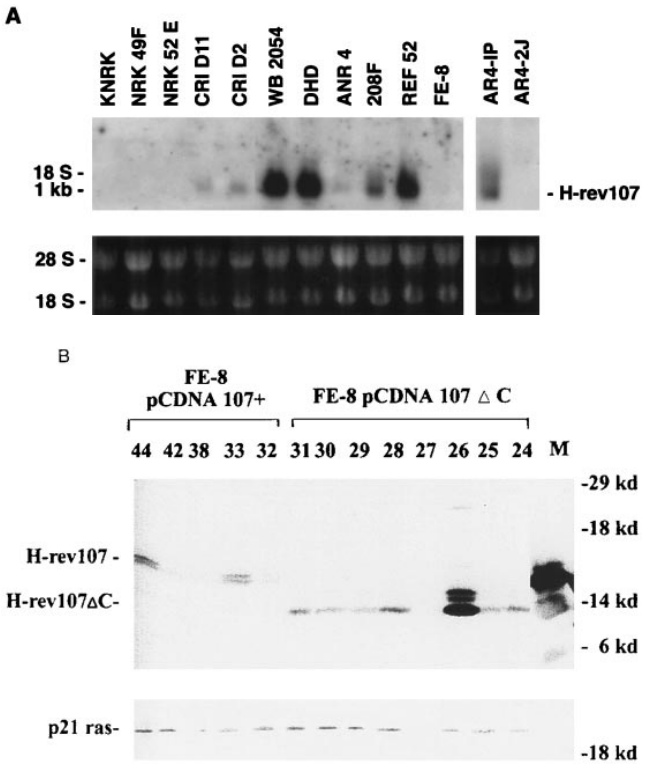


Figure 1. (*A*) *H-rev107* mRNA expression in rat tumor cell lines. *H-rev107* expression is detected in the immortal rat pancreas epithelial cell line AR4IP, in the immortal nontumorigenic rat fibroblasts 208F and REF52, and in F9, a phenotypic revertant cell line, derived from FE-8. No or low *H-rev107* mRNA is present in ANR4 (*EJ-RAS* transformed rat liver cell line), AR42J, CRI-D2, CRI-D11(rat pancreatic tumor cell lines), KNRK (Ki-RAS transformed rat kidney cell line), NRK 49E epitheloid rat kidney cell line, NRK 52F fibroblastoid rat kidney cell line, and FE-8, *HRAS* transformed derivative of 208F. High expression of *H-rev107* mRNA is detected in DHD and WB2054 (rat colon carcinoma cell lines). The 1-kb *H-rev107* mRNA transcript, 18S, and 28S rRNA used as size markers are indicated. (*B*) Western blot analysis of H-rev107 protein overexpression in stably transfected FE-8 cells. 100 μg of crude extract from hygromycin B-resistant clones transfected either with pCDNA107+ (clones 32, 33, 38, 42, 44) or pCDNA107 ΔC (clones 24–31) were separated by electrophoresis through a 16% SDS-polyacrylamide gel, transferred to nitrocellulose, and incubated with the H-rev107 polyclonal antiserum. The full-length H-rev107 protein of ~16 kd is detected only in clones 33 and 44; the truncated form of 14 kd is detected in clones 24–26 and 28–31. 10 ng of recombinant H-rev107 protein were loaded together with the marker (*M*) giving a strong signal of 16 kd, reactive with the H-rev107 antiserum. The same extracts were used to investigate expression of the p21 HRAS gene product, shown in the lower panel. All clones express the RAS protein except clone 27.

cDNA, and the plasmid pCDNA3 were used as controls. The cloning efficiencies of FE-8 and ANR4 cells, following the introduction of the plasmid pCDNA107+, were reduced by 62 and 75%, respectively, as compared to control transfections (Table I).

After stable transfection of FE-8 cells with pCDNA107+, only 5 of 13 isolated FE-8 clones showed significantly elevated levels of the H-rev 107 protein, as determined by Western blots. In contrast, eight out of nine clones transfected with pCDNA107 ΔC expressed the truncated form of the H-rev107 protein (Fig. 1 *B*). Immunofluorescence analysis of the five independent pCDNA107+ transfectant clones using the H-rev107 antiserum, revealed that only 20–50% of the cells were positive for the protein. Within a few weeks of culture, the fraction of H-rev107 positive cells decreased to <10% (data not shown). A significant reduction of colony formation was not observed in transfections of nontumorigenic 208F cells excluding an unspecific toxicity of *H-rev107* expression plasmids (Table I). These experiments showed that the level of *H-rev107* expression inversely correlated with the clonogenicity of *HRAS*-transformed cells transfected with the full-length *H-rev107* cDNA. Moreover, there was a strong selection against *H-rev107* expressing cells during continued culture of transfectants.

To confirm this, we also transfected the pCMVTK107+ vector, which yields very high H-rev107 protein levels in the transfected cells due to the presence of the thymidine kinase leader sequence cloned in front of the *H-rev107* cDNA. Transfection of this *H-rev107* expression vector did not yield individual stable ANR4 transfectant clones, while only few colonies were formed after selection of FE-8 cells. None of 11 clones tested expressed *H-rev107*, whereas HRAS protein expression was maintained, indicating that a very high *H-rev107* expression in the presence of HRAS does not permit permanent proliferation of transfectants in vitro. In addition to the stably transfected cells, FE-8 fibroblasts harboring the *H-rev107* cDNA under the control of a tetracycline-inducible promoter (pUHD-107), were tested for their cloning efficiencies with or without induction of the H-rev107 protein (Fig. 2). A reduction in cloning efficiency similar to that observed with constitutively expressed H-rev107 was obvious in cells with pUHD-107 (Fig. 2). Upon prolonged culture of these cells in the presence of doxycycline, there was again a selection against H-rev107 expressing cells (data not shown).

To analyze the effects of *H-rev107* overexpression on proliferation in vivo, we generated FE-8 and ANR4 transfectants with the same expression vectors as above. To minimize loss of *H-rev107* expressing cells due to prolonged culture in vitro, colonies resistant to the selective agent were trypsinized, after they had reached a size of more than 200 cells, and pooled. 8 d after trypsinization and replating we performed immunofluorescence analysis to determine the fraction of H-rev107 positive cells. A representative culture of FE-8 transfectants is shown in Fig. 3 (*A* and *C*). To quantitate the H-rev107 positive fraction of cells transfected with pCMVTK107+, we counted the cells present in several independent microscopic fields of FE-8 and ANR4 transfectants. The proportion of H-rev107 positive cells was 56% in a total of 614 cells counted. FE-8 and ANR4 cells transfected with the antisense expression vector, or transfected with a pCDNA3 plasmid directing the expression of an unrelated gene, formed progressively growing tumors after a latency period of 4–6 d. In transfected FE-8 populations, the latency period increased only marginally, while it increased to 9–13 d in ANR4 populations (Fig. 4). The tumors formed after injection of FE-8 and ANR4 cell pools transfected with the high expressing plas-

Table I. Colony Formation of Cells Transfected with H-rev107 Expression Vectors and Control Plasmids

Cell type	Transfected cDNA	Number of colonies/ 10^5 cells/μg plasmid DNA	Percentage of colony growth
ANR4	pCDNA107+	1.18 ± 0.35	25.7
ANR4	pCDNA107−	5.4 ± 0.7	117.9
ANR4	pCDNA107 ΔC	2.76 ± 0.42	60.3
ANR4	pCDNA3	4.58 ± 0.52	100
FE-8	pCDNA107+	62.5 ± 15.62	38.6
FE-8	pCDNA107−	193 ± 24.33	119.1
FE-8	pCDNA107 ΔC	111.2 ± 17.73	68.6
FE-8	pCNDA3	162 ± 7.79	100
208F	pCDNA107+	310 ± 54.2	98.4
208F	pCDNA107−	267.6 ± 11.39	84.9
208F	pCDNA107 ΔC	340 ± 43.93	107.9
208F	pCDNA3	315 ± 30	100

10^6 cells were cotransfected with 1 μg pY3, 5 μg recombinant expression vector, and 20 μg of high molecular weight carrier DNA. Stable transfectants were selected in medium containing hygromycin B. Resistant colonies were counted in triplicate cultures, and numbers were normalized to the number of colonies obtained per μg DNA in 10^5 transfected cells. Colony numbers obtained after transfection with the control plasmid pCDNA3 were arbitrarily taken as 100% colony growth. H-rev107 expression vectors were cloned as described in Materials and Methods. FE-8 cells (*HRAS*-transformed rat 208F), ANR4 cells, (*HRAS*-transformed rat hepatocellular carcinoma), and 208F cells (nontransformed, immortalized rat fibroblasts).

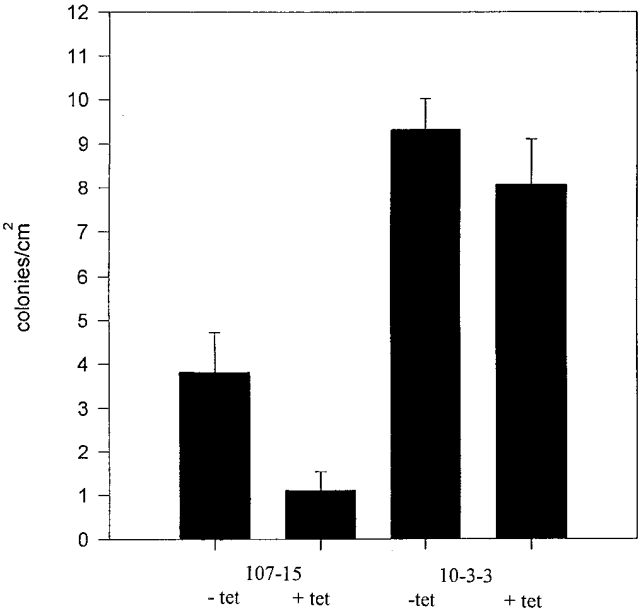


Figure 2. Tet-inducible FE-8 transfectants. Colony formation of FE-8 cells expressing H-rev107 (*107-15*) and control cells containing the vector only (*10-3-3*) after induction with doxycycline. 2000 cells/25 cm^2 tissue culture flask were plated and grown either in the presence (+) or on the absence (−) of 2 μg/ml doxycycline. Medium containing fresh doxycycline was changed every three days. Samples were analyzed in duplicates.

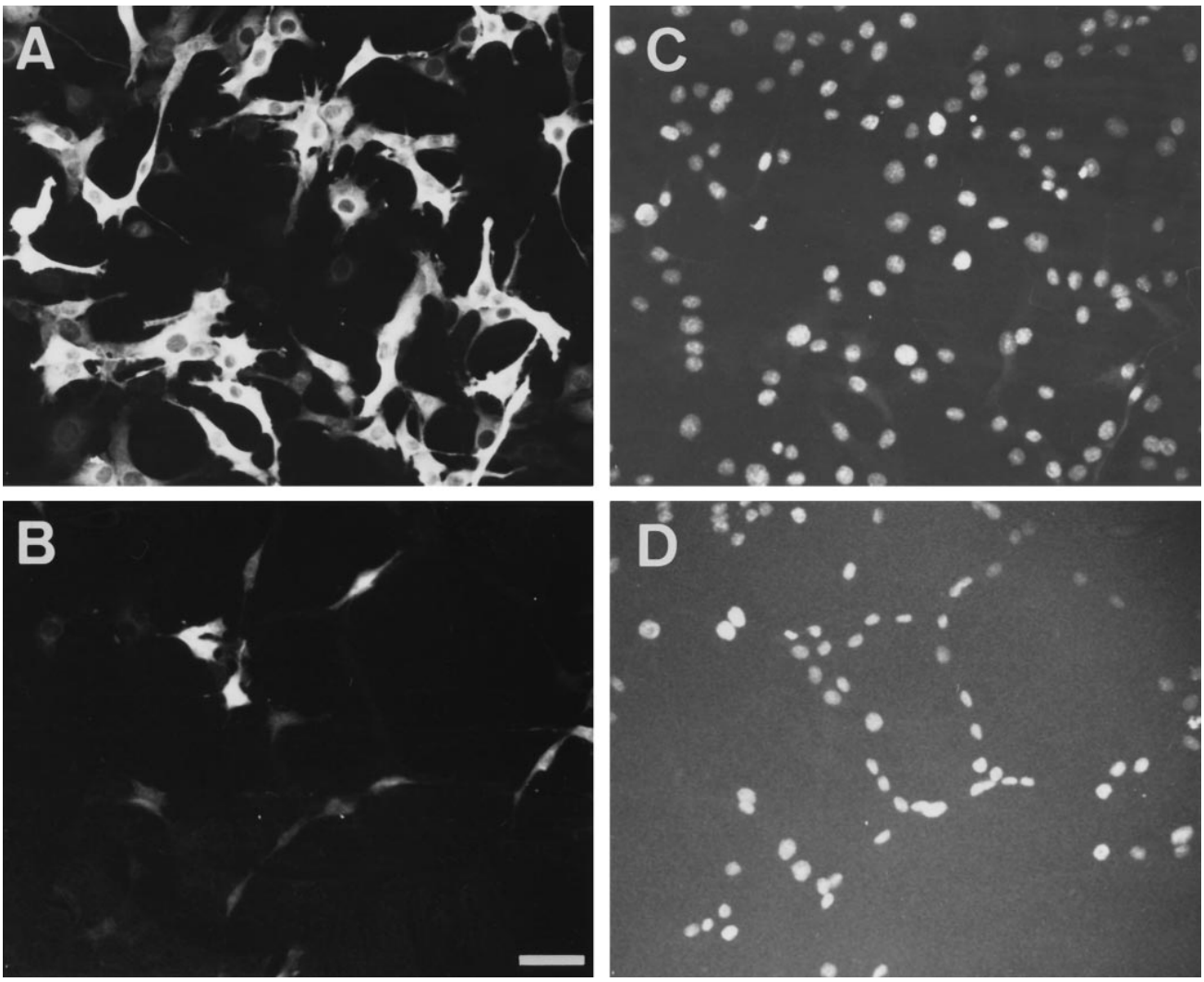


Figure 3. Loss of *H-rev107* expression during tumor growth in vivo. *H-rev107* expression in populations of FE-8 cells stably transfected with pCMVTK+ cDNA. (*A* and *B*) Cells were incubated with the *H-rev107* antiserum and stained with a DTAF-conjugated goat anti–rabbit antibody. (*C* and *D*) Nuclei were stained with Hoechst 33258. (*A* and *C*) Transfected cells prior to injection into nude mice. (*B* and *D*) Cell cultures explanted from tumor and grown in culture. Photographs of cells before injection and after explantation were taken using the same exposure times. Bar, 20 μm.

mid pCMVTK107+ enlarged more slowly than control transfectants until about day 11. Afterwards, the differences in tumor sizes originating from cells transfected either with full length *H-rev107* cDNA or *H-rev107* antisense constructs gradually disappeared. No significant differences in the size of tumors could be observed at 20 d after injection. Cells transfected with the truncated *H-rev107* cDNA (pCDNA107 ΔC) did not suppress tumor growth in vivo (Fig. 4). After 14 or 20 d the mice were killed, and cells from the tumors were explanted, grown in tissue culture, and analyzed for H-rev107 protein by immunofluorescence. The proportion of transfected tumor cells positive for H-rev107 protein was reduced as compared to the original cells. A representative culture of tumor explants is shown in Fig. 3 (*B* and *D*). Counting of independent microscopic fields of FE-8 transfectants (866 cells counted) and ANR4 transfectants (784 cells counted) revealed an average of 15% of cells staining positive with the H-rev107 antibody. These results suggest that either *H-rev107* expression was downregulated in most of the cells during in vivo passaging or that the H-rev107 expressing cells are efficiently overgrown by the nonexpressing cells.

Intracellular Localization of the H-rev107 Protein

The product of the *H-rev107* gene is an intracellular protein of 160 amino acids found either in the cytoplasmic or in the membrane fractions of cell extracts. Density-dependent growth arrest of 208F fibroblasts leads to an accumulation of the protein in the membrane fraction (Hajnal et al., 1994). To analyze the subcellular localization of this in vitro, weakly expressed protein in more detail, *H-rev107* was transiently overexpressed in COS cells or in 208F immortal rat fibroblasts. Expression of the full-length protein using the strongly expressing vector pCMVTK-107+ was analyzed by Western blot technique, after biochemical fractionation of cellular extracts, and by immunofluorescence staining and subsequent inspection by confocal laser

microscopy. Western blotting of nuclear, membrane, and cytoplasmic extracts of cells overexpressing H-rev107 revealed presence of the protein in all three fractions (Fig. 5 *A*). The cytoplasmic fraction shows a single protein band, while an additional band with slightly lower electrophoretic mobility is detected in the nuclear and the membrane fractions, suggesting posttranslational modification of the protein.

A plasmid expressing a truncated *H-rev107* cDNA (pCDNA-107 ΔC), lacking the last 25 codons, was also overexpressed in COS cells. The missing region encodes predominantly hydrophobic amino acids and is probably responsible for membrane association. Deletion of this hydrophobic region leads to a loss of the H-rev107 protein in the nuclear and in the crude membrane fractions. The shortened H-rev107 ΔC protein is entirely found in the cytoplasmic fraction (Fig. 5 *A*), suggesting that the COOH-terminal hydrophobic domain mediates the binding of H-rev107 to an intracellular membrane. In cells overexpressing full-length H-rev107, immunofluorescence analysis revealed no staining within the nucleus and at the plasma membrane. Rather, the H-rev107 protein appears to be linked to the nuclear membrane and to membranes in the perinuclear space (Fig. 5, *B* and *C*). As a considerable amount of the protein is present in the cytoplasm, staining of the free protein presumably masks the exact localization of the membrane-bound polypeptide.

Expression of H-rev107 mRNA in Normal Adult Rat Tissues, Tumor Cell Lines, and Experimental Tumors

High levels of *H-rev107* mRNA expression have been observed in the *RAS*-transformation-resistant fibroblasts REF 52 and EK3 and in the phenotypic revertant cell line F9 obtained from *HRAS*-transformed cells. To further investigate the role of *H-rev107* in neoplasia, we compared the expression of the gene in various normal rat tissues and tumor cell lines. Northern blot analysis of mRNA obtained from adult rat tissue showed ubiquitous expression of the *H-rev107* gene (Fig. 6). Strong expression was observed in brain, kidney, liver, stomach, large intestine, spleen, muscle, and heart. A weaker expression was detected in breast and small intestine, and a very weak signal was observed in placenta. The H-rev107 protein is detectable in protein extracts from epithelial tissues of adult rats (Hajnal et al., 1994). Immunohistochemistry on cryosections of normal rat tissues using the H-rev107 antibody was used to localize H-rev107 expression in the tissues. In the colon, strong H-rev107-specific staining was limited to the differentiated epithelial cells. Similarly, epithelial specific expression was found in stomach, small intestine, and esophagus, in tracheal and bronchial epithelium, in testis, and in transitional epithelium of the bladder (Fig. 7). Weaker expression was also observed in the cardiac muscle and in spleen (data not shown).

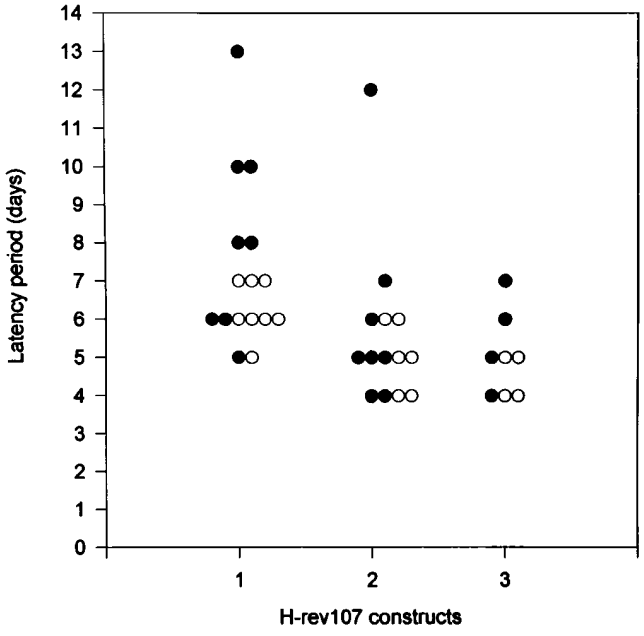


Figure 4. Attenuation of tumor growth in nude mice mediated by overexpression of *H-rev107* cDNA in FE-8 and ANR4 cells. 10^6 cells from pools of FE-8 cells (○) and ANR 4 cells (●) stably transfected with the following plasmids: 1, pCMVTK-107+ (sense); 2, pCMVTK107- (antisense); 3, pCNDA 107 ΔC (truncated), were injected subcutaneously into either side of nude mice. Latency period of individual tumors measured is the time until tumor nodules were palpable.

In contrast to the normal tissues the rat liver carcinoma cell line ANR4 expressed only low levels of *H-rev107* mRNA. No mRNA was detectable in two immortal kidney cell lines, NRK 49F and NRK 52E, or in the *Ki-RAS* transformed kidney cell line KNRK. The highest expression levels were detected in the colon carcinoma cell lines WB2054 and DHD (Fig. 1 *A*). The *H-rev107* sequences transcribed in these tumorigenic cell lines are presumably wildtype, since analysis of RNAs prepared from WB 2054 and DHD cells by RT-PCR, SSCP, and sequencing of PCR fragments did not provide evidence for mutations in the coding sequences of *H-rev107.* However, analysis of the H-rev107 protein expression using the H-rev107 antibody revealed very low levels in WB2054 and no detectable staining in DHD (data not shown). *H-rev107* mRNA was also expressed in the immortal, nontumorigenic pancreatic cell line AR4 IP and in normal human pancreas (Husmann, K., C. Sers, and R. Schäfer, manuscript in preparation). Very low levels were present in the two pancreatic carcinoma lines Cri-D11 and Cri-D2, and no *H-rev-107* mRNA could be detected in the tumorigenic pancreas line AR4 2J (Fig. 1 *A*).

To find out if *H-rev107* expression is altered in malignant tissues, we analyzed tumors derived from transgenic mice carrying an activated *HRAS* oncogene controlled by the whey acidic protein promoter. The whey acidic protein promoter is activated upon onset of lactation in the mammary epithelial cells, leading to the development of mammary tumors in certain transgenic lines (Andres et al., 1987). *H-rev107* mRNA expression was absent or very low in tumors L 25, 25.33G, and 25.72 (Fig. 8). Compared to the two control samples (Fig. 8; normal-*A*, normal-*B*), also the tumors 25.81, 25III13B, and 25.89 show a clear reduction in *H-rev107* mRNA levels. Immunohistochemical analysis of the same malignant tissues failed to detect expression of the H-rev107 protein in spite of detectable mRNA. Western blot analysis of preneoplastic 208F rat fibroblasts and mouse NIH3T3 cells had shown a weak signal derived from the endogenous protein (Hajnal et al.,

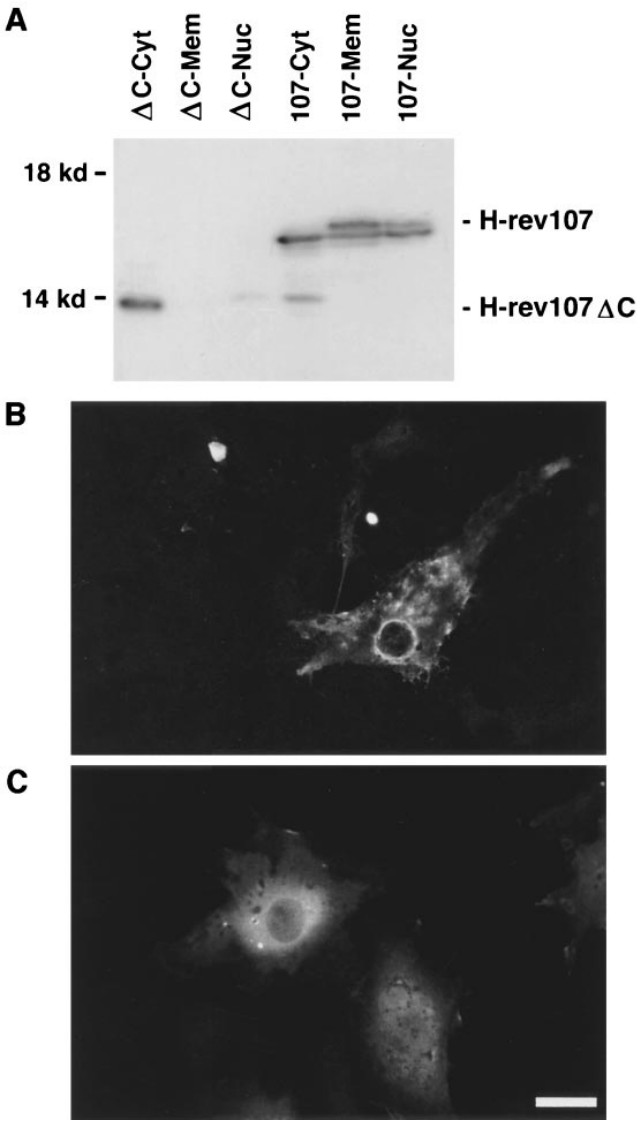


Figure 5. (*A*) Subcellular localization of the full length H-rev107 protein in the nuclear (*107-Nuc*), the membrane (*107-Mem*), and the cytoplasmic fractions (*107-Cyt*) of COS cells transiently transfected with the pCMV-TK107 expression vector. The truncated H-rev107 protein expressed after transfection with the plasmid pCDNA107 ΔC is only detectable in the cytoplasmic fraction (*ΔC-Cyt*) but absent from the nuclear (*ΔC-Nuc*) and the membrane fractions (*ΔC-Mem*). For Western blot analysis, cells were lysed 48 h after transfection and extracts fractionated as described in Material and Methods. 20 μg of each fraction were separated by electrophoresis through a 16% polyacrylamide gel, blotted, and processed using the ECL method. Molecular size markers in kd are indicated, H-rev107 proteins of ~16 kd and the truncated H-rev107 ΔC proteins of ~14 kd are indicated. (*B* and *C*) Immunofluorescence analysis by confocal laser microscopy of ectopically expressed *H-rev107* protein in 208F rat fibroblasts. Cells transfected with the plasmid pCMVTK107+ were grown on coverslips, fixed using 3% paraformadehyde and incubated with the H-rev107 antiserum as described in Material and Methods. Bar, 10 μm.

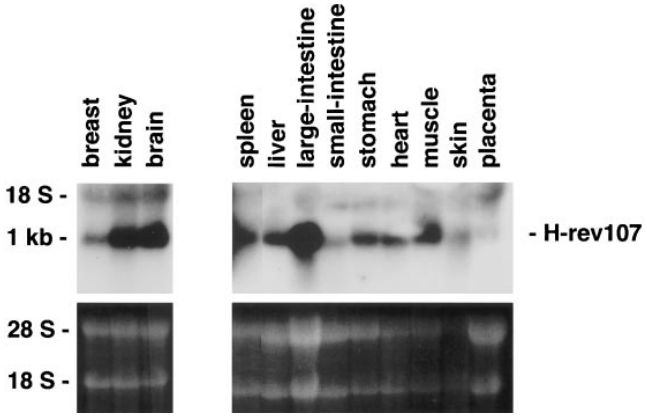


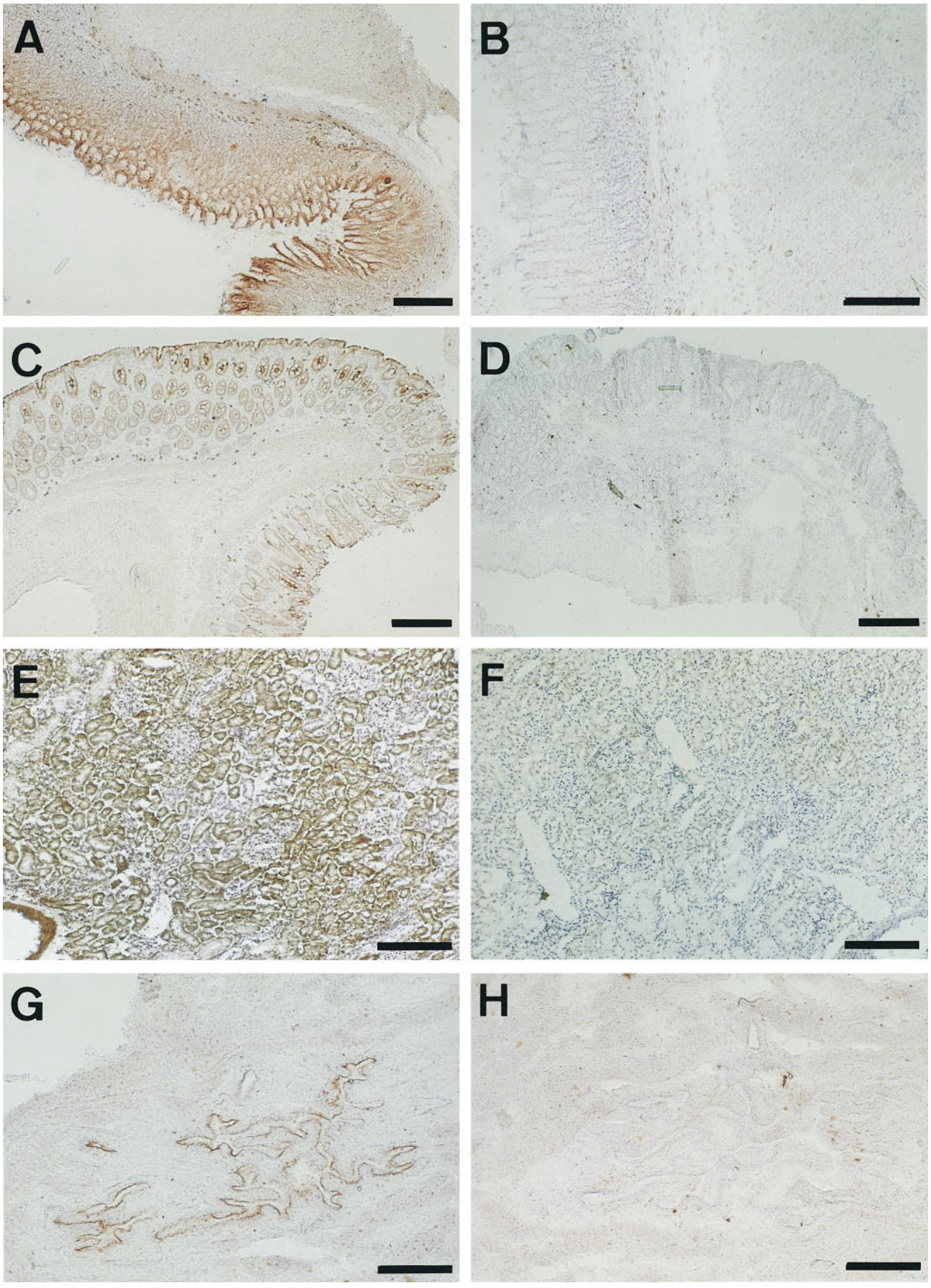
Figure 6. H-rev107 mRNA expression in various normal adult rat tissues. 15 μg of glyoxal-denatured total RNA prepared from the tissues indicated were separated by electrophoresis through a 1% agarose gel, blotted, and hybridized using a 523-bp cDNA fragment encoding part of the *H-rev107* coding sequence. The 1-kb *H-rev107* mRNA transcript, 18S, and 28S rRNA used as size markers are indicated.

1994 and Sers, C., unpublished observations), but detection of the protein in individual cells was not possible. This is most likely due to a very low protein level, a phenomenon also observed in the rat colon carcinoma line WB2054, substantiating the notion that H-rev107 downregulation can occur at the RNA and the protein level.

Discussion

Several lines of evidence suggest that *H-rev107* is a transformation-suppressing gene. We have previously reported that *H-rev107* mRNA is below the limit of detection in *HRAS* transformed cells as shown by Northern blot analysis, and that the gene was upregulated in the phenotypic revertant cell line F9 and in two different *HRAS*-transformation-resistant fibroblast lines (Hajnal et al., 1994). In this paper, we have shown that *H-rev107* downregulation is frequent in tumorigenic rodent cell lines and in murine tumors, while the gene is ubiquitously expressed in the corresponding normal tissues. Most importantly, transfection of *H-rev107* cDNA expression vectors into two different *HRAS*-oncogene expressing cell lines resulted in the suppression of colony formation. Clones permanently overexpressing the *H-rev107* protein could not be obtained. In vivo, ectopic expression of *H-rev107* leads to the attenuation of tumor growth in nude mice. Populations of explanted tumor cells did not express significant amounts of the H-rev107 protein.

What is the mechanism of growth suppression due to the overexpression of the *H-rev107* gene? Only a minor reduction of colony formation was observed in cells transfected with the truncated *H-rev107* gene (Table I). Expression of the truncated gene can be maintained stably in culture and did not result in a reduction of tumor growth (Fig. 1 *B* and Fig. 4). This suggests that the membrane attachment of the protein plays an important role in the proliferation–suppressive function in vivo. The H-rev107 protein is clearly not associated with the plasma membrane in 208F fibroblasts but rather with yet undefined intracellular mem-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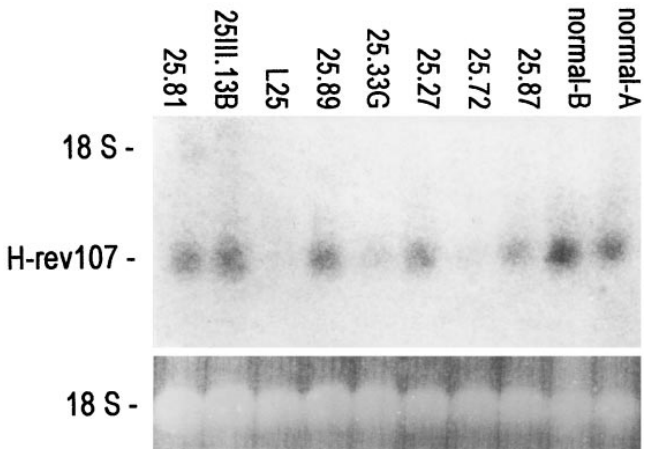

Figure 8. H-rev107 mRNA expression in the normal mammary gland and loss of *H-rev107* mRNA in a subset of mammary tumors of whey acidic protein-*RAS* transgenic mice. Numbers refer to individual tumors. The 1-kb *H-rev107* mRNA transcript, 18S, and 28S rRNA as size markers are indicated.

branes and presumably with the nuclear membrane. An attractive speculation is that the H-rev107 protein may serve as a negative regulator of an oncogenic HRAS-mediated signal by sequestration of kinases or of other growth-regulatory proteins. Members of the signal transduction kinase cascade have been shown to bind to anchoring proteins. These proteins target their catalytically active partners to specific subcellular sites and thereby control activation and phosphorylation of specific substrates (Mochly-Rosen et al., 1991; Coghlan et al., 1995). To understand the role of the *H-rev107* protein in growth suppression in molecular detail, it is important to identify the target membrane and interacting proteins.

With constitutive expression of oncogenic *RAS*, slow growing cell populations overexpressing the *H-rev107* gene are easily taken over by the fast-growing, nonexpressing cell populations. Thus, a detailed analysis of growth arrest in specific stages of the cell cycle was hampered by such an instability of H-rev107 high-expressers among *RAS*-transformants and by the low concentrations of the endogenous H-rev107 protein in cultured cells. The endogenous H-rev107 protein was detected exclusively in differentiated epithelial cells of several tissues, suggesting that it plays a role in differentiation-related growth arrest. We did not observe a significant fraction of apoptotic cells in transfected cell cultures (data not shown). Thus, the reduction of colony formation of transfected *HRAS*-transformed cells and the replacement of *H-rev107*-expressing cells by negative cells in vivo and in vitro may be explained by induced senescence, which is regarded as an important mechanism of tumor suppression (O'Brien et al., 1986; Shay and Wright, 1991).

The *H-rev107* gene can be categorized as a class II tumor suppressor gene which has lost a growth-constraining function in tumor cells due to an expression block (Lee et al., 1991). In class I tumor suppressor genes such as *TP53* or *RB* (for review see Vogelstein and Kinzler, 1992; Riley et al., 1994), loss of function results from mutation or deletion of DNA. Furthermore, the concept of two classes of tumor suppressor genes suggests that some class II genes are the targets of class I genes encoding transcriptional regulators (Lee et al., 1991). An increasing number of class II tumor suppressor genes have been identified on the basis of their downregulation in tumorigenic cells and owing to their transformation-inhibiting activity. The product of the *K-rev* gene, a Ras-like GTP-binding protein, was shown to interfere with oncogenic signaling (Kitayama et al., 1989; Frech et al., 1990; Culine et al., 1992). Other class II genes encode the cytoskeletal proteins tropomyosin (Fernandez et al., 1992) and vinculin (Prasad et al., 1993), the matrix-modifying enzyme lysyl oxidase (Contente et al., 1990; Krzyzoziak et al., 1992; Hajnal et al., 1993), gap junction proteins (Lee et al., 1992), and the invasion modulating serine protease maspin (Zou et al., 1994).

We have observed a concerted expression block to several *H-rev* genes in *HRAS*-transformed cells. Downregulation affected the *H-rev107* gene (Hajnal et al., 1994), the *H-rev142* gene encoding lysyl oxidase (Hajnal et al., 1993; Oberhuber et al., 1995), the *H-rev18* gene encoding Ril, a novel member of LIM double zinc finger proteins involved in differentiation (Kiess et al., 1995), and *Tsp-1* encoding the angiogenesis inhibitor thrombospondin (Shäfer, R., unpublished observations). These findings support the notion that continuous mitogenic signaling by mutated RAS specifically results in the coordinated downregulation of critical genes.

The repression of class II suppressor gene activity mediated by onco-proteins provides an important novel link between the activity of oncogenes and anti-oncogenes. The most intriguing feature of class II tumor suppressor inactivation is perhaps its reversibility triggered by various agents (Contente et al., 1990; Krzyzosiak et al., 1992; Oberhuber et al., 1995). Upregulation of *H-rev* gene expression in phenotypic revertants may occur in a coordinated fashion (Hajnal et al., 1993; Oberhuber et al., 1995). Several drugs have been identified that are able to reestablish the expression of downregulated genes and revert the transformed phenotype (Krzyzosiak et al., 1992; Kumar et al., 1995). The further development of those drugs may provide new therapeutic options for interfering with the activity of oncogenes commonly activated in human neoplasia.

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Figure 7. Immunohistochemical detection of the H-rev107 protein in the terminally differentiated epithelial cells of (*A*) stomach, (*C*) colon, (*E*) kidney, and (*G*) bladder. Analysis on cryostat sections of normal adult rat tissues was performed as described in Materials and Methods. The same tissues were stained after preabsorption of the antiserum with 8 μg/ml purified H-rev107 protein, showing no unspecific reactivity in (*B*) stomach, (*D*) colon, (*F*) kidney, and (*H*) bladder. Bars: (*B–H*) 50 μm; (*A*, *C*, and *D*) 100 μm.

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