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

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Abstract. The \textit{H-rev107} gene is a new class II tumor suppressor, as defined by its reversible downregulation and growth-inhibiting capacity in \textit{HRAS} transformed cell lines. Overexpression of the \textit{H-rev107} cDNA in \textit{HRAS}-transformed ANR4 hepatoma cells or in FE-8 fibroblasts resulted in 75% reduction of colony formation. Cell populations of \textit{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 \textit{H-rev107} protein, suggesting strong selection against \textit{H-rev107} expression in vitro and in vivo. Expression of the truncated form of \textit{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 \textit{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. \textit{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 \textit{RAS} transgene. In colon carcinoma cell lines only minute amounts of protein were detectable. Thus, downregulation of \textit{H-rev107} expression may occur at the level of mRNA or protein.

\textbf{R\textit{as}} gene mutations contribute to a large proportion of human cancers. Point mutations activating one of the three \textit{RAS} genes are detected in 50% of colon carcinomas and adenomas >1 cm in size (Vogelstein et al., 1988), in \textasciitilde 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, \textit{RAS} mutations are present in early stages of the neoplastic disease, suggesting a role in the initiation of malignant transformation. The normal \textit{RAS} protein is activated by GDP/GTP nucleotide exchange factors in response to the binding of different ligands to their cognate receptors. Thereby, \textit{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, \textit{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). \textit{RAS} is inactivated by hydrolysis of GTP to GDP. Mutations affecting amino acids 12, 13, or 61 within the \textit{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 \textit{RAS}. These \textit{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 \textit{NF1}. Mutations within the coding sequence of the \textit{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 \textit{RAS} (for review see Marshall, 1995). Several different protein kinases, including Raf-1, PL-3-kinase, PKC-\textit{\theta}, and MEK kinase, have been identified as downstream effectors of \textit{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.
resistant fibroblast lines (Hajnal et al., 1994). This result demonstrated 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 rg-1 (Contente et al., 1990), and the F-actin bundling proteins tropomyosin (Prasad et al., 1993) and vinculin (Fernandez et al., 1986). Clone F9 is a phenotypic revertant cell line derived from FE-8 (Schafer 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 32P 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-H-rev107+ 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-H-rev107− drives transcription of antisense H-rev107 mRNA. The pCDNA1-H-rev107 vector was constructed by PCR amplification of a 416-bp fragment obtained from pBS107C using the H-rev107 specific primers 5′ TTGAATTCTT GACCATACGAA CACACAAG 3′ and 5′ GGAGATCTTGGC CCACGTTTG A TGTCT 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 106 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 tetracycline-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 pancreatic tumor cell line 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 AR4-23 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) were immortalized, nontumorigenic rat fibroblast cell lines. The FE-8 cell line is an H-RAS transformed derivative of 208F (Griego et al., 1986). Clone F9 is a phenotypic revertant cell line derived from FE-8 (Schafer 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.
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 dithiothreitol, 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 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, diluted from the 100,000 g supernatant using methanol/chloroform (Wessel and Baltimore, 1981). The 500 g supernatant was centrifuged at 4°C for 10 min to obtain the membrane fraction. Cytoplasmic proteins were precipitated from the 100,000 g supernatant by methanol/chloroform (Wessel and Baltimore, 1981). The 500 g supernatant was centrifuged at 4°C for 10 min to obtain the membrane fraction. Cytoplasmic proteins were precipitated from the 100,000 g supernatant using methanol/chloroform (Wessel and Baltimore, 1981).

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-rev107 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. For control cells (10-3-3), H-rev107 antiserum, diluted 1:100 in PBS/1% BSA, washed in PBS, 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-rev107 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

REFS2 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 epithelial 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-rev107 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. 1B). 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 unspecified 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 plasmids were significantly smaller than tumors formed with H-rev107 expressing cells.

**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      | pCDNA107ΔC       | 2.76 ± 0.42                                 | 60.3                        |
| ANR4      | pCDNA3           | 4.58 ± 0.52                                 | 100                         |
| FE-8      | pCDNA107+        | 62.5 ± 15.62                                | 38.6                        |
| FE-8      | pCDNA107ΔC       | 111.2 ± 17.73                               | 68.6                        |
| FE-8      | pCDNA3           | 162 ± 7.79                                  | 100                         |
| 208F      | pCDNA107+        | 310 ± 54.2                                  | 98.4                        |
| 208F      | pCDNA107ΔC       | 340 ± 43.93                                 | 107.9                       |
| 208F      | pCDNA3           | 315 ± 30                                    | 100                         |

10^5 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 are 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 cm² 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.
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).

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 KNKR. 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-rev107 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 4. Attenuation of tumor growth in nude mice mediated by overexpression of H-rev107 cDNA in FE-8 and ANR4 cells. 10⁶ cells from pools of FE-8 cells (○) and ANR4 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.
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 1). 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-

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 C 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% paraformaldehyde, 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.
unspecific reactivity in (instability of H-rev107 high-expressers among specific stages of the cell cycle was hampered by such an cell populations. Thus, a detailed analysis of growth arrest are easily taken over by the fast-growing, nonexpressing growing cell populations overexpressing the mor 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; Krzyzosiak 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 (Kriess 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.

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.
