Partial reversion of the transformed phenotype in HRAS-transfected tumorigenic cells by transfer of a human gene
(suppressor gene/morphological transformation/anchorage independence/ouabain selection)

REINHOLD SCHEAVER, JAYA IYER, ELSBETH ITEN, AND ARTO C. NIKKO
Ludwig Institute for Cancer Research, Bern Branch, Inseleipital, CH-3010 Bern, Switzerland

Communicated by Elwood V. Jensen, November 6, 1987

ABSTRACT The transformed phenotype of rat FE-8 cells transfected by an activated human HRAS gene was suppressed upon fusion with normal cells. An experimental approach was developed to identify and isolate a human gene capable of suppressing the transforming activity of the HRAS oncogene in FE-8 cells. Genomic DNA from human placenta was introduced into FE-8 cells by cotransfection with the plasmid pY3 conferring hygromycin B resistance. Transfectants were selected in medium containing hygromycin B. HRAS-transformed FE-8 cells showed an increased sensitivity toward ouabain when compared to their normal counterparts. Therefore, the population of transfected hygromycin B-resistant cells was treated with ouabain to eliminate cells with a transformed phenotype. Ouabain selection resulted in a small number of cell clones exhibiting a more normal phenotype. The clones had lost the morphology of transformed cells and required anchorage for growth. The tumorigenicity of transfected cells in nude mice was reduced but not completely abolished. FE-8 revertants continued to express the p21 RAS protein. Human repetitive sequences contained in the DNA of a secondary transfectant were used for isolation of the suppressor gene from reverted FE-8 cells. The cloned DNA fragment was transfected into tumorigenic FE-8 cells and conferred a partial reversion of the transformed phenotype.

Tumorigenicity and anchorage-independent proliferation are frequently suppressed in somatic cell hybrids between tumorigenic cell lines and normal diploid cells. From these findings and the consistent detection of chromosome deletions in human hereditary tumors, the hypothesis has emerged that mutation, deletion, or rearrangements of tumor suppressor genes (recessive oncogenes or "anti-oncogenes") contribute to the conversion of a normal cell to the malignant state (for review, see refs. 1–5). These genetic alterations might be cooperative with the activation of dominantly acting oncogenes (6). It has been proposed that normal cellular growth control is reestablished in a tumor–normal cell hybrid because the functions of inactivated genes of the tumor cell are restored by genes of the normal parental cell (1–5). The normal phenotype in tumor–normal hybrids predominates even during the continued expression of transforming RAS genes (7–9). Genetic analysis of suppressed cellular hybrids and of tumorigenic segregants derived from them permitted the assignment of putative suppressor genes to specific human or rodent chromosomes (for review, see refs. 1 and 2). Recently, results from segregation analysis of human intraspecies hybrids were confirmed by microcell transfer experiments. Introduction of human chromosome 11 via microcell fusion into tumorigenic HeLa cells and into a Wilms tumor cell line resulted in the abrogation of tumorigenicity (10, 11). It is desirable to advance these studies to the molecular level and identify and characterize the genes capable of reverting the neoplastic phenotype. The use of microcell fusion or of whole-cell fusion does not allow the access to molecular clones of suppressor genes.

We established a functional assay leading to identification and molecular cloning of DNA sequences involved in genetic suppression of neoplastic transformation. Genomic DNA was prepared from normal human placenta and was transfected together with a dominant selectable hph gene (12) onto a rat cell line transformed by an activated human HRAS gene. Cotransfectants were selected in two subsequent procedures. The antibiotic hygromycin B (HMB) was used to select a cell population of several thousand individually transfected cell clones. HMB-resistant cells were subjected to treatment with ouabain to isolate cell clones that had acquired the normal (transformation-suppressed) phenotype after incorporation of donor DNA sequences. This selection is based on the increased sensitivity of RAS-transformed cells toward ouabain when compared to their normal counterparts (13). Here we report the isolation of primary and secondary DNA transfectants that exhibit a partial suppression of the transformed phenotype. The cells showed morphological reversion, inhibition of growth in semisolid agar medium, and reduced tumorigenicity, although HRAS expression was not impeded. The DNA sequence capable of counteracting the HRAS-induced transformed phenotype of rat cells was isolated from the DNA of a secondary transfectant. Suppressor gene activity resided in an 18-kilobase (kb) BamHI restriction fragment. The cloned human gene was transfected into HRAS transformed rat FE-8 cells and conferred suppression of the transformed phenotype on each of the transfected cell clones.

MATERIALS AND METHODS

Cells and Culture Conditions. FE-8 is a tumorigenic cell clone derived from neoplastic 208F rat cells by cotransfection of the human EJ-RAS gene and the pSV2neo resistance marker. It was selected in medium containing G418 ( Geneticin; GIBCO) at 400 μg/ml. These cells contain four or five copies of the transforming HRAS gene (9). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (standard medium). All cells were tested for mycoplasma contamination and were found to be negative.

DNA Transfection and Selection of Transfectants. High molecular weight DNA was prepared from human placenta as described (14). Cotransfection of human DNA and the plasmid pY3 harboring the HMB-resistance gene (hph) (12) into FE-8 cells was accomplished by calcium phosphate coprecipitation as described by Wigler et al. (15). Thirty

Abbreviation: HMB, hygromycin B.

*For reasons of simplicity, the term suppression is used throughout the text to mean partial suppression.
micrograms of genomic DNA was mixed with 500 ng of plasmid DNA, coprecipitated, and added to each 75-cm² cell culture flask containing 1 × 10⁶ cells. Twenty-four hours after transfection, cells were trypsinized and plated onto five cell culture flasks. Selection in the presence of HMB (GIBCO or Calbiochem) at 400 μg/ml was initiated 24 hr later. Transfectants were maintained in DMEM plus G418 (400 μg/ml) during HMB selection. Colonies of cells resistant to both antibiotics were visible after 10–12 days in transfected cultures. Cultures in which colonies of HMB/G418-resistant FE-8 transfecants had reached a size of 500–1000 cells were trypsinized, pooled, and replated at a density of 1 × 10⁶ cells per cell culture flask. On the following day, cultures were washed twice with serum- and K⁺-free DMEM and then refed with K⁺-free DMEM supplemented with 10% fetal calf serum and 3 mM ouabain (Sigma). After 7 days, ouabain-containing medium was replaced by standard DMEM supplemented with G418 and HMB (each at 400 μg/ml). Colonies visible after 2–3 weeks were isolated in stainless steel cylinders, gently trypsinized, and grown into mass cultures.

**Analysis of Cellular Growth Properties and Tumorigenicity.**

Proliferation in semisolid agar medium was estimated as described (16) by plating 10⁵–10⁶ cells in DMEM containing 10% fetal calf serum and 0.4% (wt/vol) Noble agar (Difco). Colonies were scored after 2–3 weeks. Tumorigenicity was determined by subcutaneous injection of trypsinized cells (10⁶ cells per site) into the flanks of 6-week-old athymic mice.

**Southern Blot Analysis, Immunoblot Analysis, and Molecular Cloning of Reversion-Active DNA Sequences.**

Transformed DNA sequences were detected by Southern blot analysis as described (17, 18). The following 32P-labeled probes were used: the 2.9-kb Sac I fragment of pEJ 6.6 (HRAS) (19), the 1.05-kb Kpn I/Sst I fragment of pY3 (12), and total human placenta DNA previously sheared to an average size of 5 kb. The oncprotein p21 was detected by immunoblot analysis of crude membrane preparations as described (20) using a p21-specific rabbit IgG antibody (supplied by R. W. Sweet, Smith, Kline & French, Swedeland, PA). A recombinant phage library was constructed by inserting BamHI-digested DNA prepared from secondary transfecnt FS9-7 into the BamHI sites of phage EMBL 3 (21). The library was screened for the presence of human repetitive DNA and hph sequences by filter hybridization (22).

**RESULTS**

**Suppression of Transformed Phenotypes in Malignant Rat Cells by Transfection of DNA from Normal Human Cells.**

Transformed FE-8 cells were derived from the phenotypically normal 208F rat cell line after cotransfection of the activated human HRAS-1 gene (EJ-RAS) and plasmid pSV2neo (9). FE-8 cells are able to proliferate in semisolid agar medium with a cloning efficiency of 23%. Individual agar colonies contain >1000 cells. FE-8 cells are highly tumorigenic in athymic mice and newborn rats. The latency period before tumors can be detected after injection of 10⁶–10⁷ cells is <5 days. The transformed and malignant properties of FE-8 cells are partially suppressible upon fusion with normal rat embryo fibroblasts. Suppression did not interfere with oncogene expression (8). The suppression of the neoplastic phenotype was shown to function across the species barrier (1). We tested whether the suppressed phenotype in FE-8 cells can be conferred by transfection of genomic DNA from normal human cells rather than by whole-cell fusion. This experiment is based on the assumption that a single gene is responsible for the suppression of the neoplastic phenotype, that it can be transferred to the acceptor cells in a functional unit by DNA transfection, and that it can be expressed and exert its function in the transfected recipient cells.

DNA from normal human placenta was chosen as a donor in transfection experiments. Its acquisition by the genome of rodent recipient cells can be visualized by hybridization to human repetitive sequences. Human placenta DNA was distributed into ~6000 independently transfected FE-8 subclones by cotransfection with the plasmid pY3 conferring HMB resistance on transfected cells. We expected that upon random integration of human DNA into the recipient cell genomes each donor gene would be represented several times in these transfecnts. The detection of rare transfecnts expressing the normal phenotype due to acquisition of a suppressor gene required a specific selection step to eliminate all transformed transfecnts. We used a selection protocol that is based on the differential resistance of normal cells toward ouabain when compared to RAS-transformed cells. This observation was previously described by Noda et al. (13) for the isolation of flat revertants from Kirsten murine sarcoma virus-transformed cells. The feasibility of ouabain selection was confirmed by monitoring the survival of preneoplastic 208F cells in mixed cultures with tumorigenic FE-8 cells present in a 10⁴-fold excess. Treatment of these cultures with 3 mM ouabain in K⁺-free DMEM for 7 days resulted in the survival of nonmalignant cells only. Subsequently, 1.3 × 10⁶ FE-8 cells (representing ~6000 independent HMB-resistant transfecnts) were treated in the same way. Three weeks after removal of the selective agent, seven clones were detected. The frequency of survivors corresponded to 0.5 colony per 10⁶ selected cells. These clones had lost the highly refractile rounded shape of malignant FE-8 cells and exhibited a flat fibroblast-like morphology (Fig. 1). No such colonies emerged after exposure to ouabain in cultures of 4 × 10⁷ FE-8 cells. Of the seven transfecnts initially isolated, two clones, designated F4 and F9, stably retained a normal flat morphology upon prolonged culture. If the phenotype of normal morphology can be conferred on FE-8 cells by acquisition of a transfected human gene, it should be possible to use the genomic DNA of primary transfecnts to confer the same phenotype on FE-8 cells in a second round of transfection. For this purpose, DNA from stable primary transfecnts F4 or F9 was transfected into FE-8 cells together with pY3 DNA. Transfected cells were again subjected to the two-step

**FIG. 1.** Morphology of tumorigenic FE-8 cells and of ouabain-selected revertants. (a) Nontumorigenic 208F cells; (b) RAS-transformed FE-8 cells; (c) primary transfected revertant F9; (d) secondary transfected revertant FS9-7. (Phase contrast, ×120.)
selection protocol involving HMB to eliminate all cells that had not incorporated exogenous DNA and ouabain as a selective agent to kill all neoplastically transformed cells. About 1500 independent HMB-resistant transfectants were obtained from $6 \times 10^6$ FE-8 cells transfected with F4 DNA. From this population, one clone was found after ouabain selection. F9 DNA was transfected onto $2.4 \times 10^7$ FE-8 cells and 33 colonies were selected from 3000 HMB-resistant clones. Only 1 clone exhibited a stable reversion of transformed colony morphology. In an independent experiment, F9 DNA was transfected into FE-8 cells without addition of the selectable marker plasmid, and 7 $\times 10^7$ cells were transfected and selected in DMEM containing HMB. The ouabain selection was omitted. Of 4 clones isolated, 3 clones, designated FS9-5, FS9-6, and FS9-7, exhibited an altered morphology of an intermediate type ranging between the one of malignant FE-8 cells and the morphologically normal F9 revertant. Secondary transfectant FS9-7 retained its intermediate morphology after prolonged culture (Fig. 1). We conclude from these results that the phenotype of a more normal morphology can be transferred from primary to secondary transfectants and stably expressed.

Primary and secondary stable transfectants exhibited reduced cloning efficiencies in agar medium ranging from 2% to $<0.001\%$. The few individual agar colonies contained <200 cells. Proliferation in standard medium on a plastic support was not affected. Transfectants were less tumorigenic than the highly malignant FE-8 recipient cells. A reduction in tumor takes and prolonged latency periods were observed. Tumors were formed in 3 of 4 nude mice after 25 days, when $10^6$ cells were injected per site, and in 26 of 36 nude mice in a period ranging from 14 to 60 days after injection of $10^4$ cells per site. This contrasts to a 100% tumor formation by FE-8 cells within <5 days at the same cell doses.

A possible mechanism for the reduction in transformed phenotypes of the ouabain-selected FE-8 cells might be the interference with the synthesis of the p21 HRAS protein. For this reason, p21 levels were quantitated and compared in the FE-8 cells and in primary and secondary transfectants with the suppressed phenotype. Increased p21 expression was found in HRAS-transfected FE-8 cells (Fig. 2, lane b) when compared to nontumorigenic 208F cells (lane a). No significant decrease in p21 protein levels can be observed in the revertants (lanes c-e). A change in p21 expression is therefore probably not responsible for the suppressed phenotype. This conclusion was supported by the demonstration that an active oncogene was present in these cells—i.e., that the oncogene had not been inactivated by a mutational event during the selection. DNA was prepared from primary transfectant F9 and was used to transfect preneoplastic mouse NIH 3T3 cells. NIH 3T3 cell transfectants were injected subcutaneously into athymic mice. Tumors appearing after a latency of 15 days were excised and DNA was prepared from them. Tumor DNAs contained a 6.6-kb BamHI fragment indicative of the transfected EJ-RAS gene originating from FE-8 cells (Fig. 3). These results confirm our notion that the phenotypic reversion of FE-8 cells is due to the expression of a transfected suppressor gene.

**Molecular Cloning of the DNA Sequence Conferring the Suppressed Phenotype on Malignant FE-8 Cells.** The transfer of the suppressed phenotype in two subsequent transfection cycles suggested to us that the altered phenotype of FE-8 transfectants had been induced by acquisition of exogenous human DNA sequences. Integration of human donor DNA and of cotransfected hph genes was confirmed by a blot analysis of DNAs from primary and secondary transfectants. Total human DNA and the 1.05-kb Kpn I/Ssr I fragment of plasmid pY3 were used as probes. DNA from primary transfectants contained multiple fragments hybridizing to both of these probes (data not shown). Unique fragments were detectable in secondary transfectants (Fig. 4). We cloned a human DNA sequence as an 18-kb BamHI fragment from DNA of secondary transfectant FS9-7. This restriction fragment also contained the cotransfected hph gene. A recombinant phage library was constructed by inserting BamHI-digested FS9-7 DNA into the BamHI sites of phage vector EMBL 3 (21). We screened 600,000 recombinants for the presence of hph sequences and human repetitive DNA by filter hybridization. Three recombinant phages with inserted 18-kb BamHI fragments (designated A97-1, A97-2, and A97-3) were isolated (Fig. 4).

**A Cloned Human DNA Fragment Confers Suppression of the Transformed Phenotype.** The preservation of an 18-kb BamHI fragment of human DNA in secondary transfectant FS9-7 and the acquisition of the suppressed phenotype in this clone suggested to us that a human gene or at least a fragment of it was contained within this restriction fragment and conferred its biological activity. To test for the presence of this gene, recombinant phage DNAs were introduced into malignant FE-8 cells via transfection. Since the coding sequence of the hph gene does not contain any internal BamHI sites, we assumed that BamHI digestion of recombinant phage DNA would not destroy its ability to confer HMB resistance on transfected cells. HMB-resistant FE-8 cells were isolated after transfection of A97-1 and A97-3 DNA. No HMB-resistant FE-8 cells were found after A97-2 transfection. The growth properties in agar medium of six HMB-resistant FE-8 clones isolated after transfection of A97-1 DNA and of four HMB-resistant clones transfected

---

**Fig. 2.** Immunoblot analysis of EJ-RAS p21 protein products in FE-8 cells and revertants. Preparation of membrane extracts, electrophoresis, and detection of p21 were as described (20). Lanes: a, nontumorigenic 208F cells; b, tumorigenic FE-8 cells; c, primary transfectant revertant F4; d, secondary transfectant revertant FS9-5; e, FS9-7; f-h, FE-8 revertants transfected with A97-1 DNA. Samples corresponding to lanes a-e and f-h were on two different gels. Amounts of membrane proteins were equal in all samples.

**Fig. 3.** Southern blot analysis of DNA from FE-8 cells and transfectants. DNA (10 µg) was digested with BamHI, fractionated by electrophoresis, blotted, and hybridized to the 32P-labeled 2.9-kb Sac I fragment of pEJ 6.6. Lanes: a, nontumorigenic 208F cells; b, tumorigenic FE-8 cells; c, primary transfectant revertant F9; d, secondary transfectant revertant FS9-7; e-h, tumors derived from NIH 3T3 cells transfected with F9 DNA; i, nontumorigenic NIH 3T3 cells. The 6.6-kb BamHI fragments indicative of the human HRAS-1 gene are marked by arrows.
with λ97-3 DNA were assayed. The clones were not treated with ouabain after HMB selection. All transfectants appeared to be strongly anchorage dependent (Fig. 5). Introduction of the hph gene by itself did not abolish the anchorage-independent phenotype of FE-8 cells. We conclude from these results that the human DNA sequence located in the 18-kb BamHI restriction fragment adjacent to the hph gene functions as a suppressor gene for the anchorage-independent proliferation of RAS-transformed FE-8 cells. Further structural analysis of the 18-kb BamHI fragment is needed to define the precise composition, organization, and the transcriptional unit contained within this fragment. The expression of the transforming p21 HRAS oncogene was measured in λ97-1 and λ97-2 transfected cell clones. The anchorage-dependent transfectants maintained a p21 expression comparable to FE-8 cells (Fig. 2, lanes f-h). This confirms our conclusion that the synthesis and expression of p21 is not the target of the suppressor gene.

**DISCUSSION**

Suppression of the neoplastic phenotype in mammalian cells transformed by activated RAS genes has been demonstrated by cell fusion with normal cells (7-9). Molecular cloning of the gene(s) involved in suppression has been hampered by lack of a functional assay allowing the transfer and subsequent detection of DNA sequences capable of inducing the suppressed phenotype in malignant recipient cells. Here we describe the DNA-mediated induction of the suppressed phenotype in RAS-transformed rat cells and the isolation of a human DNA fragment active in suppressing anchorage-independent proliferation. The successful isolation of primary transfectants with normal morphology and anchorage requirement for proliferation is based on a two-step selection protocol. In the first step, genomic placenta DNA was distributed into tumorigenic rat cells and the transfected cell population was selected. From these transfected cells, those expressing the fully transformed phenotype were eliminated in a second step. We took advantage of the finding that normal cells are more resistant toward ouabain when compared to RAS-transformed cells (13).

Induction of the suppressed phenotype by the cloned DNA sequence was 10,000-fold more efficient (0.5 colony per ng of DNA per 10⁶ recipient cells) than by human donor DNA or primary transfectant DNA. Stable FE-8 transfectants harboring genomic DNA from normal human cells or the cloned 18-kb BamHI fragment exhibited growth characteristics similar to the ones observed in suppressed somatic cell hybrids of FE-8 cells and normal rat embryo fibroblasts. In these hybrids, the ability of anchorage-independent proliferation was stably suppressed, as indicated by very low cloning efficiencies in semisolid agar medium (9). This suggests that the cloned DNA sequence that is capable of suppressing anchorage-independent growth of RAS-transformed cells functions similarly to the suppressor gene present in hybrids or is homologous to this gene. The reduction in tumorigenicity found in primary and secondary FE-8 transfectants as well as in FE-8 hybrids (9) is possibly due to retarded proliferation in vivo. We do not assume that the reduction in tumorigenicity is associated with alterations in ion transport or ATP metabolism, since the revertants did not acquire a stable ouabain resistance after gene transfer and were able to proliferate in standard medium at diminished rates. Evidence from investigations of both rodent and human cellular hybrids suggests that a different gene (or genes) controls the expression of transformed and tumorigenic phenotypes (for review, see refs. 1 and 2). Thus, more than one gene may be required to completely restore the normal phenotype of RAS-transformed FE-8 cells. Recent investigations on the role of RAS oncogenes in the process of malignant transformation based on gene transfer into cultured cells (23-26) or on the analysis of mice carrying oncogenes as transgenes (27, 28) suggest that expression of an activated RAS gene alone or of complementary RAS and MYC oncogenes is insufficient to induce malignancy in the absence of additional genetic changes. Such genetic alterations possibly involve inactivation of suppressor genes (recessive oncogenes). The molecular identification of a suppressor gene provides the means to test this hypothesis by analyzing its structure and expression at various steps in the malignant progression of human and rat cells as well as in somatic cell hybrids between tumor and normal cells.

Incorporation of the 18-kb BamHI fragment into recipient FE-8 cells did not result in a down-regulation of oncogene...
expression. Rather, the suppressor gene appears to act either at a distant step in the series of molecular events initiated by the activated RAS oncogene (RAS-induced signaling pathway) or in a cellular pathway that is functionally independent of RAS gene action. RAS proteins appear to play an essential role in the transfer of signals to cytoplasmic effectors (for review, see ref. 29). Therefore, specific inhibitors of RAS protein function would aid in defining individual steps in the signaling pathway and in identifying target molecules. The elucidation of a specific RAS-inhibitory function requires identification of the protein product encoded by the suppressor gene isolated in this study.

The retinoblastoma susceptibility gene and a recessive oncogene in *Drosophila* have been isolated by chromosome walking techniques (30–32). Transfer of the lethal (2) giant larvae gene into *Drosophila* mutants resulted in a hereditary suppression of neuroblastoma formation (33). No tumor-suppressing activity has yet been reported for the retinoblastoma gene. DNA-mediated reversion of a spontaneously transformed mouse NIH 3T3 cell clone was described earlier (34). However, an increase in the frequency of induced reversion exceeding that of spontaneous reversion occurred only after the sixth transfection cycle with unintegrated DNA as the donor, and the reversion-active sequence was not cloned. Recently, Padmanabhan et al. (35) reported a high incidence of growth inhibition in HeLa cells following transfection of DNA from quiescent human cells. We speculate that transfer of DNA from normal cells into different tumorigenic indicator cells and selection for phenotypically normal revertants may provide the basis for detection of multiple recessive oncogenes. Indicator cells harboring a defined transforming gene are preferable to spontaneously transformed recipient cells, since revertants resulting from loss or mutation of the known oncogene can be easily detected and excluded from further analysis. The suppressor gene described in this paper is tightly linked to the *hph* gene. Thus, suppressing activity can be assayed in any HMB-resistant transfectant including tumorigenic human cells. The further characterization of the molecular structure and function of recessive oncogenes will provide insights into tumorigenesis and help to define their role in relation to dominantly acting oncogenes. Analysis of the presence and expression of the suppressor gene described here in human tumors and cell lines and consistent involvement will define its significance for cancer diagnosis and therapy.

We thank Dr. B. Groner for constant support and critical discussions, Dr. R. W. Sweet for the p21-specific antibody, and P. Wegmueller for photographic work.