RESEARCH ARTICLE

Transformation of Human Bronchial Epithelial Cells Transfected by Harvey *ras* Oncogene

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Abstract. *Transfection of normal human bronchial epithelial (NHBE) cells with a plasmid carrying the* ras *oncogene of Harvey murine sarcoma virus (v-Ha* ras*) changed the growth requirements, terminal differentiation, and tumorigenicity of the recipient cells. One of the cell lines isolated after transfection (TBE-1) was studied extensively and shown to contain v-Ha* ras *DNA. Total cellular RNA from TBE-1 cells hybridized to v-Ha* ras *structural gene fragment probes five to eight times more than RNA from parental NHBE cells. The TBE-1 cells expressed phosphorylated v-Ha* ras *polypeptide p21, showed a reduced requirement for growth-factor supplements, and became aneuploid as an early cellular response to v-Ha* ras *expression. As the transfectants acquire an indefinite life-span and anchorage independence they became transplantable tumor cells and showed many phenotypic changes suggesting a pleiotropic mechanism for the role of Ha* ras *in human carcinogenesis.*

Two approaches are generally used in studies of oncogenes in human cancer. In one, DNA from human tumor cell lines is transfected (*1*) into mouse NIH 3T3 cells, with the phenotype of the human oncogenes being confirmed by subsequent isolation of the transforming DNA (*2–5*). In the second approach, human tumor cell DNA is mapped by restriction enzyme analysis in order to detect polymorphic changes in human genetic homologs of oncogenes, these oncogenes originally having been detected in avian and rodent systems (*5*).

The use of xenogeneic cells as recipients for oncogene transfection requires that the mammalian cell is capable of expressing the transforming properties of an oncogene that evolved in another species. Thus, oncogenes detected by this system must fulfill the genetic requirements of being dominant, pleiotropic genes capable of altering pathways associated with transformation in different cell types and in different species.

In addition to being sensitive to transformation by human oncogenes, mouse NIH 3T3 cells undergo low-frequency, spontaneous conversion to tumorigenicity (*2*). These cells have proved useful for the initial isolation of transforming genes, but to better understand the properties of oncogenes it would be useful to analyze them in less permissive genetic backgrounds, including primary rodent (*6, 7*) and human cells (*8–10*).

Analysis of oncogenes in human epithelial cells is of particular interest because of the epithelial origin of carcinomas, because epithelial cells undergo differentiation, and because defects frequently occur in the differentiation of carcinoma cells grown in culture (*11, 12*). These defects include escape from the G_1 resting phase of growth that is usually maintained by some types of epithelial cells undergoing terminal differentiation (*13*). Oncogene analysis in transfected normal human epithelial cells would provide information about the mechanism of transformation of a normal progenitor cell of human cancer and about the interaction of oncogenes with growth regulation and differentiation pathways.

In the studies described here we used normal human bronchial epithelial (NHBE) cells. These are the major progenitor cells for lung carcinoma. The cell donor had died as a result of traumatic injury, and an autopsy indicated no cancer-related pathology. Protoplast-fusion was chosen as the means of transfection because of its applicability to NHBE cells and because it results in stable genetic transfection of large numbers of cells, each transfected cell receiving low numbers of inserts in its genome (*14, 15*).

The native form of v-Ha *ras*, as isolated from the Harvey murine sarcoma virus (Ha-MuSV) (*16*), was used to develop a transfection assay for the oncogenic transformation of NHBE cells. The virally transduced v-Ha *ras* gene or its transforming human cellular homologs act in a genetically dominant fashion in transforming NIH 3T3 mouse cells (*3, 16*), thus fulfilling the dominance requirement for oncogenes expected to transform primary human epithelial cells. The association of altered human c-Ha *ras* genes with various types of tumors (*5*) suggests that v-Ha *ras* can transform cells of different tissue types and embryonic origin. The evolutionary conservation of the *ras* gene family and *ras* gene product (a protein of 21,000 daltons, p21) (*17–21*) is consistent with a functional involvement in control of cell growth and differentiation (*2, 18–20*). Biochemical studies of *ras* proteins indicate that the guanosine triphosphate (GTP) binding properties and membrane association of *ras* polypeptides are not altered by changes that activate the gene product (*22*). However, GTP phosphorylase activity, p21 autophosphorylation, and the interaction of p21 polypeptides with epidermal growth-factor receptors are altered by oncogenic changes in the *ras* gene (*22*). In addition, studies of tumorigenicity in animal cell test systems (*23*), the characterization of the *ras* structural gene (*24*) and p21 (*25, 26*), and the association of oncogenic polymorphism of cellular *ras* genes with human lung carcinoma make v-Ha *ras* an attractive oncogene for transfection testing of NHBE cells.

Transfection experiments with NHBE cells. Normal human bronchial epithelial cells grown in serum-free medium (*27, 28*) form homogeneous populations that can be used as transfection recipients in oncogene studies. Since epithelial cells transfected with oncogenes might be un-

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able to differentiate, it might be possible to select NHBE cells transfected with v-Ha *ras* by growing them in the presence of agents that induce differentiation (*28, 29*). For example, NHBE cells in LHC-4 medium undergo terminal squamous differentiation when exposed to small amounts of blood-derived serum (BDS) (*28*) or to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (*29*), whereas v-Ha *ras* transfected cells do not.

For our initial selection of v-Ha *ras* transfectants the cells were cultured in LHC-4 medium with 2 percent BDS for 9 days. Approximately 4×10^6 NHBE cells were transfected at 70 to 80 percent confluence in 60-mm dishes by protoplast fusion (*14, 15*) with a strain of *Escherichia coli* carrying plasmid H1. A complete transforming sequence from the 5′ side of the Ha-MuSV is carried on H1 (*20, 30*). The small size and simple genetic structure of v-Ha *ras*, compared to its oncogenic human cellular homologs, and the ability to identify v-Ha *ras* gene product because of its unique autophosphorylation (*25*), provide the most direct genetic test for Ha *ras* p21 transformation of human epithelial cells.

This selection method yielded v-Ha *ras* transfected colonies at a frequency of approximately 10^{-3}. The transfected colonies were subcultured in several media. All the transfected cell cultures were maintained to isolate potential transformants. Cultures of NHBE cells from other donors were transfected with pSV2gpt (*31*) or pSV2neo (*32*). Transfection frequency was measured by staining and counting colonies after growth in selective media and by comparison with nontransfected cultures in control dishes (*15*). The frequency of cells with the neo^+ or gpt^+ markers in the transfected cell cultures was 1×10^{-3} to 3×10^{-3} (*15*). The estimates of differentiation-resistant colonies growing in LHC-4 medium containing 2 percent BDS were consistent with an approximate total yield of 4×10^3 NHBE cells. Within 9 days of growth in LHC-4 medium containing 2 percent BDS, the untransfected control cultures were induced to differentiate.

The v-Ha *ras* transfected cells were initially subcultured in the same medium with 2 percent BDS (*28*), in MCDB-104 medium containing 5 percent BDS (M104 medium) (*33*), or in RPMI 1640 medium containing 10 percent BDS (HUT medium). M104 and HUT media were used for continued growth of the v-Ha *ras* transfected cells. When the cultures were 80 percent confluent the cells were subcultured by dissociation and reseeding at one-third of this density to test the capacity of the v-Ha *ras* transfected cells for indefinite growth. The transfected cells were subcultured three times at 3- to 5-week intervals for 3 months after the initial selection. All of these cultures, including four that were maintained at high density for the last 8 weeks of the time period, became quiescent at the end of 3 months. Toward the end of 3 months, the cultures underwent a "crisis" during which most of the transfected cells died having completed approximately 10 to 12 cell divisions after the parent culture had differentiated. Four to 8 weeks before they reached the crisis stage the cells maintained at high density formed colonies, indicating partial loss of contact inhibition; four of these colonies in three different dishes survived. These four foci were subcultured as individual colonies and have continued to grow indefinitely (Fig. 1). Each focus contained approximately 2×10^6 viable cells that have continued to grow in culture for more than a year (>120 cell generations); one of these, TBE-1, has been studied extensively (Fig. 1). During the progression of v-Ha *ras* transfected human epithelial cells to tumorigenicity, v-Ha *ras* DNA sequences, v-Ha *ras* transcripts, and phosphorylated p21 gene product were found consistently as soon as we could obtain enough cells to carry out the experiments.

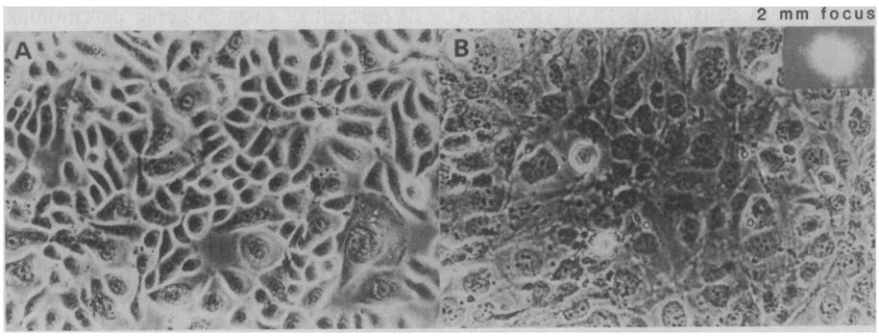

Fig. 1. Transformed focus (TBE-1) and first monolayer outgrowth of NHBE cells transfected with v-Ha *ras*. (A) Monolayer culture of NHBE cells transfected by protoplast fusion with plasmid H1 carrying v-Ha *ras* at passage 1. (B) Altered morphology of TBE-1 at the first monolayer outgrowth. Inset: One of four 2-mm foci that arose at the time the culture was undergoing senescence (10 to 12 weeks).

Phenotypic properties of TBE-1 cells. We tested TBE-1 cultures to determine their growth characteristics, response to TPA, ability to form colonies of anchorage independent cells in soft agar, and tumorigenicity in athymic nude mice. The results indicated that TBE-1 cells (i) are not induced to squamous terminal differentiation by either BDS or $10^{-7}M$ TPA; (ii) do not produce a growth factor recognized by normal rat kidney (NRK) cells (*34, 35*); (iii) do form colonies in soft agar; and (iv) do produce a TBE-1 growth factor, since population doublings per day increased by a factor of 5.0 when autogenously conditioned medium was used to supplement cell growth at clonal density (Table 1).

When athymic nude mice were injected with TBE-1 cells before an anchorage independent population had been selected, nodules less than 0.1 cm in size developed at a frequency of 2 per 16 animals tested. These nodules regressed after 14 days (Table 1). Persistent tumors, less than 0.8 cm in size, developed in 1 of every 25 animals. TBE-1 tumors were removed for histopathologic analysis at intervals up to 14 days after injection, and many mitotic figures were present in the nodule before regression (Fig. 2A). Approximately half of the mice with nodules that regressed during the first 2 months after injection of TBE-1 cells developed tumors after a 7- to 9-month latency period (Table 1).

The TBE-1SA cells were isolated by selection of anchorage independent cells (*36*) growing in soft agar cultures of TBE-1 cells. The TBE-1SA cells were characterized for tumorigenicity, karyology (Fig. 3), and isoenzyme phenotype. Tumor tissue derived from mice injected with TBE-1SA cells was subjected to immunocytochemical staining for keratin and β-human chorionic gonadotropin (β-hCG). The progression of this subpopulation of cells was indicated by their ability to form tumors that grew larger than 1.0 cm in diameter and persisted longer than 90 days in 13 of 14 athymic nude mice (Table 1). These tumors reached 2.5 cm in diameter and did not show the regression that was frequently observed when TBE-1 cells were tested for tumorigenicity (Table 1). The low frequency and extended latent period observed for TBE-1 tumorigenicity indicates that the selection of anchorage

independent cells (TBE-1SA) yielded a subpopulation of TBE-1 that had progressed to tumorigenicity. The tumorigenicity of the TBE-1SA cells was also indicated by the growth of secondary transplants of TBE-1SA tumor tissue in nude mice. The immunocytochemical staining of TBE-1SA tumors for keratin (Fig. 2B) was consistent with the epithelial origin of TBE-1SA cells. More than 70 percent of bronchogenic carcinomas contain detectable levels of β-hCG (*37*), and this hormone was present in the TBE-1SA tumor cells (Fig. 2C). The ultrastructural features of TBE-1SA tumor cells observed by electron microscopy (data not shown) included prominent and well-defined intermediate junctions, Golgi apparatus, rough endoplasmic reticulum, and abundant mitochondria. These features are similar to the ultrastructural characteristics that typify poorly differentiated tumor cells.

Isoenzymes from tumor tissue or cell extracts were assayed by gel electrophoresis and enzyme-specific staining to identify isoenzyme phenotypes (*38*). The human isoenzymes found in TBE-1 were human, lactate dehydrogenase; glucose-6-phosphate dehydrogenase; phospho-

Table 1. Summary of the phenotypic properties of NHBE cells before and after transfection with v-Ha *ras*, TBE-1, TBE-1SA, and TBE1-SAT cells isolated from tumor tissue. The differentiation response, which is shown for cells grown in LHC-4 medium supplemented with 2 percent BDS or cells grown in $10^{-7}M$ TPA, indicates resistance to squamous differentiation (+, differentiation; −, resistant to differentiation). The transfected cells did not produce a tumor cell growth factor capable of supporting the anchorage independent growth of NRK cells. However, clonal growth of TBE-1 cells required 10 to 50 percent MCDB-104, LHC-4, or RPMI 1640 medium conditioned by 3 days of growth with 50 percent confluent TBE-1 cultures. This indicates that TBE-1 cells elaborate an autogenous factor (autogenous conditioned medium response, ACMR). The ability to form colonies during growth in soft agar was used to isolate TBE-1 derivatives capable of anchorage independent growth (TBE-1SA). Early passage TBE-1 cells did not form colonies during growth in soft agar when seeded at 10^6 cells per 60-mm dish. That anchorage independence is related to tumorigenicity was shown by the increase in the tumorigenicity of TBE-1SA cells and the transplantability of TBE-1SA tumors after removal and transfer to another animal (T^t). The identity of tumor tissue was confirmed by recovery of a cell line (TBE-1SAT) from a TBE-1SA tumor and determination that the isoenzyme pattern for those cells matched the characteristics of the parent cell line. N.D., test not done.

Cell name	Differentiation response		Conditioned medium response				Anchorage independence in soft agar	Tumorigenicity	Frequency	Size (cm)	Transplantability
	In 2 percent BDS	In TPA	NRK MCDB-104	ACMR MCDB-104	LHC-4	RPMI 1640					
NHBE	+	+	N.D.	N.D.	N.D.	N.D.	−	−		0.0	
TBE-1	−	−	−	5.0 ± 0.2*	+	+	+	+^{r}†	2/16	0.1 to 0.8	
TBE-1SA	−	N.D.	−	+	+	+	+	+	13/14	>2.5	T^t‡
TBE-1SAT	−	−	N.D.	+	+	+	+	−	9/10	>1.0§	

*Ratio of population doublings per day at clonal density. †The +^{r} phenotype included regression of nodules within 14 days after subcutaneous injection of 10^6 or 2×10^7 cells. Approximately 50 percent of the TBE-1 nodules were expressed as progressively growing tumors after a 7- to 9-month latent period. ‡The tumorigenicity of TBE-1SA was supported by the transplantability (T^t) of TBE-1SA tumors. §It remains to be determined whether the TBE-1SAT cells are more tumorigenic than TBE-1SA cells.

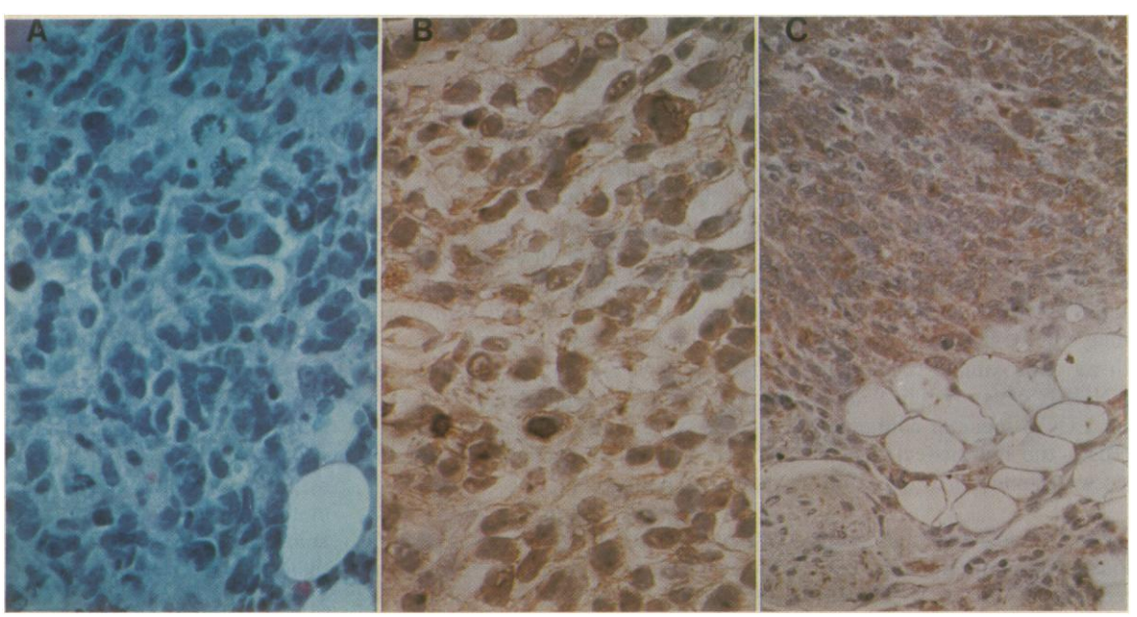

Fig. 2. The phenotypic properties of TBE-1 and TBE-1SA tumors in athymic nude mice (BALB/c). (A) Photomicrograph of tumor (stained with hematoxylin-eosin) removed 10 days after injection of 5×10^6 TBE-1 cells into mice. The cellular morphology indicates a poorly differentiated tumor with abundant mitotic figures and anaplasia. (B) Immunoperoxidase study of TBE-1SA tumor tissue with antibody to human keratin (DAKO 1:140). There is a little keratin within the tumor cells and a few cells have formed keratin networks similar to those observed in normal epithelial cells and typical carcinomas. (C) Immunoperoxidase study with antibody to β-hCG (DAKO 1:300). The antibody bound to the TBE-1SA cells (upper right) but not to the mouse nerve cells (lower left). For the immunoperoxidase studies the tumors were fixed in ethanol and embedded in paraffin. Four 5-μm sections were deparaffinized and hydrated and then treated with 1.5 percent hydrogen peroxide in 0.3 percent methanol for 30 minutes to inhibit endogenous peroxidase.

glucomutase 3; esterase D; mitochondrial malic enzyme; adenylate kinase 1 (soluble); and glyoxalase-1. The matching isoenzyme phenotype of TBE-1, TBE-1SA, and TBE-1SAT cells indicated that in each case the cells were of human origin and that they were the progeny of one donor (data not shown).

The tumors formed by unselected, early-passage TBE-1 cells frequently regressed after 14 days. That more strongly tumorigenic cells were present, however, was indicated by the appearance of progressively growing TBE-1 tumors after a 7- to 9-month latency period (Table 1). At early passages, immediately after focus formation, TBE-1 cells produced no anchorage independent colonies within the limits of detection (10^{-6}), but they did express v-Ha *ras* gene transcripts and p21 gene product during the period of growth and progression to tumorigenicity (100 to 120 population doublings).

The karyotypic analysis of TBE-1SA cells (Fig. 3) and the isoenzyme phenotype of TBE-1SA tumor tissue collected 84 days after transplantation indicated that the cells were human and that the tumor tissue isozymes corresponded with those of TBE-1SA cells. The modal distribution of chromosomes was 74 to 75 for TBE-1SA, with marker chromosomes and chromosomal abnormalities that became extensive as the transfected cells progressed to tumorigenicity in athymic nude mice (Fig. 3). The aneuploid karyotype of TBE-1 cells was an early event detectable at the first passage after the isolation of foci.

Nucleic acid hybridization and gene product analysis. The transfected v-Ha *ras* gene was detected by Southern hybridization (*39*) analysis of TBE-1 DNA with a ^{32}P-labeled plasmid probe carrying the v-Ha *ras* gene of MuSV. This probe consisted of a 2.1-kb Bam HI–Eco RI DNA fragment carried on plasmid pBR322 that includes the v-Ha *ras* structural gene but does not contain the long terminal repeat (LTR) sequences of MuSV (*40*). The TBE-1 DNA was digested with Pst I, Bgl II, or Hind III to release the transfected sequences for detection of v-Ha *ras* DNA. After hybridization these digests were compared to undigested DNA from TBE-1 cells and a lung carcinoma cell line, All46 (*41*) (Fig. 4). The 1.8-kb fragment released by Bgl II digestion of TBE-1 DNA indicated that the v-Ha *ras* structural gene sequence was present in cellular DNA (Fig. 4). The integrity of the sequences between the MuSV LTR and the v-Ha *ras* structural gene was indicated by the hybridization of HB 11 probe DNA to the 3.0-kb Hind III fragment and the 3.9-kb Pst I fragment released by digestion of TBE-1 DNA. The specificity of this hybridization for v-Ha *ras* sequences was indicated by hybridization of the probe DNA to undigested TBE-1 DNA and by the lack of hybridization to undigested DNA from the A1146 cells (Fig. 4).

The expression of v-Ha *ras* transcripts was determined by comparing the slot-blot hybridization (*42, 43*) of ^{32}P-labeled HB 11 fragment probe to total cellular RNA from TBE-1 and NHBE cells (Fig.

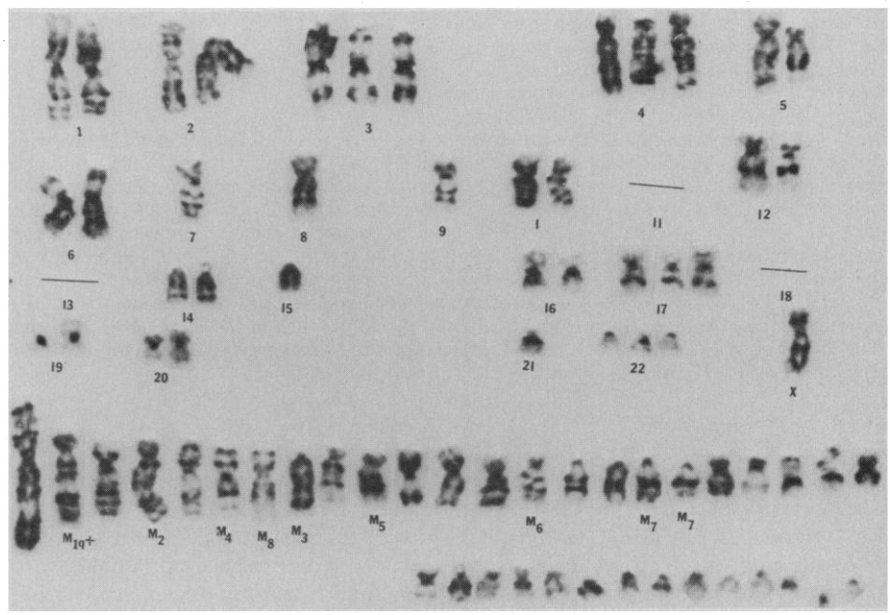

Fig. 3. The karyology of TBE-1SA cells indicate that they are from a human female. The Y chromosome is absent, and the modal number of chromosomes is 74 to 75. The chromosome ploidy distribution per 100 metaphases was 94 metaphases with 70 to 80 chromosomes, and six metaphases with greater than 140 chromosomes. Karyotypes were prepared (*36*) for chromosome numbers 68, 69, 73, 75, and 77. The karyotype for a 77-chromosome metaphase of TBE-1SA is shown in Fig. 5. Eight marker chromosomes were observed: M1+ = t(9pter → 9q33ht: : 4q14 → 32); M8 = t(15qht; 18q); M2 = 10qt; M3 = unknown; M4 = t(15q;?); M5 = del 9(p13); M6 = del 7(p12); M7 = del 11 (p11). Normal chromosome 11 was absent in all karyotypes, 9 and 18 were absent in most, and chromosomes 1, 4, 7, 8, 10, 14, 15, and X were monosomic or absent in most karyotypes.

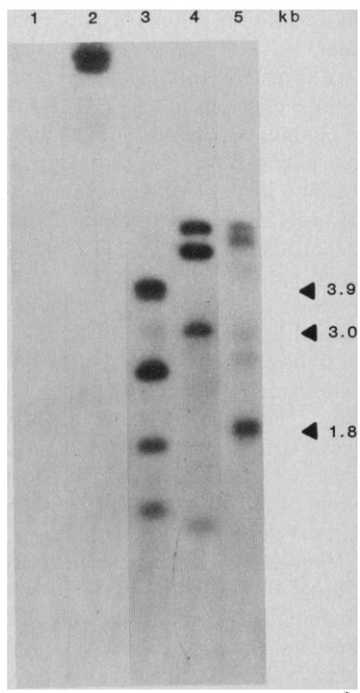

Fig. 4. Southern blot analysis of TBE-1 cellular DNA by hybridization with probe DNA that contains a 2.1-kb Bam HI–Eco RI fragment of MSV sequence on pBR322 labeled with α^{32}P-dCTP to 1×10^8 to 2×10^8 cpm/μg [HB 11 (*40*)]. The region of MSV contained on the HB 11 probe includes the v-Ha *ras* structural gene but not the LTR sequences (*20, 40*). Probe preparations of HB 11 DNA are predicted to hybridize to fragments of cellular DNA that contain sequences homologous to the region between Bam HI and Eco RI of MuSV (*20*). Therefore, the presence of an intact copy of v-Ha *ras* structural gene and the contiguous linkage of the transfected gene to the MSV LTR can be determined by hybridization of HB 11 probe DNA after digestion with (i) Bgl II, to release the 1.8 kb of v-Ha *ras*–specific fragment, (ii) Hind III, to release the 3.0-kb fragment with MuSV LTR and the v-Ha *ras* gene, and (iii) Pst I, to release the 3.9-kb fragment that also includes the MuSV LTR with the v-Ha *ras* gene (*20*). Lane 1 contained 15 μg of undigested DNA from carcinoma cell line A1146 and lane 2 contained 15 μg of undigested DNA from TBE-1 cells. TBE-1 cellular DNA was digested with (i) Pst I, which released a 3.9-kb band, lane 3, which hybridized to ^{32}P-labeled HB 11 probe DNA, (ii) Hind III, which released a 3.0-kb band, lane 4, and (iii) Bgl II, which released a 1.8-kb band, lane 5.

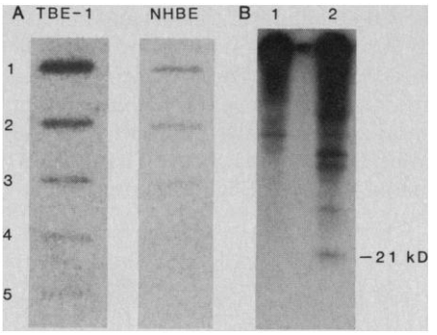

Fig. 5. Analysis of v-Ha *ras* gene expression in TBE-1 cells by slot-blot analysis of total cellular RNA to compare the level of detectable Ha *ras* gene transcription in (A) TBE-1 and NHBE cells and (B) immunoprecipitation of γ^{32}P-GTP labeled TBE-1 and NHBE cell extracts with a monoclonal antibody specific for Ha *ras* p21 (*26*). The 2.1-kb Ha *ras* fragment released by digestion with Bam HI and Eco RI was isolated by electrophoretic separation and recovery from agarose (IBI) by electroelution and chromatography on an NACS-resin column (Bethesda Research Laboratories), and the DNA fragment was collected after it flowed through a S400 (Pharmacia) column to remove any NACS-resin contaminating the preparation. The probe DNA was extracted with phenol, precipitated with ethanol, and dissolved in 10 m*M* tris and 1 m*M* EDTA, *p*H 8.0, and stored at −20°C until nick translation and denaturation for use as a probe. The 2.1-kb Ha-*ras* fragment was nick-translated with α^{32}P-dCTP and used at approximately 2×10^7 cpm per hybridization. (A) Total celluar RNA was slotted at 10.0, 3.0, 1.0, 0.3, and 0.1 μg, respectively, and densitometric peak areas were determined to compare the relative level of Ha *ras* transcription. The differences in expression relative to TBE-1 indicate five to eight times more Ha *ras* p21 because the normal cellular homolog c-Ha *ras* does not produce a phosphorylated p21 gene product (*25*). (B) Autoradiography of the p21 gene product after γ^{32}P-GTP labeling of (lane 1) NHBE cells and (lane 2) TBE-1 cells indicates that TBE-1 cells express phosphorylated p21 from the v-Ha *ras* gene whereas the NHBE cells do not.

5). Densitometric comparison of the relative amounts of HB 11 hybridized to RNA isolated from TBE-1 and NHBE cells indicate that TBE-1 cells transcribed five to eight times more Ha *ras* hybridizing RNA than NHBE cells (Fig. 5A). However, the levels of Ha *ras* RNA in TBE-1 cells showed a three- to fourfold decrease when the cell cultures were at or near confluence at the time of RNA isolation (data not shown). The transfected v-Ha *ras* RNA was first detected by hybridization of TBE-1 RNA within 4 weeks after transfection at passage 2, and the v-Ha *ras* phosphorylated p21 were measured at various times after the first isolation of v-Ha *ras* transfected foci.

Expression of the phosphorylated v-Ha *ras* gene product in TBE-1 was tested by immunoprecipitation with a monoclonal antibody specific for the p21 of Ha *ras* and by gel electrophoretic resolution of polypeptides from cell extracts labeled with γ^{32}P-GTP (*26*). The v-Ha *ras* gene product is a phosphorylated polypeptide (21 kilodaltons) that can be enriched by precipitation with monoclonal antibody Y138 followed by gel electrophoresis and detected by autoradiography. The phosphorylated v-Ha *ras* p21 band is found in TBE-1 but not NHBE cell extracts (Fig. 5B). The phosphorylation of Ha *ras* p21 is a property of the v-Ha *ras* gene product (*25, 26*).

Discussion. High-frequency transfection of NHBE cells with v-Ha *ras* results in malignant transformed cells that, during the early stages of transformation (within the first 10 to 15 population doublings), become resistant to agents that induce terminal squamous differentiation. The transfected cells progress (during 100 to 120 population doublings) to immortality and tumorigenicity, surviving indefinitely after the other cells in culture have undergone senesence and died. The isolation of oncogenic homologs of Ha *ras* from human lung cancer cells (*4*) and the frequent detection of genetic polymorphism of c-Ki *ras* homologs in association with human lung tumors suggest that altered *ras* genes play a role in lung carcinogenesis. The recent observation that expression of altered human *ras* genes in primary rodent cells can lead to immortalization and tumorigenicity of primary rat fibroblasts (*7*) provides a precedent for single oncogene transformation following Ha *ras* transfection.

Our finding that immortality and tumorigenicity were attained by transfected human bronchial epithelial cells after extensive growth and expression of the v-Ha *ras* gene is consistent with the previous difficulties reported for the transformation of primary human cells in vitro (*8–10, 44*) and the pleiotropic properties of Ha *ras* gene as an oncogene. Fibroblasts from patients with Bloom's syndrome that are transfected with a plasmid carrying v-Ha *ras* on a Pst I fragment from plasmid H1 showed altered tumorigenic properties, but the transfected cells underwent senescence (*8*). When transfected into normal diploid human fibroblasts, E_J-Ha *ras* resulted in no change (*9*), or induced anchorage independence of the cells but did not cause immortalization or tumorigenicity (*10*). The differences in the cell types used as recipient—that is, Blooms' syndrome fibroblasts (*8*) and diploid human fibroblasts (*9, 10*)—may account for these contrasting observations. These experiments also differed in the oncogene donor DNA selected for testing. The donor DNA's included (i) the human c-Ha *ras* (EJ/T24) from bladder carcinoma carried on a plasmid (*9*); (ii) total genomic DNA from hematopoietic tumors known to carry an oncogenic homolog of human c-Ha *ras* (*10*); and plasmid Ha 8 carrying a fragment of Ha-MuSV with v-Ha *ras* (*8*). In contrast, the plasmid H1 used for transfection of NHBE cells contains the Ha-MuSV complementary DNA sequence divided by restriction of the Eco RI site and ligation with pBR322 (*20*). Any of these differences could contribute to the divergent results obtained after *ras* oncogene transfection of human cells.

That cultured mouse and rat cells eventually become malignant in the absence of carcinogenic treatment whereas cultured human cells do not has long been a matter of discussion and interpretation (*9, 44*). Our observation that NHBE cells transfected with v-Ha *ras* progress to immortality and tumorigenicity without additional carcinogenic treatment suggests that both of these phenotypic states may occur as a consequence of v-Ha *ras* expression. Cryopreserved TBE-1 from early passages of nontumorigenic cells provide human epithelial cells with a known carcinogenic potential. Studies of the effects of v-Ha *ras* in these cells may reveal whether other known oncogenes are involved in the progression of TBE-1 cells to tumorigenicity.

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