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A Transforming *ras* Gene in Tumorigenic Guinea Pig Cell Lines Initiated by Diverse Chemical Carcinogens

Abstract. *Fetal guinea pig cells were transformed by treatment with four different chemical carcinogens including nitroso compounds and polycyclic hydrocarbons. As a consequence of this treatment, oncogenes capable of transforming NIH/3T3 cells became activated in each of five independently established clonal guinea pig cell lines. Molecular characterization of representative NIH/3T3 transformants revealed that the same oncogene was present in each of the cell lines tested. Moreover, detection of this transforming gene paralleled the acquisition of tumorigenic properties by these neoplastic cells.*

Cells from various human tumors have oncogenes capable of inducing malignant transformation upon transfection into morphologically normal cells (*1–8*). Molecular characterization of these oncogenes revealed that in most cases they are members of the *ras* gene family (*8–12*). However, how these sequences control the expression of malignancy remains unknown. Transforming *ras* genes have also been detected in chemically induced tumors (*13–16*).

We conducted an investigation to determine whether chemically transformed guinea pig cells also contain transforming *ras* genes. This in vitro model system has several advantages for studying the role of oncogene activation in the multistep process of carcinogenesis (*17*). For instance, nontreated guinea pig cell cultures, unlike those of rat or mouse origin, do not transform spontaneously during continuous passage. Moreover, several distinct preneoplastic stages have been observed as carcinogen-exposed guinea pig cells develop into tumor cells (*17*). Expression of tumorigenicity in this model system is always preceded by the stages of morphologic alteration and morphologic transformation. Additional phenotypic properties, including anchorage-independent growth, are often expressed along with tumorigenicity. This indicates that multiple changes are necessary for the acquisition of the complete neoplastic phenotype (*17, 18*).

Chemically transformed guinea pig cell lines used in our studies were derived after exposure of fetal cells, either in vitro or in vivo, to four different chemical carcinogens (*17*). Cell line 74-C3 was derived by treatment in vitro of cells from 48-day fetal skin with 3-methylcholanthrene (3-MCA) and cloned in agar in its 64th subculture. Cell line 104-C1 was derived by exposure in vitro of cells from a 43-day-old fetus to benzo[*a*]pyrene and cloned in agar at its 20th subculture. Cell line 107-C3 was derived by treatment in vitro of cells from the same 43-day-old fetus that was used to generate 104-C1 cells—in this case the fetal cells were exposed to *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG), and the cell line was cloned in agar in its 50th subculture. Cell line HM4-C1 was derived from cells of a 49-day-old fetus that received a transplacental injection of MNNG 48 hours before removal from its mother—the cell line was cloned in agar at its 70th subculture. The HM2-C1 cell line was derived from cells of a 38-day-old fetus whose mother had received a single injection of diethylnitrosamine (DEN)—the cell line was cloned in its 85th subculture (*17*). As controls, a nontreated guinea pig fetal cell culture GP84 (*19*) and several other guinea pig fetal cell pools were used.

Approximately 20 μg of high molecular weight DNA extracted from each guinea pig cell line was precipitated with calcium phosphate and tested for the presence of transforming activity in transfection assays in which NIH/3T3 mouse fibroblasts were used as recipient cells (*20, 21*). DNA's isolated from each of the five chemically transformed cell lines induced foci of morphologically transformed cells after 2 weeks of incubation. Transformation efficiencies ranged from 0.08 to 0.15 foci per microgram of donor DNA, a frequency comparable to those obtained in our laboratory with DNA from T24 human bladder carcinoma cells (*6*). In contrast, no detectable morphologic alteration occurred in the NIH/3T3 cultures with DNA's isolated from six different cell pools derived from untreated embryos. These results indicate that an oncogene became activated in these transformed guinea pig cell lines as they progressed from the normal to the neoplastic state.

To demonstrate that transformation of NIH/3T3 cells was due to the uptake of

Table 1. Correlation between oncogene activation and development of malignancy in carcinogen-treated guinea pig fetal cells. Transformation efficiency is expressed as number of foci per microgram of DNA. Colony formation in agar was determined as follows: trypsinized single cells (10^2 to 10^5) were suspended in 4 ml of RPMI 1640 medium containing 10 percent fetal bovine serum and 0.35 percent agar and poured onto a solidified basal layer of 4 ml of RPMI 1640 medium but containing 0.5 percent agar, in 60-mm petri dishes. Colonies consisting of more than 100 cells were scored after 10 days of culture at 37°C and 8 percent CO_2 atmosphere. Results given are means of two experiments. Tumorigenicity data were obtained from (*17*).

Mass cultures and passage number	Clonal cells	Transformation efficiency	Colony formation in agar (%)	Tumorigenicity
107-P17		0	<0.001	−
107-P40		0	<0.001	−
107-P61		0.01	1.0	+
	107-C3	0.10	4.8	+
HM2-P33		0	<0.001	−
HM2-P67		0	<0.001	−
HM2-P86		0.05	1.1	+
	HM2-C1	0.08	1.4	+
HM4-P31		0	<0.001	−
HM4-P58		0	<0.001	−
HM4-P77		0.02	0.8	+
	HM4-C1	0.09	2.0	+

donor DNA, we used nick-translated ^{32}P-labeled genomic guinea pig DNA to test for the presence of guinea pig–specific repetitive sequences in each of the transformants. Guinea pig sequences were detected in each of the NIH/3T3 transformants tested but not in control NIH/3T3 cells (Fig. 1). Confirmation that transformation of NIH/3T3 cultures was mediated by an oncogene was shown by their ability to transmit their malignant phenotype in subsequent cycles of transfection. However, the guinea pig repetitive sequences were lost after two or three cycles of transfection (data not shown). Therefore, methods that have been successful in identifying oncogenes from human tumor cells (*1*, *4–8*), were not applicable for the characterization of guinea pig oncogenes.

Most transforming genes identified in human and animal tumors are related to retroviral oncogenes (*8–12*, *15*, *16*). The possibility that NIH/3T3 transformants derived from the five chemically transformed guinea pig cell lines may also contain retrovirus-related oncogenes was investigated. Representative first-cycle transformants derived from each of the five guinea pig cell lines were submitted to Southern blot analysis (*22*) with probes specific for *onc* genes of several retroviral isolates, including MC29 virus, avian myeloblastosis virus, Rous sarcoma virus, Abelson murine leukemia virus, the BALB, Kirsten, and Moloney strains of murine sarcoma virus (MSV), the Snyder-Theilen strain of feline sarcoma virus, and simian sarcoma virus. Each retroviral *onc* gene probe was prepared from a pBR322 subclone containing only *onc* sequences [for references to each specific probe, see (*11*)]. Under standard hybridization conditions (50 percent formamide and fivefold-strength standard saline citrate at 42°C) no retrovirus-related sequences could be detected in any of the transformants tested, except for those present in control NIH/3T3 DNA. However, when hybridization conditions were relaxed by decreasing the concentration of formamide to 20 percent, v-*bas*, the *onc* gene of BALB-MSV specifically detected an Eco RI DNA fragment of about 11 kilobase pairs (kbp) in each of the NIH/3T3 transformants tested (Fig. 2). A member of the *ras* gene family of oncogenes, v-*bas* (*23*, *24*) has frequently been detected in human as well as in animal tumors (*8–12*, *15*, *16*). Under the same experimental conditions, normal guinea pig DNA also exhibited an 11-kbp Eco RI DNA fragment, thus indicating the guinea pig origin of these *ras*-related sequences. To confirm these observations, we analyzed the above-mentioned DNA's with the restriction endonuclease Hind III. Two DNA fragments of 8.6 kbp and 1.8 kbp, not present in control NIH/3T3 DNA, were detected in each NIH/3T3 transformant tested (data not shown). The same DNA fragments were present in guinea pig DNA cleaved with Hind III. These results showed conclusively that the oncogenes present in these NIH/3T3 transformants are members of the *ras* gene family and, moreover, are of guinea pig origin.

The similar size of the *ras*-related guinea pig DNA fragments detected in each of the NIH/3T3 transformants indicated that each of the five guinea pig tumorigenic cells described above may contain the same activated oncogene. To further substantiate the evidence presented above, we used different restriction endonucleases to digest DNA isolated from representative NIH/3T3 transformants derived from each of the five chemically transformed guinea pig cell lines. Bam HI had no effect on the ability of each of these DNA's to transform NIH/3T3 cells. In contrast, Hind III completely abolished their transforming activities. These findings, taken together, demonstrate that the same *ras* oncogene is activated in neoplastic cells derived from five independent guinea pig cultures exposed to four different chemical carcinogens.

These results suggested that activation of *ras* oncogenes may be a necessary step for the acquisition of neoplastic properties by carcinogen-treated guinea pig cells. Evans and DiPaolo (*18*) described the isolation of morphologically transformed, anchorage-independent clonal cells from a nonsenescent guinea pig cell line (designated 118) that, after 90 passages, had been treated with MNNG, the chemical carcinogen we used to generate the 107-C3 and HM4-C1 tumorigenic cells. These MNNG-treated cells, however, lacked detectable transforming genes. Thus, guinea pig cells can

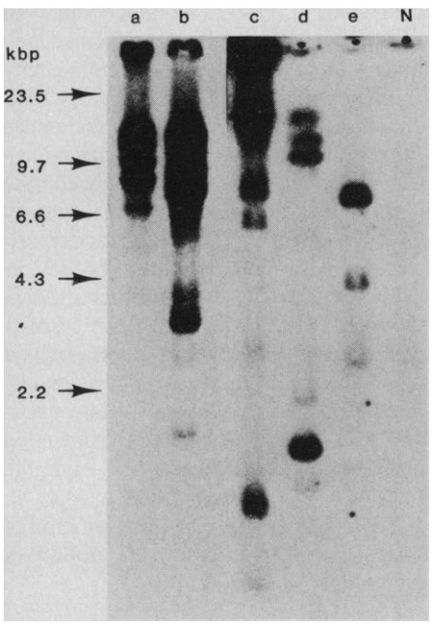

Fig. 1. Detection of guinea pig–specific sequences in NIH/3T3 cells transformed by DNA isolated from tumorigenic guinea pig cell lines. High molecular weight DNA (20 μg) isolated from NIH/3T3 transformants derived from (a) 3-MCA-treated 74-C3 cells, (b) benzo[*a*]pyrene-treated 104-C1 cells, (c) MNNG-treated 107-C3 cells, (d) DEN-treated HM2-C1 cells, and (e) MNNG-treated HM4-C1 cells, as well as DNA isolated from (*N*) control NIH/3T3 cells, were digested with Eco RI for 1 hour at 37°C, then subjected to electrophoresis in horizontal agarose (0.7 percent, weight to volume) gels (18 hours at 30 V) and blotted on nitrocellulose filters (*22*). Filters were hybridized under stringent conditions (50 percent formamide and fivefold-strength standard saline citrate at 42°C) for 40 hours to ^{32}P-labeled (2×10^7 count/min), nick-translated, genomic guinea pig DNA. Hybridized blots were exposed to Kodak XAR-5 film at −70°C in the presence of intensifier screens for 18 hours. Their sizes were deduced from their migration relative to those of DNA fragments of Hind III–digested λc1857 DNA.

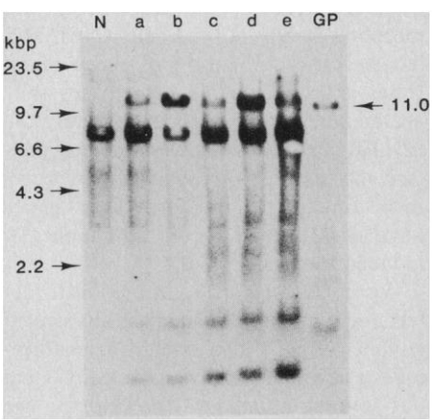

Fig. 2. Detection of *ras*-related sequences in NIH/3T3 cells transformed by DNA isolated from carcinogen-treated guinea pig fetal cells. High molecular weight DNA (20 μg) isolated from: (*N*) control NIH/3T3 cells; NIH/3T3 transformants derived from (a) 3-MCA-treated 74-C3 cells, (b) benzo[*a*]pyrene-treated 104-C1 cells, (c) MNNG-treated 107-C3 cells, (d) DEN-treated HM2-C1 cells, and (e) MNNG-treated HM4-C1 cells; and (*GP*) guinea pig GP 84 cells were digested with Eco RI, subjected to electrophoresis, and blotted on nitrocellulose filters as described in the legend to Fig. 1. Filters were hybridized under nonstringent conditions (20 percent formamide and fivefold-strength standard saline citrate at 42°C) for 40 hours to a ^{32}P-labeled (2×10^7 count/min), nick-translated 675–base pair Hind III–Bam HI insert of pHB-1, a DNA fragment that contains the *onc* gene of BALB-MSV (*35*). Hybridized blots were exposed to Kodak XAR-5 film at −70°C in the presence of intensifier screens for 3 days. Sizes were determined as described in the legend to Fig. 1.

acquire anchorage-independent growth properties by mechanisms not involving the activation of *ras* oncogenes. None of the MNNG-treated transformed cell lines derived from 118 cells were tumorigenic in vivo (*18*). These results indicate that oncogene activation may be required to drive guinea pig cells to a fully neoplastic phenotype. Yet DNA's isolated from two other tumorigenic clones, 123-C1 and HM9-C1 (*17*), have so far failed to induce transformation of NIH/3T3 cells. However, it is possible that tumorigenicity in vivo could also be acquired by mechanisms not involving activation of the *ras* oncogene.

Guinea pig cells require several months of subculturing before they acquire anchorage-independent growth (*17*). This property is usually associated with the ability of these transformed cells to induce tumors in newborn guinea pigs or nude mice (*17, 18*). To examine when oncogenes became activated during the progression of carcinogen-treated guinea pig cells toward malignancy, we analyzed parental cultures, which had been stored in liquid nitrogen, before the development of tumorigenicity. They included passages 17, 40, and 61 of the MNNG-treated 107 cell line; passages 33, 67, and 86 of the DEN-treated HM2 series; and passages 31, 58, and 77 of the MNNG-treated HM4 line. In each case, the last passage used was the mass cell culture used to derive clones 107-C3, HM2-C1, and HM4-C1, respectively (*17, 25*). Cells were thawed, expanded, and harvested for DNA extraction. DNA's were tested for transformation of NIH/3T3 cells in transfection assays. In each of the series, early nontumorigenic passages did not show detectable anchorage-independent growth, and their DNA's did not induce morphologic transformation of NIH/3T3 cells (Table 1). In contrast, each of the DNA's isolated from the cell lines at later passages, when they had developed tumorigenic properties, showed transforming activity (Table 1). These results indicate that activation of a specific *ras* oncogene in carcinogen-treated fetal guinea pig cells is associated with the acquisition of tumorigenicity.

Cellular transforming genes isolated by molecular cloning techniques can efficiently induce malignant transformation of immortalized cells (*6, 26–31*). However, they have failed to transform primary cultures or senescent cells (*32–34*). In our studies, we observed the appearance of the oncogene in fully neoplastic guinea pig cells after several passages in culture. Thus, if the oncogene became activated immediately after exposure to the carcinogen, some biological event, such as acquisition of immortality (*32–34*), ought to occur before the transforming gene can be phenotypically expressed. Alternatively, oncogene activation may represent a well-defined step within the carcinogenic process, which is triggered but not directly caused by the initiating carcinogenic event. Isolation and characterization of the *ras* oncogene specifically activated in carcinogen-treated, tumorigenic guinea pig cells may provide the necessary tool to investigate, at the molecular level, the mechanisms involved in oncogene activation and its role in the multistep process of carcinogenesis.

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Isolation, Structure, and Synthesis of a Human Seminal Plasma Peptide with Inhibin-Like Activity

Abstract. *A basic peptide isolated from pooled human seminal plasma exhibited inhibin-like activity by suppressing pituitary follicle-stimulating hormone secretion in vitro and in vivo. The peptide has been characterized and sequenced, and a 31-amino-acid synthetic replicate showed full biological activity in vitro.*

A substance called "inhibin," believed to be present in aqueous extracts of testis, was imputed (*1*) to have an important if not exclusive role in the secretion of pituitary follicle-stimulating hormone (FSH) in the male. An ovarian nonsteroidal component also exerts a similar effect in the female (*2*) and participates in the regulation of cyclical events in the hypothalamo-pituitary-gonadal axis (*3, 4*). Since the name was proposed half a century ago (*1*), activity resembling that of inhibin has been detected in various gonadal and related fluids from at least six species (*5*). Peptide or proteinaceous extracts of testis, seminal plasma, spermatozoa, rete-testis fluid, testicular lymph, ovary, ovarian follicular fluid, and culture media of granulosa and Sertoli cells have been reported to contain a factor (or factors) (*3–8*) that inhibit pituitary FSH secretion and that