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Cell Biology

Normal genetically mosaic mice produced from malignant teratocarcinoma cells

(embryonal carcinoma/teratoma/embryoid body core cells/blastocyst injection/allophenic mice)

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ABSTRACT **Malignant mouse teratocarcinoma (or embryonal carcinoma) cells with a normal modal chromosome number were taken from the "cores" of embryoid bodies grown only *in vivo* as an ascites tumor for 8 years, and were injected into blastocysts bearing many genetic markers, in order to test the developmental capacities, genetic constitution, and reversibility of malignancy of the core cells. Ninety-three live normal pre- and postnatal animals were obtained. Of 14 thus far analyzed, three were cellular genetic mosaics with substantial contributions of tumor-derived cells in many developmentally unrelated tissues, including some never seen in the solid tumors that form in transplant hosts. The tissues functioned normally and synthesized their specific products (e.g., immunoglobulins, adult hemoglobin, liver proteins) coded for by strain-type alleles at known loci. In addition, a tumor-contributed color gene, *steel*, not previously known to be present in the carcinoma cells, was detected from the coat phenotype. Cells derived from the carcinoma, which is of X/Y sex chromosome constitution, also contributed to the germ line and formed reproductively functional sperms, some of which transmitted the *steel* gene to the progeny. Thus, after almost 200 transplant generations as a highly malignant tumor, embryoid body core cells appear to be developmentally totipotent and able to express, in an orderly sequence in differentiation of somatic and germ-line tissues, many genes hitherto silent in the tumor of origin. This experimental system of "cycling" teratocarcinoma core cells through mice, in conjunction with experimental mutagenesis of those cells, may therefore provide a new and useful tool for biochemical, developmental, and genetic analyses of mammalian differentiation.**

The results also furnish an unequivocal example in animals of a non-mutational basis for transformation to malignancy and of reversal to normalcy. The origin of this tumor from a disorganized embryo suggests that malignancies of some other, more specialized, stem cells might arise comparably through tissue disorganization, leading to developmental aberrations of gene expression rather than changes in gene structure.

Teratocarcinoma (or embryonal carcinoma) cells are the malignant stem cells of transplantable mouse teratomas (1, 2). Some teratomas have been experimentally converted to a modified ascites form, termed "embryoid bodies" (3). Small-size embryoid bodies have the advantage over the solid tumors that the location of their embryonal carcinoma cells is known: the latter comprise a "core" (Fig. 3) surrounded by a yolk sac epithelial "rind". Single cells from embryoid bodies are able to form tumors containing various tissues when they are injected subcutaneously (4). Whether such cells are totipotent has remained unknown, as the solid tumors consistently lack certain tissues (5).

Abbreviations: PBS, Dulbecco's phosphate-buffered saline; DMEM, Dulbecco's modification of Eagle's medium; FCS, fetal calf serum; GPI, glucosephosphate isomerase; MUP, major urinary protein complex.

The most rigorous test possible for developmental totipotency would be significant contributions of the carcinoma cells to the normal differentiation of virtually all tissues in a mouse. For this to occur, the initially malignant cells would presumably have to be brought into association with early embryo cells so that the latter could provide an organizational framework appropriate for normal development. The experiment is analogous to production of allophenic mice (6).

The projected teratoma studies in this laboratory involve the use of mutagenized embryonal carcinoma cells for experimental analyses of genetic regulatory systems in mammalian differentiation (7). The most promising source of baseline, or nonmutagenized, cells appears to be from the cores of small embryoid bodies grown only *in vivo*, as these would be less likely to have accumulated genetic changes than would cells cultivated *in vitro*. Most *in vitro* teratocarcinoma cell lines do not in fact have a normal chromosome number and some have lost multipotentiality (8–11), whereas the tumors from which they originated were diploid (12).

We report here that teratocarcinoma cells taken from the cores of embryoid bodies grown only *in vivo* for 8 years, during which they retained a euploid chromosome complement, appear to be developmentally totipotent. The original conversion to malignancy in all likelihood did not involve mutational events and has proved to be completely reversible to normalcy. A partial report of these results has been presented (7).

MATERIALS AND METHODS

Core Cells. The OTT 6050 ascites teratoma, originally received from Dr. L. C. Stevens, has been maintained by intraperitoneal transfers every 2–3 weeks in syngeneic males of the 129/Sv Sl^J *C P* inbred strain (to be referred to as 129) (see Fig. 1). To obtain core cells, only small-size embryoid bodies were collected, in Dulbecco's phosphate-buffered saline (PBS), by filtration of ascites fluid through 100 μm Nitex mesh (13). They were subjected to light proteolytic treatment for approximately 3 min in 0.25% trypsin (crystalline, Worthington) and 1.25% pancreatin (Difco) in PBS; proteolysis was stopped in Dulbecco's modification of Eagle's medium (DMEM) with 50% fetal calf serum (FCS) (Gibco). Addition of 5 μg/ml of DNase eliminated stickiness. After about 15 min, the embryoid bodies were centrifuged and transferred to DMEM with 15% FCS and organic buffers [10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) and 10 mM morpholinopropane sulfonic acid (Mops)], and the epithelium was peeled off with tungsten needles. The cores (Fig. 3) were then partially dissociat-

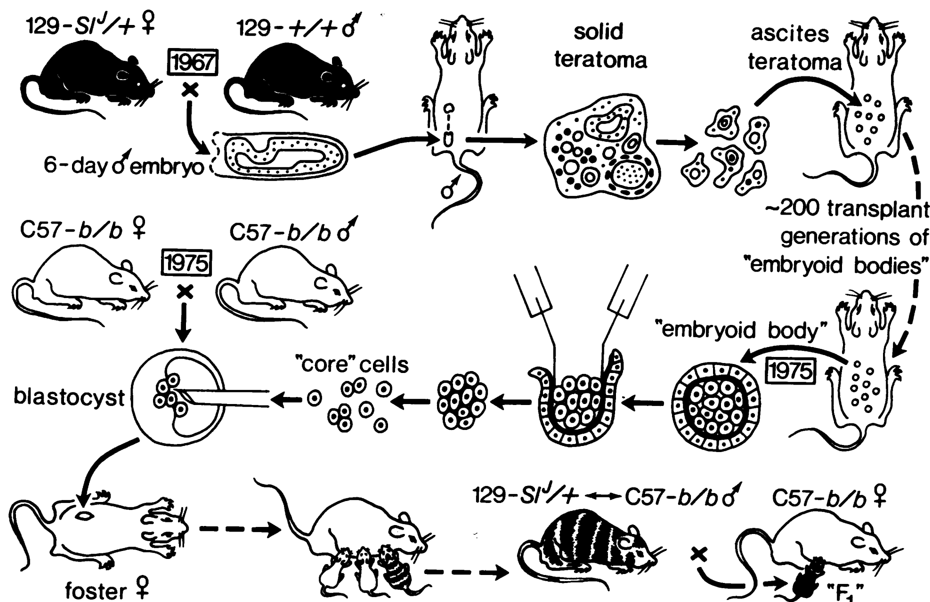

FIG. 1. The 8-year history of the experiment is diagrammed, starting at the upper left. The OTT 6050 teratoma was experimentally produced (3) in 1967 from a 6-day chromosomally male (X/Y) embryo of 129-strain (129/Sv Sl^J *C P*) *agouti black* parents. (Retrospectively, from the coat of mosaic mouse no. 1 [Fig. 2], this embryo and its mother were found to have had the *steel* [Sl^J/+] coat genotype; the father was wild-type [+/+].) The embryo was placed under a testis capsule, where it became disorganized, forming a teratoma which metastasized to a renal node. The primary tumor was minced and transplanted intraperitoneally; it became an ascites tumor of "embryoid bodies" with yolk sac "rinds" and teratocarcinoma (or embryonal carcinoma) "cores". In 1975, after almost 200 transplant generations in syngeneic hosts, the rinds of some embryoid bodies were peeled away and the malignant core cells were injected into blastocysts from parents of the *non-agouti brown* C57-*b/b* (C57BL/6-*b/b*) strain. The blastocysts were transferred to the uterus of a pseudopregnant foster mother (mated to a sterile vasectomized male). Pregnancy ensued and live normal mice were born. Some had coat-color mosaicism (striped) and/or internal tissue contributions of the 129 tumor-strain. At maturity, a mosaic male (shown in Fig. 2) was test-mated to C57-*b/b* females. Production of "F_1"-like offspring proved that he also had normal sperms derived from the teratocarcinoma cells; some progeny were Sl^J/+, some +/+.

ed in 0.25% trypsin-EDTA and Ca^{++}- and Mg^{++}-free PBS for about 2 min, rinsed in DMEM, FCS, and DNase, and gently pipetted in DMEM and 15% FCS, for final separation into single cells. Some cores were reserved for metaphase chromosome counts in colchicine-treated, air-dried, aceto-orcein-stained preparations.

Blastocyst Injections. Attempts to produce composites of core cells and normal blastomeres by the aggregation method (reviewed in ref. 6) were unsuccessful because surface differences prevented the two kinds of cells from adhering. Therefore, the method of blastocyst injection (14, 15) was used. Blastocysts were genotypically from ICR sublines of the following inbred strains: C57BL/6 (C57), C57BL/6-*b/b* (C57-*b/b*, a strain differing from C57 only by substitution, through mutation, of the *black* allele with *brown*), or WH. They were placed in a microdrop of the medium used for peeling embryoid bodies, under halofluorocarbon oil (Voltalef 10S), on a Zeiss inverted UPL microscope equipped with Leitz micromanipulators and siliconed pipettes. Each blastocyst was injected with five core cells into the blastocoel cavity near the inner cell mass. The injected blastocysts were incubated at 37° for approximately 4 hr in DMEM with 15% FCS under 10% CO_2 in air. They were then surgically transferred (6) to uteri of pseudopregnant ICR randombred albino females (or, in some experiments which were terminated prenatally, to C3H females) previously mated to sterile vasectomized ICR males.

Analyses of Tissue Genotypes. Presence of strain-specific allelic electrophoretic variants in tissue homogenates or blood cell lysates was determined by starch gel electrophoresis methods for glucosephosphate isomerase (GPI; D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9) (*Gpi-1* locus) (16) and hemoglobin (*Hbb* locus) (17). Variants of the major urinary protein complex (MUP) (*Mup-1* locus) were analyzed by acrylamide gel electrophoresis (18). MUP is manufactured in the liver and is normally excreted by mice (19). The proportions of MUP strain variants detected in the urine of a given allophenic mouse conform to the proportions of the two strains of liver parenchymal cells (but not necessarily of other cell types in the liver), histochemically visualizable by an independent marker; therefore, the liver parenchymal cell genotypes of an allophenic mouse can be indirectly but accurately diagnosed from urinary analyses of its MUP variants (20). The 7S classes of G1 and G2a strain-specific allotypes of immunoglobulins were kindly determined by Dr. Melvin J. Bosma of this Institute, by the Ouchterlony double-diffusion precipitin method. The reagents used (21) are specific for allelic differences at both the *Ig-1* and *Ig-4* loci.

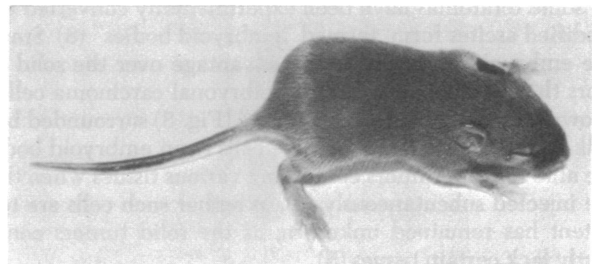

FIG. 2. Male *mosaic mouse no. 1*, produced as shown in Fig. 1. His coat is largely *agouti* and *black*, as in 129, with an overall greyish dilution (especially visible on the feet) due to the *steel* gene. Very narrow transverse stripes of *agouti* and *non-agouti* hair follicle clones are on both flanks and the crown of the head. Abbreviated wide patches of *brown* melanoblast clones are over the hindquarters, on each side of the head, and on the tail.

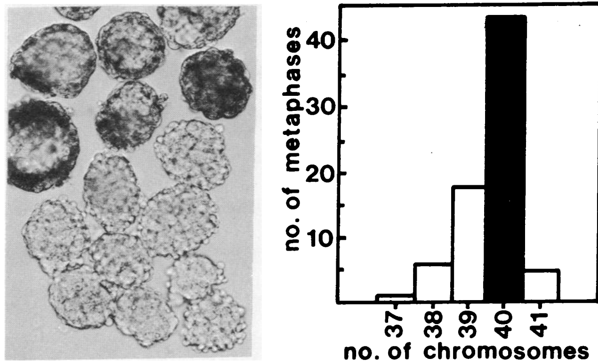

FIG. 3 (*left*). Living embryoid body "cores" (clear, bottom) of embryonal carcinoma cells after removal of the yolk sac "rinds". Intact embryoid bodies (dark, top) are shown for comparison.

FIG. 4 (*right*). The normal chromosome number of 40 is the modal number in metaphases of embryonal carcinoma cells taken from the "cores" of embryoid bodies grown only *in vivo*.

RESULTS

The isolation procedures did not diminish tumorigenicity of core cells: when tested by subcutaneous inoculation, single cores produced solid tumors with a variety of tissues.

Chromosome counts in 76 metaphases of embryoid body core cells revealed a normal modal number of 40 chromosomes, with little deviation (Fig. 4).

Of 280 total blastocysts injected with core cells, 97 were in surrogate mothers autopsied on days 8–18 of gestation; they yielded 45 living embryos or fetuses. The remaining 183 blastocysts were in mothers allowed to go to term; they delivered 48 mice, all live. All fetuses and postnatal animals appeared normal and showed no evidence of tumors.

Thus far, six fetuses and eight postnatal individuals, from injected C57 or C57-*b/b* blastocysts, have been extensively analyzed for presence of cells of the 129 tumor strain. Three of the postnatal animals have proved to be cellular genetic mosaics with substantial tissue contributions from the teratocarcinoma cell strain. The remaining 11 cases had only the C57 (or C57-*b/b*) strain.

The first of these three, *mosaic mouse no. 1* (Fig. 2), is alive, now 11 weeks of age, and is a healthy male from a C57-*b/b* (genotypically *non-agouti* and *brown*, or *a/a;b/b*) blastocyst injected with embryoid body core cells presumed, but not previously demonstrated, to be genotypically *white-bellied agouti* and *black*, or $A^w/A^w;B/B$, as in the 129 strain in which the tumor arose. This animal is very striking because he greatly resembles 129-strain controls. His coat is largely *agouti* and *black* (approximately 90% of each) and he also has the cream-colored belly characteristic of A^w/A^w. Upon closer inspection, he is partially striped and is indistinguishable from those striped allophenic mice (produced from aggregated normal blastomeres of $A^w/A^w;B/B$ and *a/a;b/b* strains) in which the *agouti* hair follicles and *black* melanoblasts happen to predominate over *non-agouti* and *brown*, respectively, in the coat (22–25). The localized striped areas (Fig. 2) are in two independently originating, but superimposed and interacting tissues. The narrow transverse bands of *agouti* and *non-agouti*, on both flanks and on the crown of the head, are hair follicle clones, each descended from a somite mesoderm cell. The few wide partial bands of *brown* melanoblasts, over each haunch and on the head, represent surviving remains of melanoblast clones, each from a neural crest-derived cell (22–25). Because mouse no. 1 has largely *agouti* hairs, his brown areas have a yellowish (phaeomelanin) cast and do not resemble the true-brown color of C57-*b/b* controls.

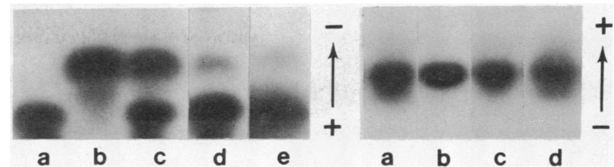

FIG. 5 (*left*). Glucosephosphate isomerase allelic strain variants in starch gel electrophoresis of blood cell lysates of 129 (slot a) and C57(b) controls, and a 1:1 control mixture (c). Circulating red blood cells (d) and white blood cells (e) from *mosaic mouse no. 1* are very largely of the 129 tumor-strain type.

FIG. 6 (*right*). *Diffuse* (129-type) (slot a) and allelic *single* (C57-type) (b) adult hemoglobins, and a 1:1 control mixture (c) from red blood cell lysates, in starch gel electrophoresis. *Mosaic mouse no. 1* (d) has largely the *diffuse* type, produced by his tumor-strain erythrocytes.

A quite unexpected observation was that the coat of this animal is also largely *steel*, as in $Sl^J/+$ heterozygotes. The *steel* gene (26) produces a slight overall dilution of coat color, especially evident in mouse no. 1 on the feet (Fig. 2), vibrissae, and belly. He has no white areas, but the small white head and belly spot seen in $Sl^J/+$ on some genetic backgrounds (26) are also absent from $Sl^J/+$ controls on the 129 background. The *steel* phenotype of mouse no. 1 was unexpected because the publication describing the origin of this tumor from a 6-day embryo (3) made no mention of the genotypes of the parents at the *steel* locus in that experimental mating, although this locus has been maintained as a segregating one by forced heterozygosity in the strain. We therefore contacted Dr. L. C. Stevens, who was able to trace in his 1967 records the parentage of the 6-day embryo in question: the mother was indeed $Sl^J/+$, the father wild-type $+/+$ (L. C. Stevens, 1975, personal communication).

The circulating red blood cells and white blood cells of mosaic mouse no. 1 are predominantly (90% or more) of the 129-strain type, as shown by their strain-specific electrophoretic variants of GPI (Fig. 5). The hemoglobin produced by the red cells is also chiefly of the *diffuse* (Hbb^d) electrophoretic type, which is the normal adult type of the 129 strain; only a minor fraction is of the C57-*b/b single* type (Hbb^s) (Fig. 6). Ouchterlony double diffusion analyses of serum immunoglobulin allotypes show only the presence of the 129 strain (Fig. 7). Serial dilutions indicate that if any C57 allotype were present, it would have to be less than 0.5–1 μg/ml, or a very small fraction of the total of approximately 4 mg/ml of both these classes (IgG1 and IgG2a).

Electrophoresis of protein from the urine, for strain-specific MUP variants, revealed presence of both the 129 and C57-*b/b* strains in mouse no. 1 (Fig. 8). He must thus have many parenchymal cells of the 129 strain in his liver.

Mouse no. 1 was mated, starting at 7 weeks of age, to females of the C57-*b/b* strain. He has thus far sired 61 offspring, all normal and all from sperms of the 129 strain, as shown from production of "F_1"-like progeny for coat, GPI, and other markers. Some progeny have received the Sl^J gene from their father and are phenotypically *steel*. Whether mouse no. 1 also has any C57-*b/b*-strain gametes should become clear from further progeny tests.

Mosaic mouse no. 2 was a female from a C57 blastocyst injected with core cells. She was killed for study at 13 days of age. Her coat was entirely like a C57 control, as with previous allophenic mice having only mosaicism of internal tissues (22–25). Her circulating red and white blood cells

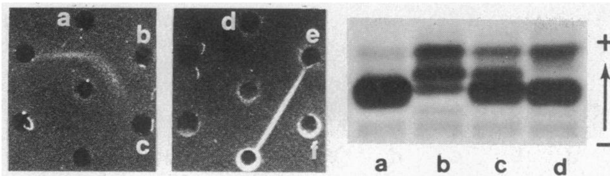

FIG. 7 (*left pair*). Ouchterlony double-diffusion analyses, showing only the 129-strain specific allotypes of 7S G1 and G2 immunoglobulin classes in *mosaic mouse no 1.* Anti-129-type serum (left, center well) reacts with serum from a 129 control (well a), and with a 1/20 dilution of serum from mouse no. 1 (b), but not with a C57 control (c). Anti-C57-type serum (right, center well) does not react with serum from a 129 control (well d), nor with undiluted serum from mouse no. 1 (e), but does react with serum from a C57 control (f).

FIG. 8 (*right*). Acrylamide gel electrophoretic patterns of allelic strain variants of the major urinary protein complex in 129 (slot a) and C57 (b) controls and a 1:1 control mixture (c). *Mosaic mouse no. 1* (d) has a mixture of both strain types, indicative of both cell strains in the liver parenchyma where the protein is produced.

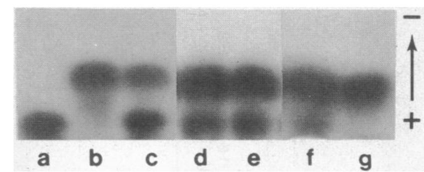

FIG. 9. Glucosephosphate isomerase strain-specific allelic variants in starch gel electrophoresis of tissue homogenates from 129 (slot a) and C57 (b) controls, and a 1:1 control mixture (c). Female *mosaic mouse no. 2* has tumor-derived (129-strain) cells in thymus (d), kidneys (e), and reproductive tract (f), but not in blood (g).

were also entirely of the C57 GPI type (Fig. 9),—a fortunate circumstance because it meant that any 129-type enzyme in homogenates of other tissues reflected the specific tissue genotype and not the blood component. As seen from analyses of the thymus and kidneys (Fig. 9), these organs must have contained a substantial proportion of cells derived from the tumor implant. The thymic cells of 129 origin in this particular animal may have been chiefly in the stroma rather than in the lymphocyte population. The entire reproductive tract, although morphologically like that of a normal (X/X) female, contained a minority of the tumor-strain cells (Fig. 9), which are known (12) to be of male (X/Y) sex chromosome constitution. Comparable cases have previously been described in X/X ↔ X/Y allophenic mice (27). Analyses of other tissues in mouse no. 2 were inconclusive because of the technical failure of a starch gel.

Mosaic mouse no. 3 was a normal male from an injected C57-*b*/*b* blastocyst. He was killed at 3 days of age, before the hairs had emerged. With the GPI marker, his blood cells were all C57-*b*/*b*, as were his brain, heart, lungs, thymus, stomach, intestines, pancreas, reproductive tract, and skeletal muscles. However, his liver included about 33% of the 129 strain-type, his spleen about 20%, and his kidneys about 33%.

DISCUSSION

Teratomas in the 129 strain may be spontaneous [from diploid spermatogonia developing *in situ* (1)]; or they may be experimentally induced, e.g., by transplanting early embryos beneath the testis capsule, where they become disorganized and form tumors. This was how the OTT 6050 teratoma was derived by Stevens (3), in 1967, from a transplanted 6-day chromosomally male (X/Y) embryo. The tumor, which had metastasized, was transplanted subcutaneously and then converted to the embryoid body ascites form by injecting minced fragments into the body cavity (3).

After this long period, the embryonal carcinoma cells in the cores (Figs. 1 and 3) of small embryoid bodies have retained their normal diploid chromosome number *in vivo* (Fig. 4), as in the tumor of origin (12). The low incidence of euploidy and the wide variability in chromosome numbers previously found for whole embryoid bodies (10, 11) may, therefore, be due to their rinds of yolk sac cells, which are not multipotential, and/or to inclusion of older cystic embryoid bodies in the preparations.

Eight years and almost 200 transplant generations of a tumor that has remained highly malignant have separated the mating of the 129-strain first pair of parents, whose embryo gave rise to the tumor, from recent matings of the C57- or C57-*b*/*b*-strain parental pairs whose blastocysts were successfully inoculated with the embryonal carcinoma cells (Fig. 1). The tumor itself generally kills its host by 3–4 weeks after transplantation. Yet our oldest mosaic animal (mouse no. 1), consisting very largely of tumor-derived cells, is now 11 weeks old and appears healthy and vigorous.

The malignant carcinoma cells, after introduction into normal blastocysts, have given rise to a large variety of normal tissues in three mosaic mice (including one animal still quite incompletely analyzed). The tissues thus far known to have differentiated from the carcinoma implants include melanoblasts, hair follicle dermis, erythrocytes, leucocytes of diverse kinds, liver, thymus, kidneys, and, perhaps most striking of all, sperms. Normal function of these tissues is attested to by production of the 129-strain-specific adult type of hemoglobin, by erythrocytes; immunoglobulins, by plasma cells; MUP, by hepatocytes; glucose-phosphate isomerase, by various tissues; black eumelanin, by melanocytes; elicitation of the phaeomelanin effect, by hair follicle cells; and production of progeny, from eggs fertilized by tumor-derived sperms. According to conventional classifications, all germ layers are included in these differentiations. However, as the old germ-layer concept undervalues flexibility in development (28, 7), it is perhaps more relevant to emphasize that the results show differentiation of teratocarcinoma cells into many developmentally unrelated tissues. Some of these tissues, e.g., liver, thymus, and kidney, have never been seen in mouse teratomas (ref. 5; and L. C. Stevens, 1975, personal communication). The differentiation of such tissues may depend on inductive interactions between embryonic components (29); if in fact the components form at all in the chaotically arranged tumors, or in cultures, they may fail to be brought into the proper associations.

The results strongly support the probability that embryonal carcinoma core cells from OTT 6050 embryoid bodies always grown *in vivo* are developmentally totipotent. It seems highly unlikely that the five cells injected per blastocyst had each already been partially committed along a different specific path of development, especially in light of the spectrum of differentiation obtained.

The conclusion of probable totipotency of the cells tested here cannot be generalized to other sources of embryonal carcinoma cells, which must be similarly tested. In the only previously reported tests, Brinster (30) injected cells from completely dissociated embryoid bodies (cores plus rinds) into blastocysts of a randombred strain. Albinism was the only known marker. He obtained a single mouse (out of 60) with a few small stripes of pigmented hairs on an albino ground. While this was encouraging, the limited evidence of

a 129-type contribution left open the possibility that one of the injected cells was already determined as a melanoblast and that there were no totipotent cells.

In the present experiments, orderly expression of many genes (e.g., immunoglobulin, hemoglobin, MUP, *agouti* genes) has occurred *in vivo* after they had been "silent" or undetected in the tumors for 8 years, as well as in cultures of teratocarcinoma cells. Differentiation has also uncovered, as an organismic phenotype, a hitherto cryptic gene, *steel*, whose presence was unsuspected. Thus, embryonal carcinoma cells from embryoid body cores (or, preferably, from a transplantable core-cell line) offer new possibilities for studies of mammalian regulatory systems: The carcinoma cells could first be experimentally mutagenized and selected during a brief *in vitro* sojourn and then "cycled" through mice via blastocyst injections. Participation in differentiation of a mosaic individual would permit developmental and biochemical analyses of the mutations; conversion of some cells to gametes would enable genetic analysis and mapping of the mutated regions through recombination and segregation during meiosis. Among the advantages over conventional germ-cell mutagenesis *in vivo* are the possibility of subjecting mutagenized cells to selective screens; of bypassing the problems of sterility and dominant lethals; of obtaining mutant clones of desired size; and of biochemically analyzing early-stage mutants.

These studies also offer a new means of analyzing the basis for malignancy. The capacity of embryonal carcinoma cells to form normally functioning adult tissues demonstrates that conversion to neoplasia did not involve structural changes in the genome, but rather a change in gene expression (7, 31). Maintenance of a proliferative stem-cell way of life, hence commitment to malignancy, appears to have been due solely to an organizational disturbance during development. Its complete reversibility in a normal environment is unequivocal, i.e., not attributable to selection for nonmalignant cells. The results thus support the hypotheses of Pierce (2) and Stevens (1, 12) concerning the origin of these tumors. A comparable nonmutational basis for neoplastic conversion, involving changes in gene expression due to tissue disorganization, may apply to some malignancies of partially specialized stem cells of particular tissues.

Note Added in Proof. More recent analyses have disclosed additional normal mosaics: two postnatal males and two fetuses with carcinoma-derived cells in some tissues, including heart and skeletal muscle in one case. Tumor-derived cells were also detected in three normal placentas (from fetuses of solely blastocyst origin).

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