ABSTRACT

Mouse teratocarcinoma cells from the OTT6050 ascites tumor were established in tissue culture and selected for 5-bromodeoxyuridine (BrdUrd) resistance. The embryonal carcinoma cells grew without a feeder layer, remained deficient for thymidine kinase (EC 2.7.1.75), and differentiated like the original tumor into various tissues after subcutaneous injection into 129 mice. We fused the BrdUrd-resistant mouse teratocarcinoma cells with HT1080-6TG human diploid fibrosarcoma cells deficient in hypoxanthine phosphoribosyltransferase (EC 2.4.2.8) and selected for hybrid cells in hypoxanthine/aminopterin/thymidine medium. The resulting hybrid cells segregated human chromosomes quickly and retained one to three human chromosomes including chromosome 17 that carries the human genes for thymidine kinase and galactokinase (EC 2.7.1.6). Single hybrid cells from five independent clones containing human chromosome 17 were injected into mouse blastocysts bearing several genetic markers that affect the coat color phenotype and strain-specific enzyme variants in order to detect tissue differentiation derived from the injected cells. After the injection of single hybrid cells into a total of 103 experimental blastocysts that had been surgically transferred to pseudopregnant foster mothers, 49 mice were born and 2 of them clearly revealed coat mosaicism. In 5 of 17 mice thus far analyzed, the injected hybrid cells proved to be capable of participating substantially in development of seven different organs. However, human gene products have not yet been detected unequivocally in those tissues and weak human-specific galactokinase activity could be recovered only from two mosaic tissues.

Our results demonstrate that, after in vitro culture and selection, at least some of the mouse–hybrid mouse cells still retain their in vivo potential to differentiate and become functionally integrated in the living organism. It now seems feasible to cycle mouse teratocarcinoma cells carrying human genetic material through mice via blastocyst injection to study human gene expression during differentiation.

Mouse teratocarcinomas are tumors that either develop spontaneously in the gonads or can be produced experimentally by grafting early embryos into the testis of syngeneic hosts (1, 2). They contain various aberrantly differentiated and chaotically organized tissues that include derivatives of all three germ layers. Usually these tumors are benign and nontransplantable, and they are then referred to as “teratomas.” Occasionally, however, in addition they contain embryonal carcinoma cells, the stem cell population of highly malignant teratocarcinomas (3). These tumor cells scattered throughout the solid tumor or propagated as “embryoid bodies” in an ascites form continue to divide and proliferate, and sometimes even invade and metastasize, thereby retaining the malignant properties of transplanted tumors.

Recently, it was shown that, after microinjection into mouse blastocysts, embryonal carcinoma cells participated in normal development (4) and clonally contributed to virtually all major adult tissues (5, 6). It therefore appears as if the initially malignant teratocarcinoma cells always grown in vivo retain developmentally totipotent and are able to express their genetic repertoire normally after being exposed to the appropriate embryonic environment. In a similar bioassay, in vitro cultured cells derived from other teratocarcinomas were also capable of contributing to several somatic tissues, although most of the chimeric mice additionally developed tumors in various anatomical sites (7). Furthermore, it was recently found that, even after in vitro selection for a particular genetic defect, the mutant teratocarcinoma cells still retained their in vivo potential to a remarkable extent (8). It thus seemed logical to find out whether foreign genetic material can be introduced into embryonal carcinoma cells via somatic cell hybridization in order to reveal its developmental and biochemical phenotype in the living organism via blastocyst injection.

In the experiments reported here, mouse teratocarcinoma cells of the OTT6050 tumor origin first were selected in vitro for thymidine kinase (TK; ATP:thymidine 5’-phosphotransferase, EC 2.7.1.75) deficiency and then fused with HT-1080-6TG human fibrosarcoma cells deficient in hypoxanthine phosphoribosyltransferase (HPRT; IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) (9). Only the interspecific hybrid cells can grow in hypoxanthine/aminopterin/thymidine (HAT) selective medium (10). On the contrary, TK- and HPRT-deficient parental cells, which lack the enzymes required for the incorporation of thymidine and hypoxanthine, respectively, do not survive in this medium. Under these selective conditions, the viable hybrid cells, which quickly lose human but not mouse chromosomes, retain at least human chromosome 17 that carries the locus for TK (11). This particular chromosome also carries a second known gene that is closely linked to TK and codes for galactokinase (GLK; ATP: D-galactose 1-phosphotransferase, EC 2.7.1.6) (12). The latter enzyme, with its characteristic electrophoretic mobility quite different from the equivalent mouse enzyme, serves as another useful biochemical marker for detecting the presence and normal expression of the human gene product in the hybrid cells.

In the present report, we describe the successful transplantation of in vitro cultured human–mouse hybrid cells into early mouse embryos, demonstrate their integration and normal contribution to several somatic adult tissues, and discuss the possible use of this in vitro approach to follow human genes through mammalian development.

Abbreviations: BL6, C57BL/6j; B10SJLJ, C57BL/10Wt x SJL/Wt; 129, 129/Sv SL C P mouse strain; DMEM, Dulbecco’s modification of Eagle’s medium; Hepes, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; CFI, glucosephosphate isomerase (EC 5.3.1.9); TK, thymidine kinase (EC 2.7.1.75); GLK, galactokinase (EC 2.7.1.6); HAT, hypoxanthine/aminopterin/thymidine; HPRT, hypoxanthine phosphoribosyltransferase (EC 2.4.2.8); BrdUrd, 5-bromodeoxyuridine.
MATERIALS AND METHODS

Selection of Hybrid Clones. TK⁻ OTT6050 mouse teratocarcinoma cells were fused with HT1080-6TG human fibrosarcoma cells (9)–10⁷ of each cell type—in the presence of β-propiolactone-inactivated Sendai virus at pH 8.0 according to established procedures (13). The hybrid cells were then selected in RPMI medium containing 10% fetal calf serum and the HAT components (10). Colonies that appeared within 2–3 weeks were picked by using glass rings and EDTA/trypsin and grown in T25 Falcon flasks in RPMI/HAT medium. Cells derived from different colonies were then cloned by placing single cells in microwells of Limbro plates. These clones were subsequently transferred to Falcon flasks and maintained in culture under selective conditions.

Karyologic Analysis. The parental and hybrid clones were stained for chromosome banding by a trypsin/Giemsa staining technique (14). At least 15 different metaphases of cells from each clone were studied in order to identify individual mouse and human chromosomes by their characteristic banding patterns.

Collection of Embryos and Cells. Inbred C57BL/6J (BL6) and SJL/J females were selected in proestrus (15) and mated with males of the same strains. The next morning, the females with vaginal plugs were separated and then killed on postcoitus day 3. Embryos at the early morula stage were flushed from the oviducts and cultured in modified Whitten’s medium (16) until the blastocyst stage. After this in vitro incubation, a group of five well-expanded BL6 blastocysts were transferred into a small drop of Ca²⁺-free medium (Dulbecco’s modification of Eagle’s minimal essential medium, DMEM) supplemented with bovine serum albumin (10 mg/ml) and 10 mM N-2-hydroxyethylpiperezine-N’-2-ethanesulfonic acid (Hepes) on a special microscope slide. The medium had previously been covered with halofluorocarbon oil (Voltalef 10 S).

In order to collect hybrid cells, the original medium in a culture flask was replaced by Ca²⁺-free medium. After 20-min incubation at 37°C, the cells were gently flushed from the bottom of the flask and transferred to a siliconized depression slide and stored under paraffin oil. For each experimental series, about 30 cells were selected and placed near the recipient blastocysts for subsequent injection.

Micromanipulation. The injection pipets were prepared from 1.2-mm (outside diameter) Pyrex tubing (Corning Glass) that had been pulled to a 15-μm tip and then sharpened on a special grinding wheel and a Defonbrune microforge. After thorough cleaning in sulfuric acid, the pipets were siliconized (Siliclad) and stored under sterile conditions. For microinjection, the capillary was connected to a syringe (Hamilton) containing paraffin oil, and the distal part was filled with halofluorocarbon oil (Voltalef 3 S). The holding pipet, with a blunt (60 μm) tip, was connected to another syringe system and its distal half was filled with Ca²⁺-free medium. Both glass capillaries could be controlled by Leitz micromanipulators equipped with a Zeiss inverted UPL microscope.

The injection pipet, into which a single cell had been sucked together with a small amount of medium, was slowly pushed through the zona pellucida and trophoblast into the inner cell mass of blastocyst, properly attached to the holding capillary. After injection, the pipet had to be withdrawn very gently in order to prevent the implanted cell from being misplaced in the blastocoel of the recipient. All injected blastocysts were incubated in Whitten’s medium for about 2 hr at 37°C before transfer to foster females.

Seven experimental BL6 and three control SJL blastocysts were surgically transplanted to the uterus of a pseudopregnant female. After injection, the pipet had to be withdrawn very gently in order to prevent the implanted cell from being misplaced in the blastocoel of the recipient. All injected blastocysts were incubated in Whitten’s medium for about 2 hr at 37°C before transfer to foster females.

FIG. 1. Mouse teratocarcinoma containing differentiated tissues such as cartilage (c), glandular epithelium (g), muscle (m), and neural rosettes (n), as well as undifferentiated embryonal carcinoma cells (e). Samples of in vitro cultured OTT6050 cells deficient for TK were injected subcutaneously into nude or syngeneic 129 mice. In both instances, the same kind of pluripotent tumors formed in vivo (×93).

C57BL/10Lt × SJL/Wt (B10SJLF1) mouse at postcoitus day 3 or 4. These females had been selected in proestrus and mated to sterile (vasectomized) B10SJ/LLEF1 males. Analyses of Tissue Genotypes. Strain-specific allelic variants of mouse glucosephosphate isomerase (GPI; d-glucose-6-phosphate ketolase) and mouse muscle glyceraldehyde-3-phosphate dehydrogenase (GpK; EC 1.1.1.42) were used to identify individual mouse chromosomes by their characteristic banding patterns.

Results

In previous studies, mouse teratocarcinoma cells of OTT6050 tumor origin were established in tissue culture and selected for BrdUrd resistance. After subcutaneous transplantation to syngeneic 129 mice, the mutant teratocarcinoma cells still expressed their malignant as well as pluripotent properties (Fig. 1), similar to the original ascites tumor.

We found that, after microinjection into mouse blastocysts, some of these TK⁻ teratocarcinoma cells became integrated during normal development and contributed substantially to several internal organs—e.g., liver, lung, heart, brain, and skeletal muscle. However, the frequency of tissue mosaicism (about 10%) in adult chimeric mice was reduced compared with the rate of integration (about 25%) obtained from embryonal carcinoma cells of the original tumor (cf. 6; unpublished data). Nevertheless, these results provided good evidence that teratocarcinoma cells selected in vitro for TK deficiency were still able to differentiate in vivo into various different tissues and should therefore be useful carriers for introducing and analyzing foreign genetic material in the living organism.

TK-deficient teratocarcinoma cells were then hybridized to HT1080-6TG human fibrosarcoma cells deficient in HPRT and
the hybrids were selected in HAT medium. Cells derived from five independent colonies were grown and cloned. One clone derived from each of the five colonies was analyzed karyotypically for the presence of human chromosomes by trypsin/Giemsa banding staining for chromosomal identification. The hybrid cells lost most of their human chromosomes but retained human chromosome 17 (Table 1). This particular chromosome could be identified in more than 90% of the metaphases of the hybrid clones.

From a total of 121 blastocysts, each injected with a single human–mouse hybrid cell, 105 were surgically transferred to pseudopregnant foster mothers that delivered 49 mice, 20 females and 29 males (48% survival).

Two of the experimental progeny clearly showed coat mosaicism. Chimeric mouse A, a male originating from a clone 3 cell-injected blastocyst and autopsied at 3 weeks of age, had a large and phenotypically white hair follicle clone extending dorsally from the midlateral side to the ventral area and several smaller patches, also white in phenotype, on the hindquarters, tail, and feet (Fig. 2). The second coat-mosaic mouse C, a female still alive and derived from a clone Z cell-injected blastocyst, developed a distinct white patch on the crown of the head.

Thus far, 17 mice, including chimeric mouse A but not C, have been analyzed for internal tissue contributions from the hybrid cell implant by biochemical assays for strain-specific electrophoretic variants of GPI. Two of these experimental mice turned out to have substantial hybrid cell-derived contributions in several internal organs, ranging from 10% to 60% (Fig. 3). The remote possibility that the tissue mosaicism might have resulted from spontaneous aggregation of experiential BL6 with control SJL blastocysts in utero was excluded by the fact that the heart, muscle, and kidney of chimeric mice A and B expressed only the BL6 (and 129) electrophoretic form of phosphoglucomutase (Pgm-1) and isocitrate dehydrogenase (Id-1) and not the SJL form. The presence of heterodimeric GPI activity in skeletal muscle attested to regular fusion of myoblasts between BL6 host and 129-type injected cell. None of the seven mosaic tissues of mouse A examined histologically in serial sections revealed any morphological abnormalities or teratoma formation. On the contrary, the subcutaneous injection of hybrid cells into athymic nude mice (20) resulted in tumors (Fig. 4).

**DISCUSSION**

It has been known for some time that single embryonal carcinoma cells, the stem cell population of malignant teratocarcinomas, remain pluripotent after subcutaneous transplantation to syngeneic mice because they can give rise clonally to the various tissue types of these well-differentiated tumors (21). The developmental plasticity of embryonal carcinoma cells, which resemble early embryonic cells morphologically, immunologically, and biochemically to some extent (22), has further been attested in the living organism by means of microinjection into mouse blastocysts (4–8). For as yet unknown reasons, the transplanted stem cells of in vitro propagated teratocarcinomas derived from either a male embryo (OTT6050) or a parthenogenetically activated ovarian egg (LT72484) lose their tumorigenic properties during the course of development and contribute normally to all major somatic tissues or, in a few instances, even differentiate into functional sperm (OTT6050) or eggs (LT72484) in chimeric mice (refs. 5 and 6; unpublished data). It had therefore been proposed earlier (23) that teratocarcinomas might provide use with a unique kind of cell which can be selected in vitro for a given somatic mutation and then cycled through mice via blastocyst injection for further in vitro analysis. Following such an experimental scheme, different sources of cultured teratocarcinoma cells that were selected either for HPRT deficiency (8) or for TK deficiency (unpublished data) seemed to retain their in situ developmental potential to a remarkable extent.

In the present series of experiments, only part of which has yet been analyzed, we carried this bioassay a significant step further and combined it with somatic cell hybridization techniques in order to introduce foreign genetic material into embryonal carcinoma cells and eventually to study its phenotypic expression in the organism. Along these lines, fusion of mouse teratocarcinoma deficient for TK with human fibrosarcoma cells deficient for HPRT resulted in interspecific hybrid cells that, as shown in Table 1, lost the great majority of human chromosomes and retained only one to three human chromosomes, including human chromosome 17 which carries the genes for human TK and GLK.

Even after in vitro selection and culture for a period of several months, at least some of the human–mouse cells still became functionally integrated during embryogenesis and participated in orderly differentiation of various internal tissues. Normal hybrid-cell derived contribution could be demonstrated directly in skeletal muscle and, most strikingly, in the coat. In addition, the normality of the mosaic tissues could be assessed by histological analysis which was consistent with earlier ob-

**Table 1. Human chromosomes present in hybrids between mouse teratocarcinoma and human fibrosarcoma cells**

<table>
<thead>
<tr>
<th>Hybrid</th>
<th>Mouse chromosomes in hybrids* no.</th>
<th>Metaphases with human chromosomes/total analyzed</th>
<th>Human chromosome:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone 1</td>
<td>39</td>
<td>1/15</td>
<td>7</td>
</tr>
<tr>
<td>Clone 2</td>
<td>38</td>
<td>0/17</td>
<td>11</td>
</tr>
<tr>
<td>Clone 3</td>
<td>40</td>
<td>0/16</td>
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<tr>
<td>Clone 4</td>
<td>39</td>
<td>0/16</td>
<td>17</td>
</tr>
<tr>
<td>Clone 5</td>
<td>38</td>
<td>0/15</td>
<td>14/15</td>
</tr>
</tbody>
</table>

* All the parental teratocarcinoma and the hybrid cells contained two metacentric chromosomes that resulted from Robertsonian centric fusion. Thus, the number of individual mouse chromosomes present in the hybrids was between 40 and 42.

![Fig. 2. Coat mosaicism in chimeric mouse A, developed from a BL6 blastocyst after microinjection of a human–mouse hybrid cell. In addition to the expected black coat phenotype of the recipient, this mouse also exhibited several white patches on the back, tail, and feet and a large white clone extending from the midlateral to the ventral side, all of which derived from the injected hybrid cell and comprised about 20% of the total coat.](image-url)
tumor became apparent and was separated from the host tissue and histologically.

and centrifuged to a small pellet that was injected into the kidney (k) become hemizygous that, during teratocarcinoma, original coat of (6). The phenotypic mosaicism coat normally with myoblasts mosaic mouse, and (A

FIG. 3. Electrophoretic analysis of strain-specific allelic variants of GPI in blood cell lysates and tissue homogenates from two chimeric mice (A and B). Control represents a 50:50 mixture of slow-migrating 129 and fast-migrating BL6 types of GPI in blood cell lysates. In each genetically mosaic mouse, clonal descendants of the injected human–mouse hybrid cell contributed substantially to several internal organs and also fused normally with myoblasts of the host, as judged from heterodimeric enzyme expression in skeletal muscle. In addition, mouse A showed conspicuous coat mosaicism (Fig. 2) whereas chimeric mouse B did not.

servations (6). The phenotypic pattern of white patches in the coat of two chimeric mice appeared unexpectedly because the original teratocarcinoma, heterozygous for the steel gene, had previously exhibited a diluted agouti phenotype (5). It is conceivable that, during in vitro culture, the hybrid cells happened to become hemizygous for steel (that is, phenotypically white as homozygous steel does) because of a small deletion of mouse chromosome 10 that carries the wild-type allele for this locus. Alternatively, a spontaneous mutation or interference of the introduced human genetic material at the albino locus on mouse chromosome 7 could have caused this phenotypic change in coat color. Further investigations including ultrastructural analysis of hybrid-cell derived skin areas should help to resolve this problem.

Although the human–mouse hybrid cells were found to contribute up to 60% in some organs of the two chimeric mice, as judged from the GPI analysis, the human gene products have not yet been detected unequivocally from those mosaic tissues. Weak human-specific GLK activity could be recovered only from the heart of mouse A and from the kidneys of mouse B. Both tissues showed the highest participation of hybrid cells in the GPI test. The failure to disclose any human GLK in all other mosaic tissues might have resulted from (i) use of assay techniques not sensitive enough for the recovery of minor enzyme activity, (ii) loss of human chromosome 17 during in situ cell divisions in the absence of selective pressure to retain this chromosome, or (iii) tissue-specific restriction in gene activity.

In spite of the fact that somatic cell hybrids still participate in normal mouse development, it is obvious that the problems related to the retention and recovery of human genes have to be solved first before we can effectively utilize our in vitro system for cycling human genetic material through mice.

We thank Dr. R. T. Prehn for providing us with excellent working facilities at Highsea, Dr. L. C. Stevens for tremendous help in tissue

FIG. 4. Rhabdomyosarcoma (r) derived from hybrid cells between human HT1080-6TG fibrosarcoma and mouse OT/310 glioblastoma teratocarcinoma. At least 10⁹ hybrid cells were collected from the culture flasks and centrifuged to a small pellet that was injected into the kidney (k) capsule of a nude mouse at age 3 weeks. About 1 month later, the tumor became apparent and was separated from the host tissue and examined histologically. (×93.)
type analysis, and D. Dorr, P. McBreen, and G. Illmensee for excellent technical assistance. This investigation was supported by National Institutes of Health Grants GM20700, CA20741, CA21124, CA21069, CA16685 (C.M.C.), CA02662 (L.C.S.), and RR05545 (Jackson Laboratory), by Grant 1-77-42 from the National Foundation—March of Dimes, and by Grant VC2220 from the American Cancer Society.