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Stable transformation of mouse teratocarcinoma stem cells with the dominant selective marker *Eco.gpt* and retention of their developmental potentialities

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Transformation of PCC4 mouse teratocarcinoma stem cells was obtained using a dominant selective marker, the enzyme xanthine-guanine phosphoribosyltransferase (XGPRT), coded by the bacterial *Eco.gpt* gene placed under the control of the early SV40 genes in the vector pSV2gpt. An average of 20 colonies of transformed cells was obtained, using the calcium phosphate technique, 10 μg DNA vector, no carrier DNA and 1 x 10^6 recipient cells. Five independent *Eco.gpt*-transformed PCC4 cell lines were propagated in selective medium and assayed for XGPRT activity. All of them had the ability to convert [^{14}C]xanthine to xanthine monophosphate. pSV2gpt sequences were present and associated with high mol. wt. cellular DNA. pSV2gpt sequences and XGPRT activity were both conserved in the three clones that were propagated in non-selective medium for 30 generations. The transformed PCC4 cells retained their ability to produce, in host mice, teratocarcinoma tumors composed of embryonal carcinoma and various differentiated tissues. Thus, pSV2gpt can be used as a dominant marker to select teratocarcinoma stem cells co-transformed with genes that are not selectable by themselves.

Key words: dominant selective marker/*Eco.gpt*/teratocarcinoma/transformation

Introduction

Mouse embryonal carcinoma (EC) cells, the stem cells of teratocarcinomas, can undergo normal development upon injection into mouse embryos. Cells derived from the injected EC cells may participate in the formation of one or several somatic tissues of the mice derived from the recipient embryos (Mintz and Illmensee, 1975; Papaioannou *et al.*, 1975). Experimental insertion of exogenous genes into the EC cell genome, followed by the incorportion of the recipient EC cells into mosaic animals may provide a useful system in which the exogenous genes undergo all steps of ontogenetic development and may possibly be functional in specific somatic tissues.

The yield of stably transformed cells upon introduction of DNA into recipient cells using calcium phosphate/DNA precipitates is very low. A selective marker is therefore required to isolate such transformed cells. Since most genes do not bring a selective advantage to the recipient cells, co-transformation experiments are necessary. To date, the most widely used selective marker is the thymidine kinase (*tk*) gene of herpes simplex virus (HSV) (Wigler *et al.*, 1977). This gene allows transformed cells to grow in HAT medium (Littlefield, 1964) and necessitates the utilization of thymidine kinase deficient (TK^-) cells.

The extent of the mutagenesis required to obtain TK^- EC cells in which both *tk* alleles are altered gives a high probability of other changes including the loss of the ability of EC cells to participate in normal development upon their injection into embryos. To overcome this difficulty, we have investigated whether or not a selective dominant marker can be employed to select transformed EC cells. Here we report the isolation of EC cell clones that were stably transformed by *Eco.gpt* and retained their developmental pluripotentialities.

Results

PCC4 and L cell cultures were treated with a calcium phosphate/DNA precipitate prepared with DNA from the pSV2gpt vector which contains the *Eco.gpt* gene. *Eco.gpt*, which codes for *Escherichia coli* xanthine-guanine phosphoribosyltransferase (XGPRT), has been placed under the control of SV40 early sequences (Mulligan and Berg, 1980). Only cells in which *Eco.gpt* is active can use xanthine for GMP synthesis and therefore survive in medium containing xanthine and mycophenolic acid (Mulligan and Berg, 1981a). Control cultures of L cells and PCC4 cells treated with calcium phosphate precipitates without DNA, or with salmon sperm DNA, gave no colonies in the selective medium, most cells dying within 5 days. When cell cultures received pSV2gpt DNA, growing colonies were observed within 7-10 days. Most dishes were fixed with methanol, stained with Giemsa, and the colonies counted (Table I). Transformation with pSV2gpt was ~10 times more efficient with L cells than with PCC4 teratocarcinoma cells. PCC4 cells had a lower cloning efficiency than L cells (15% and 56%, respectively), but these figures do not entirely account for the difference in the observed respective yields of transformed colonies (Table I).

Presumptive transformed PCC4 colonies were composed of typical carcinoma cells indistinguishable from the untransformed PCC4 cells (Figure 1). Colonies were picked at random and subcultured in selective medium.

XGPRT activity

Five PCC4 and nine L cell lines derived from independently transformed colonies and maintained in selective medium were assayed for XGPRT activity. All of them had the ability to convert [^{14}C]xanthine to xanthine monophosphate (XMP)

Table I. Frequency of colonies growing in selective medium upon transformation with pSV2gpt

Experiment no.	Recipient cell line	Transformed colonies	
		Number/dish[a] (extreme values)	Average frequency x 10^{-6}
1	PCC4	9-38	19
	L	122-168	142
2	PCC4	4-13	9
	L	182-285	223
3	PCC4	5-45	32
	L	234-459	331
4	PCC4	5-33	14

[a]Ten dishes were counted in each series.

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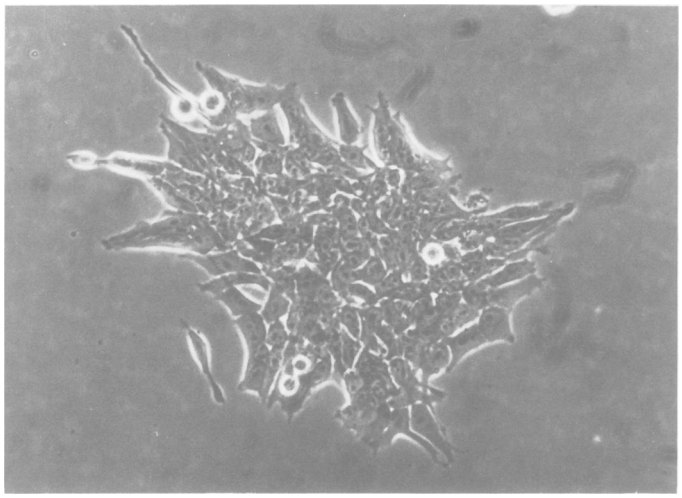

Fig. 1. Primary colony of pSV2gpt-transformed PCC4 teratocarcinoma stem cells. Phase contrast microscopy (x 600).

Table II. XGPRT activity in cell extracts of pSV2gpt-transformed cells

Clones	XMP formed (nM/min/mg of protein)
PCC4 (untransformed)	0.09
P 1	0.22
P 2	0.44
P 3	1.26
P 4	0.23
P 9	0.27
L (untransformed)	0.09
L 1	0.96
L 2	0.53
L 3	1.45
L 4	0.96
L 5	0.63
L 6	0.46

(Table II). Cell extracts from untransformed PCC4 and L cell lines both showed a low background (corresponding to a pseudo-activity of 0.09 nM [^{14}C]XMP produced/min/mg of protein) that did not increase after 1 h incubation of the reaction mixture at 37°C and was not suppressed when the reaction mixture was boiled for 5 min before incubation. This background, also detected in extracts of PCC4 cells deficient in hypoxanthine phosphoribosyltranferase (not shown), was therefore considered as non-enzymatic.

Histology of the tumors

Twenty-one out of the 22 mice injected with cells from one of three different clones (P1, P3, P4) of pSV2gpt-transformed PCC4 cells developed tumors within 2 weeks. Examination of histological sections from 14 different tumors grown from the pSV2gpt-transformed cells revealed that all the tumors were carcinomas containing both carcinoma cells and a variety of differentiated tissues representative of the three primitive germ layers, including primitive ectoderm, neuroepithelium, mesenchyme, muscle, various epithelia and parietal yolk sac (Figure 2). The range of differentiated tissues

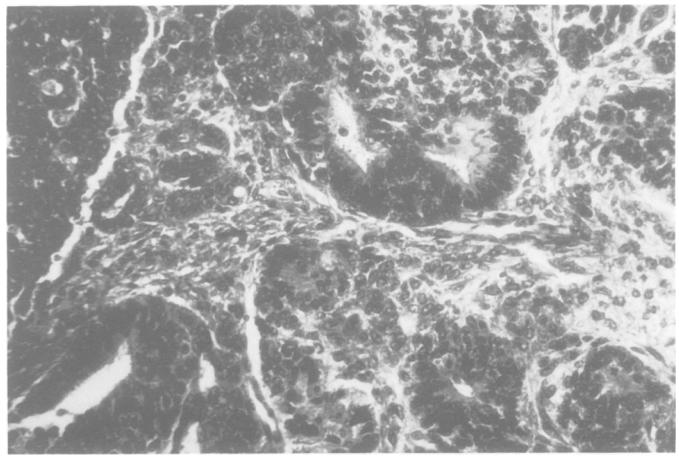

Fig. 2. Histological section of a tumor obtained upon injection of clone P3 of pSV2gpt-transformed PCC4 teratocarcinoma stem cells. Neuroepithelium, mesenchyme and embryonal carcinoma are visible. Hematoxylin-eosin (x 300).

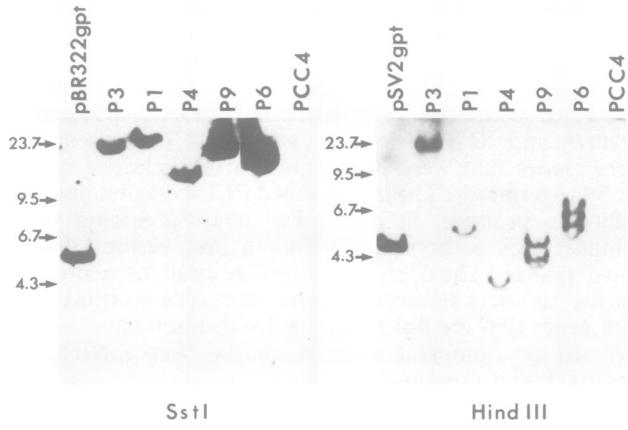

Fig. 3. Identification of pSV2gpt sequences in DNA fragments generated by *Sst*I (left) or *Hind*III (right) digestion of pSV2gpt-transformed PCC4 clones. DNA fragments were fractionated on 1% agarose gels, blotted onto nitrocellulose and hybridized with nick-translated ^{32}P-labelled pBR322gpt. Size markers were from *Hind*III-cleaved phage λ DNA. P3, P1, P4, P6 are transformed PCC4 clones. PCC4 DNA gave no hybridizing band.

and the amount of necrosis in the tumors derived from the transformed cells were comparable with those found in the tumors obtained with the untransformed PCC4 cells.

Detection of pSV2gpt sequences in the transformed cell lines

DNA from pSV2gpt-transformed PCC4 and L cell lines was digested with either *Sst*I or *Hind*III endonuclease, run on agarose gels, blotted onto nitrocellulose paper and hybridized with ^{32}P-labelled pBR322gpt as a probe. The enzyme *Sst*I does not have any recognition sites in the pSV2gpt; *Hind*III cuts only once and linearizes the vector.

Figure 3 shows the results of the hybridization of DNAs prepared from five independent transformed PCC4 teratocarcinoma cell clones. When DNA is digested with *Sst*I (Figure 3, left), the five clones exhibit a single band of high mol. wt. This suggests a single insertion site, but does not prove it since resolution in this size range is poor, and in some cases several bands could be indistinguishable. The size of the labelled fragment is different in each clone and varies from 15 to 30 kb. *Hind*III-digested DNAs from two clones (P3 and P1) (Figure 3, right) have only one band that is larger than 5.1 kb and that hybridizes to the radioactive probe. This pat-

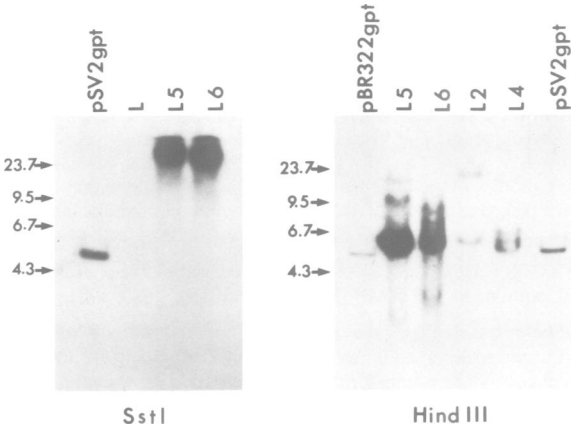

Fig. 4. Identification of pSV2gpt sequences in DNA fragments generated by *Sst*I (left) or *Hind*III (right) digestion of pSV2gpt-transformed L cell clones. DNA fragments were fractionated on 1% agarose gels, blotted onto nitrocellulose and hybridized with nick-translated ^{32}P-labelled pBR322gpt. Size markers were from *Hind*III-cleaved phage λ DNA. L2, L4, L5, L6 are transformed L clones. L cell DNA gave no hybridizing band.

Table III. Persistence of XGPRT activity [a] in pSV2gpt-transformed teratocarcinoma stem cells

Clone	Original activity in selective medium	Activity upon 30 generations in non-selective medium
P 1	0.22	0.21
P 3	1.26	0.25
P 4	0.23	0.19

[a]Expressed as nM XMP formed/min/mg of protein.

tern suggests that a single copy of pSV2gpt was inserted in high mol. wt. DNA. The absence of a second labelled DNA fragment might result either from the insertion of the plasmid near its *Hind*III site, or from a deletion in the plasmid involving the *Hind*III site. In the three other clones P4, P6 and P9, *Hind*III generates three fragments that hybridize to the probe, as expected for insertion at the same site of multiple tandemly arranged copies. The absence of the expected 5.1-kb fragment in two of the three clones (P4 and P6, Figure 3, right) can be explained by the presence of three pSV2gpt copies after a deletion which left only one *Hind*III site. Thus, partial deletion of the integrated pSV2gpt sequence involving the *Hind*III site may have occurred in four out of the five transformed PCC4 clones.

Blotting patterns of *Sst*I-cleaved DNA from two transformed L clones (L5 and L6) are shown in Figure 4 (left). Only a single high mol. wt. band, slightly different in each case, is observed. When digested with *Hind*III, DNAs prepared from L4, L5 and L6 clones revealed several bands (four, four and six, respectively) hybridizing to the probe, including a heavy band the size of the linearized pSV2gpt (at 5.1 kb). This suggests that multiple copies of the full length plasmid are tandemly integrated at the same site (Figure 4, right). The same conclusion appears valid also for clone L4. However, for L4, L5 and L6, one expects three bands. The additional bands may result either from deletion and/or multiple site insertion. The large amount (10 μg) of transforming DNA used without carrier may account for an increase of insertion frequency within the same cell. *Hind*III-digested DNA from clone L2 has two bands hybridizing with the probe, both

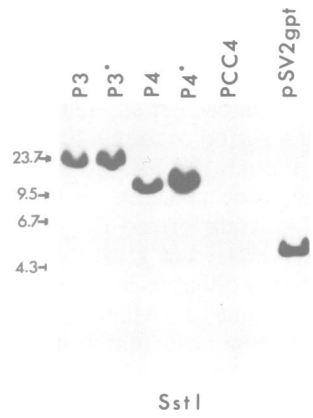

Fig. 5. Stability of pSV2gpt sequences inserted in DNA of transformed PCC4 clones. DNA prepared from two transformed clones before (P1 and P3) and after (P1* and P3*) culture in non-selective medium for 30 cell generations, was digested with *Sst*I, subjected to electrophoresis on 1% agarose gels, blotted onto nitrocellulose and hybridized with nick-translated ^{32}P-labelled pBR322gpt.

larger than 5.1 kb, which indicates that a single copy is integrated in cellular DNA.

Stability of Eco.gpt transformation

The stability of *Eco.gpt*-transformed teratocarcinoma cells was tested by growing three of the transformed PCC4 cell lines in non-selective medium for 30 generations. Cell extracts were then assayed for XGPRT activity. In two clones, the enzymatic activity did not vary; in the third clone (P3), the activity which originally was the highest on several assays, decreased to a value comparable with that of the other clones (Table III). However, the intensity of the band associated with high mol. wt. cellular DNA was maintained (Figure 5).

Discussion

The present study shows that PCC4 teratocarcinoma stem cells can be stably transformed by the *E. coli Eco.gpt* gene, inserted in the plasmid pSV2gpt, without impairing their capacity to differentiate. We have regularly obtained transformed EC cells by pSV2gpt using either PCC4 or F9 EC cells as recipients and using either pSV2gpt as it is, or including an additional sequence, e.g., a human β-globin gene. The results described here are also in agreement with those recently reported by Wagner and Mintz (1982).

The possibility that the transformed cells that were selected were not representative of the PCC4 cell population has to be considered since *Eco.gpt* is placed under the control of the SV40 early promoter in pSV2gpt. It is known that teratocarcinoma stem cells do not support expression of SV40 T-antigen, while their differentiated progeny do (Swartzendrubber and Lehman, 1975; Boccara and Kelly, 1978; Segal and Khoury, 1979). Differentiated cells, e.g., endodermal cells, can spontaneously evolve from the EC stem cells in culture (Sherman and Miller, 1978). However, our results indicate that the pSV2gpt-transformed EC cells are indeed stem cells. All the transformed colonies selected had the EC cell morphology characteristic of the PCC4 recipient cells. The transformed cells also produced typical teratocarcinoma tumors upon injection into hosts. This rules out the possibility that we selected differentiated cells derived from the stem cell line. Moreover, we did not select a minority of stem cells capable of supporting T-antigen expression, as revealed by immunofluorescence studies performed 48 h after SV40 in-

fection of cultures from eight transformed EC clones (data not shown).

Upon SV40 infection (Segal *et al.*, 1979) or SV40 DNA transformation (Linnenbach *et al.*, 1981) of teratocarcinoma stem cells, the early region of the SV40 genome is apparently transcribed even though the cells do not contain detectable levels of the early gene proteins. In contrast, the *Eco.gpt* message in pSV2gpt-transformed EC cells is translated and the XGPRT is synthesized at a rate sufficient to allow the cells to survive and proliferate in the selective medium (see Results, and also Wagner and Mintz, 1982). This discrepancy might result from some post-transcriptional block in SV40-transformed EC cells. According to Linnenbach *et al.* (1981), T-antigen mRNA is correctly processed. The *Eco.gpt* mRNA could by-pass the EC cell block because of its different sequence. Alternatively, the block could be related to the efficiency of transcription. The activity of the SV40 early gene promoter may be weak in EC cells. If this were the case the results obtained here could be explained if, in teratocarcinoma stem cells, *Eco.gpt* transcription is not actually under the control of the SV40 early promoter in pSV2gpt. Roberts and Axel (1982) have reported selection of TK^+ L cells upon transformation with a promoterless HSV *tk* gene. In these cells, TK protein is translated from truncated mRNA that results from ectopic promoter activity. This mechanism cannot by itself account for our results since it would induce a lower rate of transformation than the rate we obtained using pSV2gpt (see Table I). Banerji *et al.* (1981) have shown that SV40 genome early sequences can enhance transcription of a rabbit β1-globin gene when present in the plasmid vector, independent of its position and orientation relative to the β-globin gene itself. A similar mechanism might be involved in *Eco.gpt* expression. The SV40 early sequence present in the pSV2gpt vector may enhance a low rate of transcription of *Eco.gpt* from, for example, the sequence TATAATC present at the *Bgl*II site on the 5′ end of the *Eco.gpt* sequence inserted in pSV2gpt (Mulligan and Berg, 1981b).

Whatever the case, it is clear that vectors relying on the *Eco.gpt* dominant selection system may be used to select teratocarcinoma stem cells co-transformed with genes that are not in themselves selectable, such as globin or insulin.

Materials and methods

Cells

The PCC4 teratocarcinoma stem cell line (Jakob *et al.*, 1973) was derived from the transplantable teratocarcinoma OTT 6050 obtained by grafting a 6-day embryo of strain 129/Sv into the testis of an adult mouse (Stevens, 1970). When injected into hosts, PCC4 cells produce tumors which contain both EC cells and a variety of differentiated tissues (Jakob *et al.*, 1973). Pluripotent PCC4 EC cells were chosen for these experiments instead of the EC cell lines we use to obtain mosaic mice because they are easier to handle in DNA transformation experiments since, unlike the latter, they grow without feeder layers. LM fibroblasts (L cells) derived from NCTC clone 929 (Sanford *et al.*, 1948) were obtained from the American Type Culture Collection (CCL 1.2.). Both cell lines were found free of mycoplasma infection by staining cell cultures with the 33258 Hoechst fluorochrome (Chen, 1977). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 8% fetal calf serum (growth medium).

Transformation experiments

Transformations were performed using the pSV2gpt vector (Mulligan and Berg, 1980), kindly provided by P. Berg. 24 h after seeding 5 x 10^5 cells into 100 mm diameter tissue culture dishes, a calcium phosphate DNA precipitate prepared with 10 μg undigested pSV2gpt DNA, and without any carrier DNA, was added to the culture medium (Wigler *et al.*, 1977). The cells were incubated with the calcium phosphate/DNA precipitate at 37°C for 24 or 48 h. The medium was then removed, the cells were washed once with phosphate buffered saline (PBS), dispersed with a solution of 0.025% trypsin and 2 mM EDTA in PBS, counted (at that time, ~ 2 x 10^6 PCC4 cells per dish and 1 x 10^6 L cells were usually found), and plated at 5 x 10^5 cells per 100 mm dishes in growth medium. 24 h later, growth medium was replaced by the selection medium, i.e., growth medium supplemented with 100 μM hypoxanthine, 2 μM amethopterin, 16 μM thymidine; 1.6 mM xanthine, and 78 μM mycophenolic acid (Mulligan and Berg, 1981a). Mycophenolic acid (MPA) was a gift of the Lilly Company. The medium was changed every 3 days. Colonies of putative transformants were visible within 7–10 days, and were picked after 14–21 days and grown in selective medium.

XGPRT assay

The XGPRT activity in cell extracts was assayed by the conversion of ^{14}C-labelled xanthine to [^{14}C]XMP (Mulligan and Berg, 1980, 1981a).

Inoculations

129/Sv syngeneic or nude mice were injected s.c. with 5 x 10^6 or 1 x 10^7 PCC4 cells or pSV2gpt-transformed PCC4 cells. The growing tumors reached 1–2 cm after 2–4 weeks; they were removed, fixed in Bouin's solution, and processed for histology. Sections were stained with hematoxylin-eosin.

DNA isolation and blot hybridization

High mol. wt. DNA was isolated from cultured cells by standard procedures (Gross-Bellard *et al.*, 1973), 20–40 μg was digested to completion with either *Sst*I or *Hind*III restriction endonuclease. The digests were run on 1% or 0.7% agarose slab gels by vertical electrophoresis at 1.5 V/cm for 16–18 h. DNA was partially depurinated, denatured *in situ* and transferred onto nitrocellulose paper (Southern, 1975). The DNA probe used was pBR322gpt (Mulligan and Berg, 1980), labelled with [^{32}P]dATP and [^{32}P]dCTP (3000 Ci/mM, Amersham) by nick-translation (Rigby *et al.*, 1977) to a specific activity of 2–3 x 10^8 c.p.m./μg. The filters were hybridized to the probe (Jeffreys and Flavell, 1977), washed (1 x SSC, 65°C) and exposed to X-ray film. Under the conditions of stringency used, no hybridization to endogenous sequences was observed.

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