

## Establishment of 'normal' nervous cell lines after transfer of polyoma virus and adenovirus early genes into murine brain cells

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**Brain cells from murine embryos were transfected with the polyoma virus large T or the adenovirus 5 E1A gene and, simultaneously, with the phosphotransferase coding *Neo<sup>R</sup>* gene. The efficiently transfected cells were selected for their resistance to Geneticin (G418) and their ability to clone at low cell density. Subsequently, most of the selected cells could be sub-cloned and continuously grown for 6–18 months so far. Their doubling time varied between 18 and 72 h. From independent transfections, more than one hundred cell lines were established. They did not exhibit a transformed phenotype, but subsequent transfection with the polyoma middle T gene induced their oncogenic transformation. The maintenance and expression of the transferred genes were verified. Most of the analyzed cell lines retained glial properties. These results suggest that the lines obtained as well as a further extension of this *in vitro* system should be of interest for the study of nervous cell interactions, differentiation and functions.**

**Key words:** immortalization/oncogenes/nervous cells/differentiation/brain

### Introduction

The tumorigenic conversion of fibroblasts results from a multi-step process. Schematically, at least two separate steps have been shown to be involved in this transformation. The first, usually called immortalization, can be induced by the polyoma virus large T gene, the adenovirus E1A sequence and the viral or rearranged cellular *myc* genes (Rassoulzadegan *et al.*, 1982, 1983; van der Elsen *et al.*, 1982; Ruley, 1983; Land *et al.*, 1983a,b). The transfer of these genes confers unlimited growth capacity to primary cultured fibroblasts without inducing the other transformation steps which lead to the expression of transformed or tumorigenic characteristics.

The question has arisen as to whether highly specialized cells or their precursors can also be immortalized by the above mentioned genes. In this respect, the cells of the central nervous system are of special interest. Glio-glia and glio-neuronal interactions are essential for the differentiation and functions of nervous cells (Denis-Donini *et al.*, 1984; Edelman, 1984; Noble *et al.*, 1984; Sobue and Pleasure, 1984). However, the molecular mechanisms involved in nervous cell interactions, differentiation and functional regulations remain little understood. Besides the complexity of the system, this is largely due to the difficulty of establishing nervous cell lines. Most of the lines which have been described are tumorigenic and have been obtained from spontaneous or chemically induced tumors (Schubert *et al.*, 1974) or by transformation of primary cultures with oncogenic viruses (de Vitry *et al.*, 1974; Pessac *et al.*, 1983; Mallat

*et al.*, 1986). A few others were derived from long-term primary cultures (Alliot and Pessac, 1984). It is reported here that the transfection of murine brain cells with polyoma large T sequences or with the adenovirus E1A region allows the immortalization of untransformed cells which could be cloned at low cell density and be grown indefinitely. More than 100 independent cell lines were derived from separate transfections. Partial analysis indicated that most of them had integrated the introduced genes and had retained properties of the glial lineage, as expected from the nature of the transfected cells. In addition, our results show for the first time that the polyoma large T and adenovirus E1A genes can also immortalize differentiated cells other than fibroblasts.

### Results

#### *Selection of immortalized clones*

Secondary cultures of cells from murine embryo forebrain or brain hemispheres were transfected by the calcium phosphate precipitation procedure (Graham and van der Eb, 1973) with several plasmids carrying early genes of polyoma virus and adenovirus 5. The polyoma virus-derived plasmids carried the large T gene (pPyLT1: Zhu *et al.*, 1984; pAX2: N.Glaichenhaus and F.Cuzin, personal communication). In the plasmids pVV12LT1 and pVV12MC9, these genes were associated in the same plasmid with a *Neo<sup>R</sup>* sequence (L.Lemieux and F.Cuzin, personal communication). The plasmid pAd5 E1A contains the entire E1A region of adenovirus 5 (M.Perricaudet, personal communication). The above-mentioned genes immortalize fibroblasts without inducing the expression of a transformed phenotype (Rassoulzadegan *et al.*, 1983; van der Elsen *et al.*, 1982). When plasmids which do not carry the *Neo<sup>R</sup>* sequence were used, this sequence was introduced by co-transfection with pSV2-TK-*Neo-β* globin, which carries the *Neo<sup>R</sup>* gene downstream of the thymidine kinase promoter and which is devoid of functional coding genes of SV40 (Nicolas and Berg, 1983).

After transfection, the cells were incubated for 48 h in standard culture conditions. They were then trypsinized, seeded at  $10^6$  cells per 100-mm dish and, 24 h later 150  $\mu$ g/ml Geneticin (G418) were added. The cells were grown for 6 weeks in the selective medium and the colonies were picked individually. They were sub-cloned at low cell density ( $3 \times 10^2$  cells per 100-mm dish). As shown in Table I, in most experiments, the combined frequency of appearance of both Geneticin-resistant and immortalized colonies was  $\sim 3 \times 10^{-6}$ , with the exception of the E1A-rat brain cell system which led to a frequency of  $3 \times 10^{-5}$ . The systematic control transfections with pSV2-TK-*Neo-β*-globin, pAT153 and pPyMT1 (a plasmid carrying the polyoma middle T gene: Treisman *et al.*, 1981) did not give rise to any immortalized colonies.

These transfection experiments led to the isolation of more than 100 independent clones. The nomenclature that we use for the present cell lines indicates the transfecting oncogene (LT, MC or E1A for the large T, truncated large T or E1A sequences

**Table I.** Frequency of G418-resistant forebrain immortalized cells ( $\times 10^6$ )

Plasmids	Rat embryo	Mouse embryo	Glioma C6 cell line
pVV12-LT1	ND	3.1	ND
pVV12-MC9	2.5	3.7	175
pAX-2	2.6	2.4	ND
+pSV-TK-Neo- $\beta$ -globin			
pAd5-E1A	37	3.2	ND
+pSV2-TK-Neo- $\beta$ -globin			
pAT153	0	0	ND
+pSV2-TK-Neo- $\beta$ -globin			
pPyMT1	0	0	ND
+pSV2-TK-Neo- $\beta$ -globin			
pSV2-TK-Neo $\beta$ -globin	0	0	32

The frequencies represent means from 5–10 independently transfected dishes. ND = not determined. For the control cell line C6, the indicated values refer to the frequency of the G418-resistant colonies.

respectively), the rat (R) or mouse (M) cell origin and the serial number of clone and sub-clone.

#### Growth properties of the immortalized cells

The selected clones have been grown continuously for 6–18 months without showing any crisis period. Ten clones isolated in separate experiments were more extensively analyzed. Their doubling times ranged between 18 and 72 h. The plating efficiency of the immortalized cells was 10–20% when seeded at  $3 \times 10^2$  cells per 100-mm dish, whereas secondary cultured glial cells did not form any colonies even when seeded at  $10^4$  cells per 100-mm dish. In most cases, their saturation density was about  $7 \times 10^5$  cells per 100-mm dish, with a few exceptions of small-sized cells (7  $\mu$ m in diameter) yielding  $5 \times 10^6$  cells per 100-mm dish. About one third of the clones were diploid.

The immortalized cells were not tumorigenic even in nude mice, they did not grow in agarose medium (Figure 1a and b) and the clones displayed a flat morphology in the standard culture medium (Figure 1e and f). To assay their sensitivity to the polyoma middle T antigen, they were submitted to a second transfection with the plasmid pPyMT1. The transfer of this plasmid was observed to induce anchorage-independent multiplication (Figure 1c and d) and the emergence of foci (Figure 1g and h) with a frequency of  $2 \times 10^{-4}$ . Therefore, unless they were subsequently transfected with an oncogene of the second class, the cell lines, established here, lack a transformed phenotype, at least on the basis of these criteria.

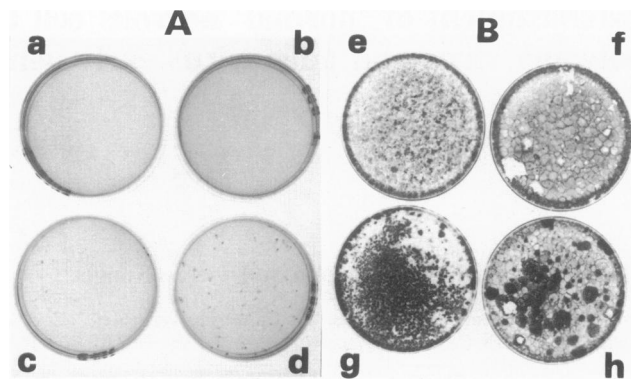
#### Maintenance and expression of the transferred genes

Southern blot hybridizations (Figure 2A and B) indicated that the immortalizing sequences were integrated in the genome of most of the lines derived from transfection with the truncated large T or the E1A sequences. In the case of the mouse cell lines obtained after transfection with the entire large T, the results were ambiguous concerning the episomal or integrated state of the introduced sequences and further investigations are being carried out to clarify this point.

As shown in Figure 2C, the Northern blots for E1A expression revealed the synthesis of cytoplasmic RNA of molecular weight higher than expected.

#### Morphological features of the immortalized cells

Besides the flat morphology of the clones, which is in accordance with their untransformed phenotype, the immortalized cells displayed various shapes which did not allow them to be characterized unambiguously as nervous cells. However, the cells



**Fig. 1.** Polyoma middle T gene-mediated transformation of immortalized clones. The cells were transfected with pPyMT1 in conditions described for the transfections with the other plasmids. (A): Colonies in agarose medium; 48 h after transfection,  $3 \times 10^5$  cells were seeded in F12/DMEM medium containing 0.3% agarose over an underlayer of 0.5% agarose. The colonies were allowed to grow for 3–4 weeks; (a, b): mock-transfected MC-R-1-1 and E1A-R-4-1; (c, d): same clones after transfection with pPyMT1. (B): Formation of multilayers foci; the cells were allowed to grow in standard conditions for 4 weeks and Giemsa-stained; (e, f): mock-transfected MC-R-1-1 and E1A-R-4-1; (g, h): same clones after transfection with pPyMT1.

of some clones showed strikingly different shapes according to whether they were incubated in serum-supplemented or serum-free medium (Bottenstein and Sato, 1979). For instance (Figure 3), when transferred to defined medium, cells from clones LT-M-1-1 and LT-M-2-1 developed long processes like those described by other authors (Schubert *et al.*, 1974; Michler-Stuke *et al.*, 1984). However, although marked, these morphological changes are insufficient by themselves to characterize the cells. Some properties of nervous cells were therefore examined.

#### Uptake of $\gamma$ -aminobutyric acid

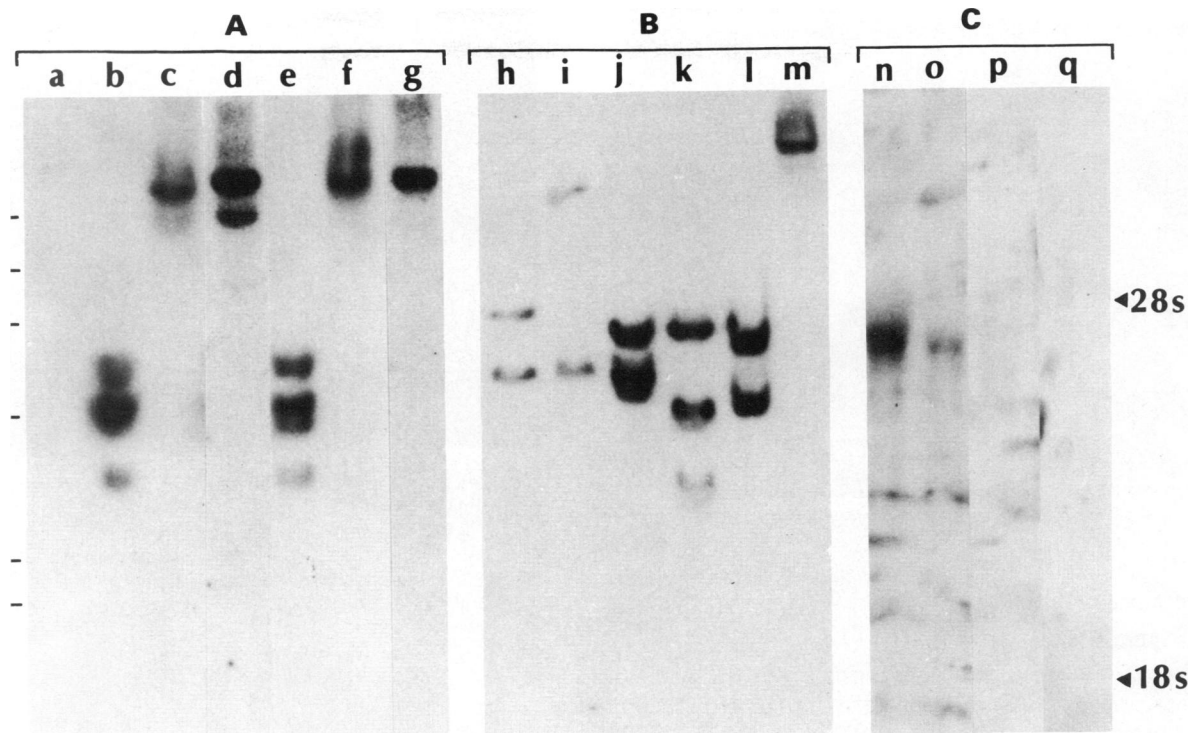
It has been reported (Iversen and Kelly, 1975; Kelly and Dick, 1978) that the uptake of  $\gamma$ -aminobutyric acid (GABA) by glial cells is preferentially inhibited by  $\beta$ -alanine, whereas this uptake by neurones is selectively inhibited by diaminobutyric acid (DABA). As shown in Table II, for most lines the uptake of GABA was preferentially inhibited by  $\beta$ -alanine.

#### Interactions between neurones and the immortalized cell lines

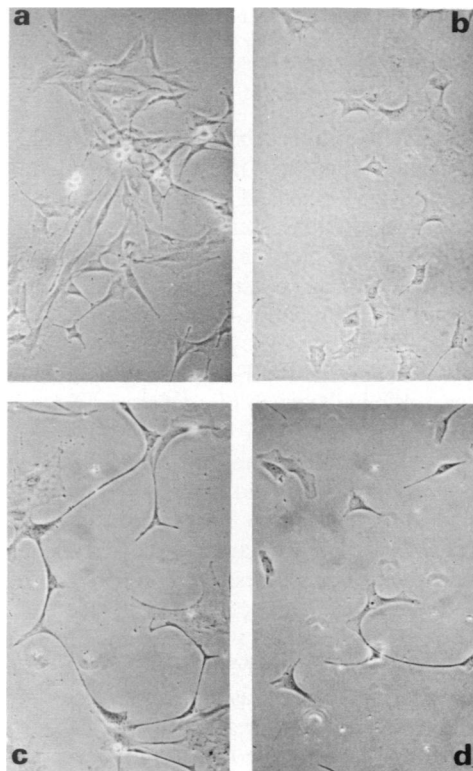
Other studies concerning glio-neuronal interactions have shown that neurones are induced to develop neuritic processes when they are plated on monolayers of glial cells, but not on fibroblastic or meningeal cells (Sensenbrenner and Mandel, 1974; Denis-Donini *et al.*, 1984; Noble *et al.*, 1984). Our cell lines were assayed for this functional property of glial cells. As illustrated in Figure 4, all the examined clones were able to establish glio-neuronal interactions.

#### Induction of glial fibrillary acidic protein (GFAP)

The maturation of astrocytes and the expression of GFAP (Bignami *et al.*, 1972; Raff *et al.*, 1978) have been shown to be inducible by dibutyryl-cAMP and by astroglial growth factors (Sensenbrenner *et al.*, 1980; Pettman *et al.*, 1985; Weibel *et al.*, 1985). In most of the uninduced immortalized clones examined here, only rare cells were labeled by antibodies to GFAP, whereas several clones could be induced to express GFAP (Figure 5). Some of them, such as E1A-R-4-1 were induced either by db-cAMP or by AGF, form 2 (AGF 2); others such as E1A-M-4-1, were not inducible by db-cAMP (1 mM) but 100% of the cells could be induced by a 9-day treatment with 3 ng/ml AGF 2. The morphological features of the GFAP-positive cells



**Fig. 2.** Integration and expression of the transferred genes. (A): 20  $\mu$ g genomic DNA from dissociated control brain cells (a), clones MC-R-1-1 (b, c, d) and MC-R-11-1 (e, f, g) were digested with *Bam*HI (a, b, c): one cut in pVV12MC9) or with *Eco*RV (c, d, e, f): no cut in pVV12MC9) or with *Xba*I (d, e, f, g): no cut in pVV12MC9). After electrophoresis and blotting, they were probed with the 1.8-kb *Bam*HI–*Eco*RI fragment of pPyLT1. The bars indicate the following sizes, in kb; from top to bottom: 23, 9.5, 6.5, 4.4, 2.3, 2.0. (B): 20  $\mu$ g genomic DNA from clones E1A-R-4-1 (h, i), E1A-R-2-1 (j, k) and E1A-M-4-1 (l, m) were digested with *Eco*RI (h, j, l: one cut in pAd5-E1A) or with *Xba*I (i, k, m: one cut in pAd5-E1A). The probe was the 1.4-kb *Eco*RI–*Xba*I fragment from pAd5-E1A. (C): 20  $\mu$ g cytoplasmic DNA were subjected to electrophoresis in 1.4% agarose-formaldehyde gel. The probe was the 1.4-kb *Eco*RI–*Xba*I fragment from pAd5-E1A. The lanes n, o, p, q, correspond respectively to clones E1A-R-4-1, E1A-R-2-1, E1A-M-4-1, and dissociated rat brain cells.



**Fig. 3.** Phase contrast microscopy for clones LT-M-1-1 (a, c) and LT-M-2-1 (b, d) in serum-supplemented standard medium (a, b) and in serum-free (Bottenstein and Sato, 1979) medium (c, d). (Magnification: 100 $\times$ ).

**Table II.** Uptake of [ $^3$ H] $\gamma$ -aminobutyric acid

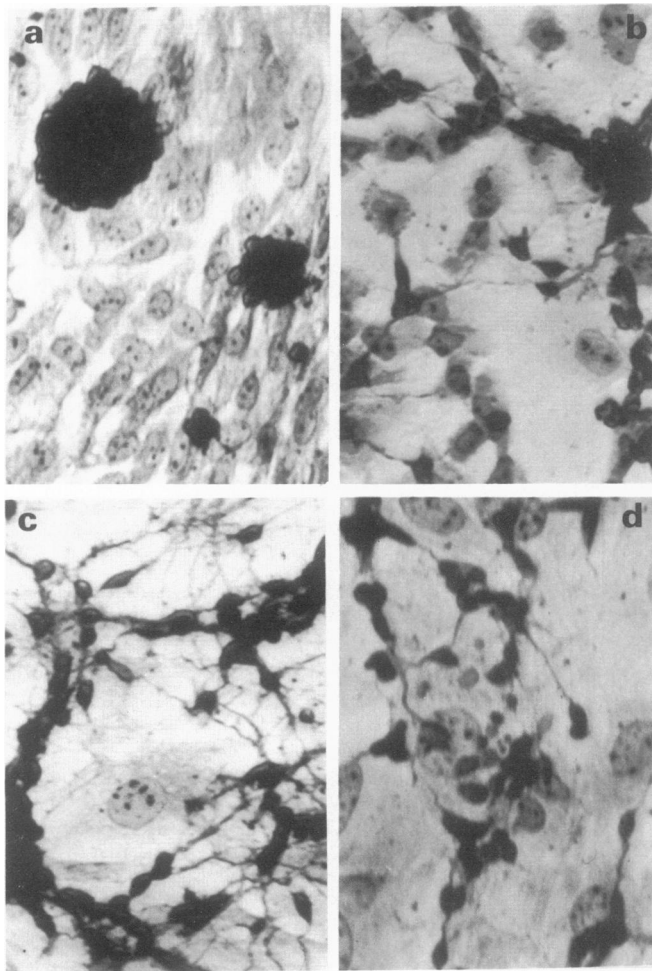
Cell origin	[ $^3$ H]GABA c.p.m. per well ( $\times 10^{-3}$ )	[ $^3$ H]GABA c.p.m. per mg protein ( $\times 10^{-3}$ )	DABA % inhibition	$\beta$ -Ala % inhibition
LT-M-1-1	1 $\pm$ 0.1	18 $\pm$ 3	24 $\pm$ 3	81 $\pm$ 7
LT-M-2-1	2 $\pm$ 0.2	44 $\pm$ 5	39 $\pm$ 4	32 $\pm$ 4
LT-M-3-1	0.7 $\pm$ 0.1	34 $\pm$ 5	<5	36 $\pm$ 4
LT-M-4-1	3 $\pm$ 0.3	60 $\pm$ 6	27 $\pm$ 3	60 $\pm$ 5
MC-R-1-1	4 $\pm$ 0.4	93 $\pm$ 10	18 $\pm$ 3	78 $\pm$ 6
E1A-R-2-1	2 $\pm$ 0.2	30 $\pm$ 3	35 $\pm$ 4	64 $\pm$ 6
E1A-R-4-1	1.5 $\pm$ 0.2	20 $\pm$ 3	30 $\pm$ 4	55 $\pm$ 6
E1A-R-8-1	0.4 $\pm$ 0.1	8 $\pm$ 2	N.D.	N.D.
Brain cell secondary cultures	41 $\pm$ 5	820 $\pm$ 70	78 $\pm$ 8	88 $\pm$ 8
FR-3T3	0.3 $\pm$ 0.1	4 $\pm$ 1	<5	<5

The values indicated are the means from four independent experiments  $\pm$  SD.

varied from clone to clone. For instance, it should be stressed that E1A-M-4-1 cells displayed large cell bodies without long processes whereas E1A-R-4-1 cells showed small cell bodies and highly stained long processes. They are reminiscent of protoplasmic and fibrillary astrocytes, respectively.

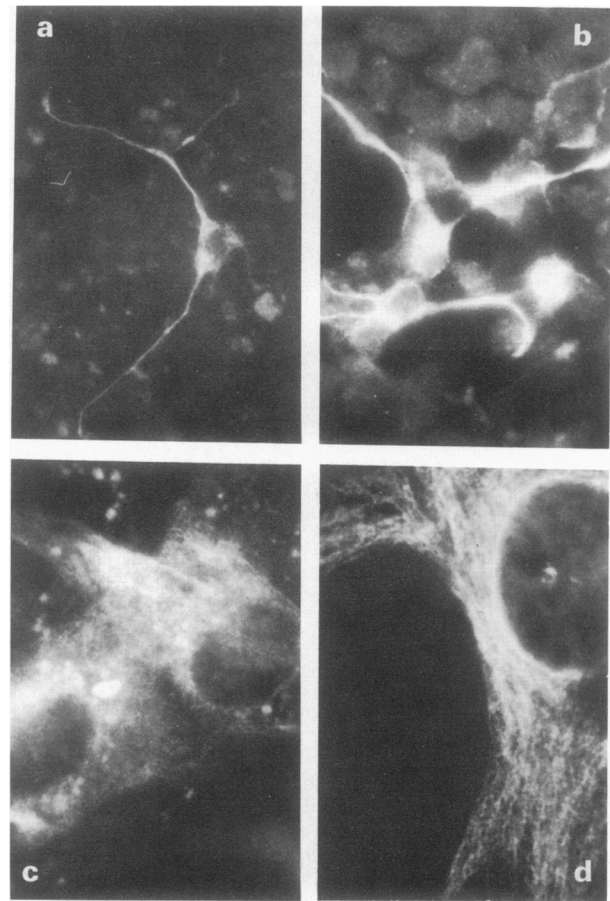
### Discussion

The results of this study show that the transfection of murine embryo brain cells with the cloned polyoma virus large T gene or its truncated form, or with the adenovirus 5 E1A sequence,



**Fig. 4.** Interactions of neurones with immortalized cells. Striatal neurones were plated at low density on monolayers of immortalized cells; they were fixed and silver-stained 48 h later. (a), FR-3T3 taken as control; (b, c, d), clones MC-R-1-1, E1A-R-4-1, LT-M-3-1, respectively. (Magnification: 200×).

can lead to the immortalization of these cells. In most experiments, the *Neo<sup>R</sup>* gene was introduced into the cells, either on the same plasmid as the above oncogenes, or by co-transfection with pSV2-TK-*Neo*- $\beta$ -globin; this plasmid is devoid of any functional coding sequence of SV40 and allows the expression of the aminoglycosyl phosphotransferase (3') II in eucaryotic cells, inducing a resistance to G418 (Colbere-Garapin *et al.*, 1981; Nicolas and Berg, 1983). This provided a rapid and convenient selection of the efficiently transfected cells. In the case of co-transfections, the ratio between the plasmid carrying the *Neo<sup>R</sup>* gene and the plasmid carrying the immortalizing sequences was 1:10, ensuring that the G418-selected cells also received the oncogene. The frequency of G418-resistant colonies was  $3 \times 10^{-6}$  in practically all cases, except  $3 \times 10^{-5}$  for the E1A-rat cell system. Consequently, the surviving cells were in clonal conditions (3–40 colonies per 100-mm dish) which allow the growth only of immortalized cells. Indeed, non-transfected brain cells cannot be cloned even when plated at  $10^4$  cells per 100-mm dish in non-selective medium. Moreover, no G418-resistant colony was detected in control transfections with pSV2-TK-*Neo*- $\beta$ -globin, either alone or combined with pAT153 or pPyMT1. Furthermore, nearly all the colonies obtained after the G418-selection could also be grown indefinitely. Thus, in our conditions, the



**Fig. 5.** Induction of GFAP. The cells were incubated, in their standard medium, either for 9 days with 3 ng/ml AGF2 or for 15 days with 1 mM db-cAMP. (a), E1A-R-4-1, AGF2 (1000×); (b), E1A-R-4-1, db-cAMP (1500×); (c), E1A-M-4-1, AGF2 (1500×); (d), E1A-M-4-1, AGF2 (2500×).

appearance of colonies reflects both their resistance to G418 and their immortalization and very likely depends on the combined expression of the *Neo<sup>R</sup>* gene and of the co-introduced oncogene. Indeed, in the constructions used, these genes were controlled by different promoter regions, even when they were carried by the same plasmid. Although constructs including a complete large T antigen gene should replicate in mouse but not in rat cells, the immortalization frequencies were the same for both species. The higher frequency for the E1A-rat cell combination might be related to a relative tropism of adenovirus 5 for brain cells (Graham *et al.*, 1978).

Most of the clones obtained have now passed 100 generations. They were not tumorigenic, did not grow in agarose medium and displayed a flat morphology. In addition, about one-third of them exhibited normal diploid karyotypes. Taken together, these data show that the present clones can reasonably be regarded as immortalized normal cell lines.

When the immortalized cells were submitted to an additional transfection with plasmid pPyMT1 carrying the transforming middle T gene of polyoma virus (Treisman *et al.*, 1981; Ras-soulzadegan *et al.*, 1983), they acquired the capacity to grow in agarose medium and to give rise to multilayer foci. This is consistent with the untransformed phenotype of the present immortalized cells and shows that oncogenic cooperations are not restricted to fibroblasts but can also take place in highly specialized cells. Furthermore, these observations indicated that our im-

mortalized brain cells are sensitive to subsequent transfections and are suitable for studying the tissue-specificity and regulation of the expression of various transfected genes in nervous cells.

The lines which derive from transfections with the polyoma truncated large T antigen gene or with the E1A region have integrated these sequences. For the cells which were transfected with the complete large T gene, this sequence appears to be maintained preferentially in an episomal state. However, in this case, some ambiguity remains: it might be related to an excision-integration process involving interactions between DNA and large T antigen as described in fibroblasts (Pelligrini *et al.*, 1984; Sylla *et al.*, 1984).

The level of expression of the transfected genes varied considerably among different cell lines, without correlation with gene copy number, as illustrated by the clones E1A-R-4-1 and E1A-R-2-1 (compare Figure 2, B and C). In addition, E1A expression leads to the synthesis of cytoplasmic RNA of molecular weight higher than expected. Since the transfected plasmid carries the termination signals of the E1A region (Gilardi and Perricaudet, 1984, and M. Perricaudet, personal communication), the RNA size could reflect rearrangements during or following the integration step, possibly leading to the absence or unavailability of the termination signals. However, in the present cell lines, the E1A products are functional at least on the criteria of their immortalizing effect.

The immortalized cell lines were analyzed for some properties of nervous cells. Taken together, the data indicate that most clones have retained glial properties, as could be expected from the nature of the transfected secondary cultures which were cleared of neurones after the trypsinization step. It should be underlined that monolayers of all the examined clones were able to interact with primary cultured neurones and to induce the development of neuritic processes. All were negative for neuronal and oligodendrocyte markers (neurofilaments, receptors to tetanus toxin, proteolipids, galactocerebrosides). Some clones were inducible for GFAP expression by db-cAMP and by AGF 2; they belong very likely to the astroglial lineage. Therefore, the present immortalized lines, which represent homogeneous untransformed cell populations, constitute a system suitable for the biochemical analysis of the functions of glial cells, of glioneuronal interactions and of the mechanisms involved in astroglial differentiation.

Even if the question remains as to whether post-mitotic non-dividing cells, such as maturing neurones, can be induced to proliferate after transfection with immortalizing genes, the present results provide evidence that normal nervous cell lines can be obtained readily from murine brain cells. This might open a new field for the study of brain nervous cells.

## Materials and methods

### Plasmids

Plasmids pPyLT1 and pMC1 carry respectively the genes coding for the polyoma virus large T antigen and its truncated form which codes for the amino-terminal 40% of the protein (Rassoulzadegan *et al.*, 1983; Zhu *et al.*, 1984). In pAX2 the truncated large T sequence is downstream from the simian virus 40 (SV40) *ori* region (L. Lemieux and F. Cuzin, personal communication). Plasmids pVV12-LT1 and pVV12-MC9 contain either the polyoma large T gene or its truncated form and the *Neo<sup>R</sup>* sequence downstream of the herpes virus thymidine kinase promoter (N. Glaichenhaus and F. Cuzin, personal communication). Plasmid pPyMT1 codes for the polyoma middle T antigen (Treisman *et al.*, 1981). Plasmid pAd5 E1A carries the adenovirus 5 E1A region (0–5.0 map units) cloned in pML2 (Lusky and Botchan, 1981) as described for the adenovirus 2 E1A region (Gilardi and Perricaudet, 1984, and M. Perricaudet, personal communication). Plasmid pSV2-TK-*Neo*- $\beta$ -globin is devoid of any functional coding sequence of

SV40; it contains the SV40 *ori* region, the thymidine kinase promoter upstream of the *Neo<sup>R</sup>* gene and the second intervening sequence of the  $\beta$ -globin gene (Nicolas and Berg, 1983).

### Cells, gene transfer and selection

Forebrain or brain hemisphere from 14–18 day embryos of Albino Swiss mouse and Wistar rat were dissected and meninges were carefully removed. The dissociated cells were plated at a density of  $10^7$  cells per 100-mm dish; the dishes were previously coated with gelatin and poly *L*-lysine (Loudes *et al.*, 1983). They were cultured for 5–6 days in F12-DMEM (1/1) medium containing 10% FCS (Gibco). After trypsinization, they were seeded at  $2 \times 10^6$  cells per 100-mm dish so that  $\sim 10^6$  cells were attached 24 h later.

Transfections were carried out using the calcium phosphate precipitation procedure (Graham and van der Eb, 1973). For  $10^6$  cells, either 25  $\mu$ g plasmid DNA carrying both an oncogene and the *Neo<sup>R</sup>* sequence or a mixture of 25  $\mu$ g DNA carrying the oncogene and 2.5  $\mu$ g DNA carrying independently the *Neo<sup>R</sup>* sequence were used. The cells were trypsinized 48 h later and seeded at  $10^6$  cells per 100-mm dish. On the next day, 150  $\mu$ g/ml G418 was added. The selective medium was changed every 4 days. For the transfected glioma cell line C6 (Benda, 1978), used as control, the G418-resistant cells were selected at 500  $\mu$ g/ml G418. The clones appeared 6 weeks later; they were individually trypsinized within cloning cylinders and transferred into gelatin-coated 15-mm wells. Subsequently, they were sub-cloned and expanded to mass cultures.

### Southern and Northern blot hybridizations

High molecular weight DNA (Wahl *et al.*, 1979) was digested with restriction enzymes and subjected to electrophoresis in a 0.6% agarose gel. After blotting (Southern, 1975), the nitrocellulose filters were hybridized with the following nick-translated (Rigby *et al.*, 1977) probes: the 1.8-kb *Bam*HI–*Eco*RI fragment from pPyLT1 or the 1.4-kb *Eco*RI–*Xba*I fragment from pAd5-E1A (specific radioactivity:  $10^8$  c.m.p./ $\mu$ g).

Cytoplasmic RNA (Favaloro *et al.*, 1980) was subjected to electrophoresis in a 1.4% agarose–formaldehyde gel and blotted onto nitrocellulose filters (Maniatis *et al.*, 1982) which were hybridized (Wahl *et al.*, 1979) with the above-mentioned probes.

### Uptake of [<sup>3</sup>H]GABA

The cells were grown to confluence in 15-mm wells and were pre-incubated for 15 min at 37°C with 0.5 mM  $\beta$ -alanine or 1 mM DABA, in 300  $\mu$ l PBS containing 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> and 33 mM glucose (Iversen and Kelly, 1975). Then, they were incubated for 15 min at 37°C in the presence of 30 nM [<sup>3</sup>H]GABA (1850 GBq/mmol, CEA). After extensive washing with PBS, the cells were treated with 250  $\mu$ l 1% Triton X-100, 0.1 M NaOH. The extracts were taken and the wells were washed with 250  $\mu$ l 1% Triton X-100, 0.1 M HCl. The washes were added to the extracts for radioactivity counting.

### Plating of neurones on cell monolayers

Striatal cells were dissociated from 15-day mouse embryos and plated at low density ( $8 \times 10^5$  cells per 35-mm dish) on monolayers of immortalized cells in the presence of 10 mM arabinosyl-cytosine (Sensenbrenner and Mandel, 1974; Noble *et al.*, 1984). After incubation for 48 h at 37°C, they were extensively washed with PBS at 4°C, fixed with 2.5% glutaraldehyde, rinsed with PBS and silver-stained.

### Immunofluorescence assay for GFAP

The cells were washed with PBS and fixed in acetone for 10 min at –20°C. The following steps were performed in PBS supplemented with 10% FCS. The cells were rinsed and incubated with rabbit antibodies to GFAP (1:10, a gift from C. Jacque). After washing, they were incubated for 30 min at 37°C with Texas red-conjugated donkey anti-rabbit immunoglobulins (1:100, Amersham). They were extensively washed and mounted in PBS-glycerol (20:80).

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