Special Issue

The immortalisation of rat hepatocytes by transfection with SV40 sequences

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Abbreviations: EGTA – ethylene bis(oxyethylenenitrilo)-tetraacetic acid; F12 – Ham's F12; FBS – foetal bovine serum; HBSS – Hank's balanced salt solution; HDM – hormonally defined medium; HEPES – 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid; NBS – new born calf serum; WME – Williams' medium E.

Introduction

Techniques are currently available to allow liver cells to be isolated and cultured *in vitro*. Such cultures can be maintained, but the cells do not proliferate, and the phenotype of the cells is unstable. In particular, expression of the drug metabolising enzymes alters within 48 h after cell isolation (Fry and Bridges, 1979; Croci and Williams, 1985; Grant *et al.*, 1985; Padgham and Paine, 1993). Established hepatoma cell lines have also stopped expressing these key aspects of liver function. This paper describes the approach of oncogene immortalisation as a means of isolating differentiated liver cell lines using a technique which has been used successfully with a variety of different primary cells (MacDonald, 1992).

There have been a number of reports describing the isolation of a hepatocyte-derived cell line using either adenovirus (Woodworth and Isom, 1987), polyoma virus (Hering *et al.*, 1991) or SV40 sequences (Isom *et al.*, 1980; Georgoff *et al.*, 1984; Isom and Georgoff, 1984; Woodworth *et al.*, 1986; Bayad *et al.*, 1991; Jacob *et al.*, 1992; Miyazaki *et al.*, 1993; Pfeifer *et al.*, 1993). No attempt has been made in these reports to optimise the transfection conditions or to examine and attempt to modulate the phenotypic variation which can occur in cell lines isolated even from within a single experiment. In most cases the characterisation of a single line is reported, and in the earlier reports the differentiated liver functions were not retained (Isom *et al.*, 1980). In this paper we describe both the mor-

phology and cytoskeletal properties of a number of immortalised cell lines and correlate these properties with expression of the liver-specific marker albumin.

Materials and methods

Rat hepatocyte isolation and culture

Rat hepatocytes were isolated by perfusing the liver in two steps (Williams *et al.*, 1977): firstly with calcium and magnesium-free HBSS (Gibco-BRL, Life Technologies) supplemented with 0.5 mM EGTA (Sigma) and 10 mM HEPES (Sigma), followed by a 0.05% (w/v) solution of collagenase type IV (Sigma) in Williams' medium E with glutamine 2 mM, HEPES 10 mM, and gentamycin 50 µg/ml (all Gibco-BRL, Life Technologies). The hepatocytes were washed four times in Williams' medium E and where applicable, layered onto a HBSS-based Percoll (Pharmacia LKB) solution, density 1.08 g/ml, and centrifuged at 400× g for 20 minutes, twice to yield cells with a viability >95% as measured by Trypan Blue dye exclusion.

Isolated hepatocytes were plated onto either 25 cm² culture flasks coated with a hydrated collagen lattice, HCL, (Elsdale and Bard, 1972), or onto Primaria (Falcon) 25 cm² culture flasks in a 1:1 mix of WME and F12 (Gibco BRL) with 5% FBS (v/v), glutamine 2 mM, and gentamycin 50 µg/ml.

Plasmid constructs

The plasmid pSVE contains the early region sequence of SV40 virus with an intact origin of replication (Brzeski *et al.*, 1986). Plasmids p6-1 and pUK42 which contain the entire SV40 genome with a 6 base pair deletion at the viral origin of replication (Gluzman, 1980) have been described elsewhere (Kreuzburg-Duffy and MacDonald, 1994).

Transfection of hepatocytes

Transfections were carried out using a modification of the procedure detailed by Woodworth *et al.* (1986). The calcium-phosphate/DNA precipitate was formed by mixing plasmid DNA, 20 µg of carrier DNA, and 50 µg of 2M calcium chloride made up to 250 µg with water, with 250 µg of HEPES-buffered saline pH 7.15. The HEPES-buffered saline was added dropwise to the DNA-calcium solution in the presence of bubbling air and the precipitate allowed to stand for 30 minutes. Hepatocytes which had been incubated for 18–24 h in WME/F12 + 5% FBS were exposed to 0.5 ml of the precipitate for five h, incubated for a further 24 h with fresh WME/F12 + 5% FBS, then transferred to hormonally defined medium (HDM) based on a 1:1 mix of WME and F12 (Isom and Georgoff, 1984) and maintained in culture with medium changes every three days until transformants appeared. Control transfections were routinely carried out in parallel with only carrier DNA and no plasmid.

Subculture of immortalised rat hepatocytes

Single colonies were sub-cultured from collagen-coated flasks by careful removal of a small section of the HCL into a pasteur pipette tip, resuspension in WME/F12 + 5% FBS and seeding onto a fresh collagen-coated flask. Alternatively, the culture medium was replaced by HBSS + EGTA for 10 minutes at 37 °C then with 0.05% (w/v) collagenase for a further 10 minutes to break down the collagen lattice. The collagenase was removed by centrifugation through three changes of culture medium (10 minutes, 400 x g) to prevent breakdown of the HCL on the new flask. In both procedures cells were allowed 5 to 20 h to adhere to the collagen-coated flask before being returned to HDM. Transformants isolated on Primaria flasks proved difficult to subculture, the most efficient method being mechanical disaggregation with a cell scraper. After several passages immortalised cells grown on Primaria

Table 1. Number of transformed colonies isolated after transfection of rat hepatocytes grown on collagen in defined medium with p6-1 and pSVE

Plasmid	Amount of DNA	No of colonies
p6-1	1 µg	7
pSVE	1 µg	1
p6-1	2 µg	14
pSVE	2 µg	2
p6-1	5 µg	4
pSVE	5 µg	0

flasks could be subcultured by incubating the monolayer in HBSS + EGTA for 10 minutes, or until cells began to 'round-up'. Many cell lines could be removed at this point by vigorous pipetting, but some of the small epithelial-like cells were resistant to the effects of HBSS + EGTA and could only be subcultured using a cell scraper.

Biochemical characterisation

Immunocytochemical detection was carried out on cells fixed using the method of Vielkind and Swierenga (1989) using rabbit anti-rat albumin and sheep anti-rat alphafetoprotein polyclonal antisera from Nordic Immunology (Tilburg, The Netherlands), mouse anti-vimentin and mouse anti-cytokeratin monoclonal antisera (Amersham), mouse anti-human fibronectin antiserum (Scottish Antibody Production Unit (SAPU), Carlisle) and mouse anti-SV40 large T antigen monoclonal antibody from Professor D. Lane. FITC-conjugated sheep anti-mouse IgG and donkey anti-rabbit IgG, HRP-conjugated sheep anti-mouse IgG, donkey anti-rabbit IgG and goat anti-sheep IgG polyclonal antisera were all obtained from SAPU. Primary antisera were used at a working dilution of 1:50 to 1:100 and second antisera at 1:30 to 1:50. All antisera were diluted in PBS. Cells were incubated with first antibody for 1 h, washed three times in PBS and incubated with second antibody for another h followed by three more rinses. The HRP substrate contained the chromogen 3-amino-9-ethylcarbazole (AEC, Sigma) and was used as previously described (Kreuzburg-Duffy and MacDonald, 1994).

Results

Transfection and immortalisation of hepatocytes

Immortalisation by origin-deleted SV40 virus DNA

On average 14 colonies were obtained when hepatocytes were transfected with 2.0 μg of p6-1 DNA (Table 1). If all of the 1×10^6 cells plated out initially survived the first 24 h of culture this would imply that 1 in every 71000 cells had been immortalised. However, the presence of dead cells and the leakage of lactate dehydrogenase into the culture medium suggests that the frequency of transformation may be higher than this estimate. Colonies of immortalised epithelial cells became visible to the naked eye after between 4–5 weeks in culture in most cases, rising to 6–7 weeks in the case of slow-growing cells.

Morphology of immortalised cell lines

The cell morphologies ranged from small, tightly-associated epithelial sheets to large, spreading cells which formed only loosely-associated colonies with an irregular border. Morphologies of transfected colonies included fibroblast-like cells with spindle-like processes in which the boundary of each cell is clearly visible; small epithelial-like cells with a very high nucleocytoplasmic ratio and occasional bi-nucleate cells with between 2 and 4 dark nucleoli in each nucleus. Other cells had a more polygonal shape and appeared to have formed cytoplasmic processes at the periphery of the colony. A third type of small epithelial cell was diamond-shaped and formed groups of cells orientated in one direction. The colonies had a smooth border with no processes emanating from the peripheral cells. These four cell types do not represent all the variations in cellular morphology observed, but highlight the variety of cell types obtained from a single preparation of hepatocytes transfected with the same plasmid.

Effect of purifying the hepatocytes prior to transfection

The hepatocytes were purified on Percoll density gradient medium at a density of 1.08 g/ml. Non-parenchymal liver cells have a density which can range from 1.04–1.06 g/ml whereas hepatocytes have a density of 1.07–1.09 g/ml. The difference in density between the two cellular components was confirmed by loading freshly isolated cells onto a discontinuous gradient consisting of three layers of Percoll solution at densities of 1.03, 1.05 and 1.08 g/ml respectively.

Hepatocytes were found in the pellet whereas non-parenchymal cells and some hepatocytes were found in the pellet whereas non-parenchymal cells and some hepatocytes were located at the 1.05/1.08 g/ml interface. Non-viable cells remained in the 1.03 g/ml solution.

The transfection of purified cells yielded less than a tenth of the number of transformants compared with the non-purified cells (Table 2), perhaps reflecting the lower survival rate of the purified cells in long term culture. Non-parenchymal cells were still present in the cultures of purified cells because the density gradient centrifugation is an enrichment rather than an absolute separation. In order to investigate whether overgrowth of non-parenchymal cells could be prevented, a selectable marker was introduced by co-transfection of a plasmid (pRSVneo) carrying the *neo* gene, so that the transfected cells could become resistant to the antibiotic G418 (Gorman *et al.*, 1983). Monolayer cultures of rat liver non-parenchymal cells at passage 1 were screened for sensitivity to G418 and found to be sensitive to a 50–100 $\mu\text{g}/\text{ml}$ G418. Hepatocytes did not show any visible signs of stress when incubated with this concentration of G418 but as the hepatocytes do not proliferate in culture there was no requirement for a selection procedure to eliminate them. The plasmid p6-1 was co-transfected with the *neo*-carrying plasmid pRSVneo under identical conditions to the control transfections with p6-1 alone. Two days after transfection G418 was added to the culture medium at 100 $\mu\text{g}/\text{ml}$. The number of non-parenchymal cells in these cultures was observed to be dramatically reduced, and the hepatocytes appeared to be unaffected. The hepatocytes in these cultures deteriorated in the same way as the Percoll-purified cells, however once again colonies of immortalised cells were isolated although the frequency of transformation was greatly reduced compared to the control p6-1 transfected cells (Table 2). The colonies produced in these cultures yielded small epithelial-like cells, eg the line C4-I-B-2.

A combination of purification of the hepatocytes prior to transfection, and selection for immortalised cells in G418-containing medium was tested to maximise the likelihood of obtaining immortalised cells derived from hepatocytes. This procedure was so stringent, however, that no transformants were isolated (Table 2) possibly because the combined purification/selection *completely* removed non-parenchymal cells from the cultures, thus removing the hepatocyte-non-parenchymal cell interactions required to maintain hepatocytes in culture. In a later experiment immor-

Table 2. Comparison of the effect of plasmid type, amount of DNA, type of medium and treatment of cells on the transfection efficiency

Plasmid type	Amount (μ g)	Medium type	cell type	Colonies/ 10^6 cells
p6-1	1.0	Defined	Mixed	6.5
	2.0		13.5	
	5.0		3.5	
p6-1	1.0	Defined	Purified	1
	2.0		0	
	5.0		1	
p6-1	1.0	Serum	Purified	2.5
	2.0		0	
	5.0		5*	
pSVE	1	Defined	Mixed	1
	2		2	
	5		0	
p6-1/pRSVneo	1.0/1.0	Defined	Mixed	1
	2.0/2.0	+G418		1
	5.0/5.0		0	
p6-1/pRSVneo	1.0/1.0	Defined	Purified	0
	2.0/2.0	+G418		0
	5.0/5.0		0	

talised cells were obtained from a culture of cells transfected with a plasmid construct (pUK42) carrying both the origin-deleted SV40 sequences from p6-1, and the *neo* gene from pRSVneo (not shown).

Effect of immortalisation in serum-supplemented medium

Percoll purified cells were transfected and grown in culture medium supplemented with 5% FBS. The hepatocytes deteriorated rapidly with few viable cells being visible after the first week in culture, supporting the findings of Enat *et al.* (1984) that non-homologous serum is cytotoxic to hepatocytes. By the third week of culture the flasks were so overgrown with non-parenchymal cells that subculturing was required to rescue the cells. On passaging, colonies of rapidly growing immortalised fibroblast-like cells were detected.

Characterisation of immortalised rat hepatocytes

Clone C4-1-B2

Clone C4-1-B-2 was isolated from a monolayer culture of rat hepatocytes co-transfected with plasmids p6-1 and pRSVneo, and selected in HDM containing 100 μ g/ml G418. A single colony of small tightly-associated epithelial cells was picked from the

collagen-coated flask and reseeded. This procedure was repeated three times. The cells grew only as colonies, single cells rarely being observed. Attempts at dissociation of the colonies with chelating agents such as EGTA failed, although collagenase could be used to remove the network of collagen from around the colony. Trypsinisation was also ineffective, although it removed cell from the periphery these cells were non-viable and did not adhere on transfer. The only means of transfer during the early passages was by mechanical destruction of the colony, although the colony could not be broken up into more than a few fragments without causing irreparable damage to the cells.

The C4-1-B-2 cells have a high nucleocytoplasmic ratio and an oval shaped nucleus with an average of two or three prominent dark nucleoli. Colonies always exhibited a smooth, clearly defined border with cell division occurring both in the interior and at the periphery of the colony and it was difficult to distinguish individual cells (Figure 1). No overgrowth of cells was observed in the colonies, contact inhibition of cell growth occurring.

Cell growth was very slow up until about the fifth passage and the plating efficiency remained low up to this point, transfer requiring care not to damage the cells. A split ratio of only 1:2 or 1:3 was possible. With continued culture the cells began to adopt a more

Figure 1. A colony of C4-1-B-2 cells after 3 cloning steps. The cells were cultured in hormonally defined medium on a collagen-coated surface. The cells have a high nucleocytoplasmic ratio, oval nuclei and form colonies with a smooth border. Dividing cells are present throughout the colony. Magnification is $\times 520$.

fibroblast-like morphology, individual cells became visible and the border of the colonies became irregular. By passage 12 individual cells were frequently observed, dissociation to yield single cells was possible with a combination of a chelating agent and collagenase and a split ratio of 1:10 was routine. Figure 2 shows bi-nucleate cells, a characteristic of freshly isolated hepatocytes with a decreased nucleocytoplasmic ratio, each nuclei having two or three prominent nucleoli. The buoyant density of C4-1-B-2 cells was measured by loading cells on to a discontinuous Percoll gradient and centrifuging at $400\times g$ for 20 minutes. The cells formed a band at an approximate density of 1.05–1.06 g/ml.

C4-1-B-2 cells expressed SV40 T-antigen on the nuclear membrane (not shown). When stained with the mouse monoclonal anti-vimentin the cells (Figure 3) had a diffuse network of fibres which delineate the cell nucleus, occasionally crossing over it to reveal

Figure 2. A colony of cells from C4-1-B-2 at passage 12. The cells were cultured in hormonally defined medium on a collagen-coated surface. The nucleocytoplasmic ratio has decreased and the borders of the original cells are now visible. The colony has an overall epithelial morphology although cells at the periphery have formed cytoplasmic processes. Bi-nucleate cells are present. Magnification is $\times 520$.

that the fibres are not linear but actually have a 'wavy' appearance. Although there was clearly a positive reaction with the anti-vimentin antiserum with peroxidase staining, the actual distribution of the fibres within the cells was not obvious. When the HRP-conjugated second antiserum was replaced with an FITC-conjugated antiserum most cells displayed a characteristic crescent shaped staining, vimentin-positive material being concentrated to one side of the cell cytoplasm. Fibroblast-like processes from the cells appeared to be rich in vimentin. Control cultures of 3T3 cells showed no staining for vimentin.

Cell surface fibronectin was detected in clone C4-1-B-2 as a cell surface fibrous network. This network extended between cells without crossing over the nuclei, indicating that in monolayer culture the

Figure 3. Clone C4-1-B-2 cells at passage 6 stained by immunoperoxidase for the expression of vimentin. Mouse monoclonal anti-vimentin antiserum was used at 1:100 and HRP-conjugated sheep anti-mouse IgG at 1:50. Vimentin-positive material appears as a network of fibres criss-crossing the cells. Magnification is $\times 1040$.

fibronectin binding sites are concentrated at the cell-cell boundaries.

The synthesis of albumin by C4-1-B-2 cells was detected by an anti-rat albumin antiserum. Control cultures of 3T3 cells showed a background staining did not display the specific perinuclear localisation pattern found with the C4-1-B-2 cells nor was it as strong. This may demonstrate albumin synthesis in the endoplasmic reticulum concentrated around the cell nucleus, but this interpretation is complicated by the high cross-reactivity and background found with 3T3 cells which are known not to produce albumin.

Characterisation of clone C8-IV

Clone C8-IV was isolated from a monolayer of rat hepatocytes grown in defined medium and transfected with plasmid pSVE. A single colony of loosely associated, large epithelial-like cells was picked from the

collagen-coated flask and reseeded. No further cloning was carried out after transfer of this first colony because the population of cells appeared to be homogenous. C8-IV cells grew as loosely-associated colonies with an irregular border, and the individual cells in the colony were clearly delineated. These cells could be dissociated easily using a chelating agent such as EGTA followed by collagenase. Cells grown on Primaria could be passaged with the chelating agent alone. Individual cells formed numerous cytoplasmic processes when cultured on collagen, but on plastic they adopted a large and spreading epithelial-like morphology with a low nucleocytoplasmic ratio. Individual cells have numerous blebs on the surface which suggest that the cells cannot form cytoplasmic processes because of the nature of the culture surface. These cells are distinct from dividing cells which have a bright 'halo' surrounding the periphery.

C8-IV cells grow rapidly and exhibit contact inhibition at confluence. The cells form spindle-like cytoplasmic processes when cultured on a collagen-coated surface and at passage 12 a split ratio of 1:20 was routine. Although the cells around the periphery of the colony have a fibroblast-like appearance, the cells in the body of the colony appear to be epithelial.

Cells from clone C8-IV expressed SV40 T-antigen on the nuclear membrane and showed dark nuclear staining. All the cells were positive with little variation in the intensity of staining.

C8-IV cells stained positive for the presence of vimentin and this vimentin-positive material was restricted to a system of fibres which have a polar distribution in many of the cells. The bundles of fibres are orientated to a focus which is either a point on one side of the cell or more commonly a perinuclear focus. Fibres emanate from the focal point in 'wavy' strands and extend to the cellular periphery, always following a radial pathway and never running perpendicularly to the periphery of the cell. Such a distribution does not suggest that the vimentin-positive material is associated with stress fibres (Figure 4). C8-IV cells did not show any reaction with the anti-cytokeratin antisera.

C8-IV cells are criss-crossed by a network of fibres of fibronectin which run the length of several cells. The fibres appear to be concentrated at the cell-cell boundaries as with C4-1-B-2, however, the network is looser and more widely spaced with longer fibres. The presence of areas of albumin-producing cells in the cultures of C8-IV is indicated by the perinuclear localisation of the albumin-positive material. The cells form a more tightly associated epithelial sheet suggesting that albu-

Figure 4. A colony of C8-IV cells at passage 6 immunoperoxidase stained for the presence of vimentin. Mouse monoclonal anti-vimentin antiserum was used at 1:100 and HRP-conjugated sheep anti-mouse IgG at 1:50. These cells clearly demonstrate the distribution of vimentin-positive fibres within the cell. Magnification is $\times 1040$.

min expression is increased at confluence when contact inhibition stops the cells from growing further. As C8-IV cells had a high growth rate this would result in low levels of albumin in the culture medium unless the cells reached confluence before assay.

Characterisation of clone SV40RH1

Clone SV40RH1 was isolated by transfecting a monolayer of rat hepatocytes with SV40 virus DNA, growing in HDM on Primaria and removing a single colony of small tightly-associated cells. Early passage SV40RH1

cells growing on a collagen-coated culture surface as colonies and not as single cells. Dissociation of these colonies was not possible with any combination of chelating agent, collagenase or trypsin. Washing the colonies with a solution of EGTA before scraping the colonies from the culture surface with a cell scraper facilitated transfer and increased plating efficiency. Cells could be cultured on both collagen-coated and Primaria flasks but those grown on collagen were easier to subculture. The colonies of SV40RH1 were more robust than those of C4-I-B-2, surviving transfer with a cell scraper to a greater degree.

SV40RH1 cells form very tight cell-cell bonds and the boundary of individual cells is not visible. Even up to passage 5 the cells continued to form tightly-associated epithelial colonies. At this point the nuclei of individual cells were still not clearly visible, their presence only being suggested by the dark nucleoli. Unlike C4-I-B-2, spindly processes often protruded from cells on the boundary of the colonies. Single cells which split from the main colonies often gave rise to new colonies, however, the growth rate still remained low. Single cells were only obtained when they detached from a colony, they could not be isolated by the dissociation of a colony. When cultured on Primaria plastic the cells have spread out more, cell-cell boundaries are still very hard to visualise and the cytoplasm of the cells remains dense revealing very little about the morphology of the cells.

The only immunocytochemical test carried out on the cell line SV40RH1 was immunofluorescent staining for vimentin, and vimentin-positive cells were present although only in a small proportion of cells. Those cells expressing vimentin showed a discrete, strongly-polarised pattern. The bundles of fibres only emanate from a focal point on one side of the nucleus in most of the cells. The SV40RH1 cell line is the only immortalised cell line which contains vimentin-negative cells, all others are strongly positive despite differences in the pattern of staining.

Characterisation of clone C3-II derived cell lines

The cells derived from clone C3-II were isolated from a monolayer culture of rat hepatocytes transfected with the plasmid p6-1, and grown on a collagen-coated surface in defined medium. The parent flask C3 contained numerous colonies of cells and was the only flask of cells on which a colony of cells was observed at a very early stage of growth. The colony of immortalised cells is composed of dense, small epithelial cells. No bile

canaliculi are present in this colony, confirming its immortalised phenotype and suggesting that the loss of the ability to form bile canaliculi may occur as a prerequisite to growth.

Single colonies of cells were removed from the flask C3 to give rise to the C3-I and C3-II lines. C3-II yielded the most epithelial-like cells and colonies were picked to give clones, one of which, C3-II-B produced colonies of tightly-associated cells. Two further cloning steps resulted in four clones, two of which had distinct morphologies. One of these clones, C3-II-B-2, at passage 5, which was considered pure when a single colony of tightly-associated epithelial cells, was obtained on transfer. On culture, however, numerous cells changed from a characteristic epithelial sheet morphology to single fibroblast-like cells. The cells themselves still appear dark, the nucleus being difficult to visualise, only the shape and the presence of discernible cell boundaries appears to have been lost. A second clone C3-II-B-2-3 analysed later was found to be more stable, remaining epithelial in nature up to passage 8 (not shown).

Cells from clone C3-II-B-2-3 were examined for the presence of vimentin by immunofluorescence. Each epithelial cell contains a clear focus of vimentin-positive material located adjacent to the nucleus. This pattern of staining appears to be more discrete where cells have an epithelial morphology, fibroblast-like cells have a more diffuse pattern of staining. Vimentin-positive material may thus be either associated with fibres involved in the maintenance of cell shape, or may itself be important in the determination of the cellular morphology.

Characterisation of cell line P9

Cell line P9 was isolated from a monolayer culture of Percoll-purified rat hepatocytes transfected with plasmid p6-1, and cultured in serum-supplemented medium. The colonies of cells grew rapidly and had an indistinct morphology. The cells have not been cloned, but this could only be achieved by dilution cloning because the cell line does not readily form colonies. The cells have a fibroblast-like morphology in culture and grow rapidly to form large sheets of contact-inhibited cells. At confluence the cells align to form patterns of cells rather than epithelial sheets. The cells have been cultured up to passage 20 with no change in morphology, a split ratio of 1:20/1:40 being routine. On reaching confluence many of the cells become elongated and occasionally grow over adjacent cells. These

cells may be passaged by treatment with versene followed by trypsinisation although if allowed to reach confluence the sheet of cells becomes difficult to dissociate perhaps due to the presence of an extracellular reticulin-like network. When cultured on a collagen-coated surface in serum-supplemented medium these cells adopt a very dark and spindly fibroblast-like morphology and the cells are elongated with cytoplasmic processes at the axis.

All of the cells in the P9 cell line expressed T-antigen and all stained with equal intensity. The cells also showed diffuse cytoplasmic immunoperoxidase staining for vimentin with the cell nuclei being delineated by the criss-crossing fibres in the cell cytoplasm.

Discussion

We describe here an optimised method for immortalising rat hepatocytes to yield proliferating parenchymal cell lines. The best results have been obtained with SV40 DNA containing a deletion at the origin of replication and using 2 μ g of DNA/25 cm² flask of cells in hormonally defined medium cultured on collagen. This is consistent with previous reports by Small *et al.* (1982) that origin-defective SV40 virus vectors enhance the number of immortalised human fibroblasts. It was important to reduce, but not completely abolish the number of non-parenchymal cells in the culture and this was achieved either by antibiotic selection or by density gradient purification. This requirement for the presence of epithelial cells for the expression of the differentiated phenotype is consistent with the work of Guguen-Guillouzo *et al.* (1983).

The parenchymal cell origin of the immortalised lines has been confirmed by the detection of albumin in the medium in the case of four lines (SV40RH1, C3-II-B-2-3, C4-1-B-2 and C8-IV) and by bilirubin-UDP-glucuronosyltransferase activity in the case of two lines (SV40RH1 and P9, Grant *et al.*, 1992). SV40RH1 and P9 have also been shown (Nairn *et al.*, 1990; MacDonald *et al.*, 1994) to be capable of resynthesising glutathione from methionine and cysteine and to have low levels of the phase II enzymes γ glutamyltransferase and π glutathione-S-transferase.

The methods described here have resulted in the isolation of five independent cell lines whose morphology and cytoskeletal proteins have been compared. All of the immortalised lines contained cells which expressed vimentin, an observation consistent with the report by Francke *et al.* (1981) that hepatocytes and

hepatoma cells in culture begin to express vimentin, although hepatocytes do not express vimentin *in vivo*. One cell line, SV40RH1, contained vimentin-negative cells, as did the MH1C line analysed by Francke *et al.* (1981), indicating that vimentin is not proliferation *in vitro*.

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