A Mutational Analysis of the Transforming Functions of the E8 Protein of Bovine Papillomavirus Type 4

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The E8 protein of BPV-4 contributes to transformation of primary bovine cells (PalFs) by inducing anchorage-independent growth and by down-regulating gap junction intercellular communication, likely due to its binding to 16K ductin. We show here that, in addition, E8 confers on PalF cells the ability to grow in low serum and to escape from contact inhibition (focus formation). E8 also transactivates an exogenous human cyclin A gene promoter, suggesting that overexpression of cyclin A is responsible for the transformed phenotype. Mutant forms of E8 were generated to establish whether the transforming functions of the protein could be segregated. Mutations were introduced both in the hydrophobic domain and in the hydrophilic C-terminal "tail", and chimeras with BPV-1 E5 were constructed. Cells expressing either wild-type E8 or mutant forms were analyzed for their ability to grow in low serum and in suspension and to form foci. Wild-type E8 and its mutants were also analyzed for their ability to transactivate the cyclin A promoter. We show here that the transforming functions of E8 can be segregated and that both the hydrophobic C-terminal tail and the residue at position 17 in the hydrophobic domain are crucial for E8 functions and for the transactivation of the cyclin A promoter. These results support the hypothesis that the different aspects of cellular transformation brought about by E8 might be due to interaction with different cellular targets. They suggest that E8 might function differently from BPV-1 E5 and demonstrate that the separate domains of E5 and E8 are not functionally interchangeable.

Key Words: BPV-4; cell transformation; E8; cyclin A.

INTRODUCTION

Papillomaviruses are small DNA tumour viruses with a closed circular double-stranded genome. The genome of papillomaviruses contains at least two genes responsible for cell transformation in vitro. The main transforming genes of human papillomavirus type 16 (HPV-16) are represented by the E6 and E7 open reading frames (ORFs)¹ (Crook et al., 1996), with the E5 ORF assisting in transformation (Banks and Matlashewski, 1996) while the two transforming genes of bovine papillomavirus type 4 (BPV-4) are the E7 and E8 ORFs (Jackson et al., 1996). Primary bovine fibroblasts (PalFs) transformed in vitro by BPV-4 are capable of growing independent of attachment to substrate. Previous work has shown that this property is dependent on the presence and expression of the E8 ORF; cells transformed by E7 alone are not capable of anchorage-independent growth (Pennie et al., 1993). Although located at the 5’ end of the early region (Jackson et al., 1991), rather than at its 3’ end like the E5 ORF of BPV-1 (Schiller et al., 1986) or HPV-16 (Halbert and Galloway, 1988), the BPV-4 E8 ORF encodes a polypeptide that belongs to the E5 family: it is only 42 amino acids (aa) long, with a putative transmembrane domain comprising the highly hydrophobic N-terminal 30 residues and a hydrophilic C-terminal tail of 12 residues (Jackson et al., 1996); it is localised to the cell membranes (Pennie et al., 1993) and is expressed in the deep layers of early stage papillomas of the upper alimentary canal (Anderson et al., 1997). In addition, E8 binds 16K ductin in vitro and induces down-regulation of gap junction intercellular communication (GJIC) in transformed PalFs (Faccini et al., 1996). When expressed in an established murine fibroblast cell line (NIH-3T3) E8 alone is transforming; E8-3T3 cells can grow in low serum and in suspension, and these cellular phenotypes are associated with the ability of E8 to transactivate the cyclin A gene promoter, to increase cyclin A protein level and cyclin A-associated kinase activity, and to inhibit the functions of the negative regulator of cell cycle, p27kip1 (O’Brien and Campo, 1998).

In this study we have further characterised the transforming activities of E8 in PalFs to establish whether, as...
in NIH-3T3 cells, E8 can deregulate expression of the cyclin A gene. We show that, in addition to their ability to grow in suspension and to down-regulate GJIC, PalFs expressing E8 can grow in low serum and are not contact inhibited and that E8 transactivates the cyclin A gene promoter in PalF cells. Using a panel of E8 mutants and chimeras constructed with BPV-1 E5, we show that the different transforming functions of E8 can be segregated and demonstrate that both the residue at position 17 in the hydrophobic domain and the hydrophilic C-terminal tail of the molecule are crucial for E8 functions.

The results obtained also suggest that E8 acts differently from BPV-1 E5 to bring about cell transformation and demonstrate that the separate domains of E5 and E8 are not functionally interchangeable.

RESULTS
Construction of E8 mutants and E8–E5 chimeras

In an attempt to segregate the different transforming functions of E8 and therefore to define its functional domains, several mutations were introduced in the predicted α-helical domain and in the C-terminus, and chimeras were constructed between BPV-4 E8 and BPV-1 E5 (Fig. 1).

Studies by others have shown that binding of BPV-1 E5 to ductin requires the N-terminal hydrophobic domain (first 32 residues) and that glutamine at position 17 is important for ductin interaction and for the transforming function of the protein (Goldstein et al., 1992; Kulke et al., 1992; Sparkowski et al., 1994, 1996). We sought to test whether the equivalent residue, asparagine, in the E8 polypeptide of BPV-4 is also crucial for cell transformation. To define the role of the C-terminal hydrophilic tail of E8, we constructed E8T, which consists of the first 32 residues, comprising the membrane-localised region of E8. A similar mutant form was produced for BPV-1 E5 to create a truncated form of this protein (E5T; residues 1–31).

Two chimeras were constructed between E8 and E5 to test the relative contribution each domain of E8 made to cell transformation and to test whether these domains were functionally interchangeable between the two peptides. One form, E8N–E5C, retains the N-terminus of E8 (residues 1–29) but the carboxyl end is replaced by residues 30–44 of BPV-1 E5. The reciprocal chimera, E5N–E8C, retains the N-terminus of BPV-1 E5 (residues 1–31) but the carboxyl end is replaced by residues 32–42 of BPV-4 E8 (Fig. 1).

Production of cell lines

PalF cells can be transformed by BPV-4, or by the E7 and E8 ORFs, only in the presence of an activated ras gene (Jaggar et al., 1990); cell immortalisation is, however, achieved only with the addition of the E6 ORF of HPV-16 (Pennie et al., 1993; Scobie et al., 1997). The cell lines described here contain BPV-4 E7 ORF, an activated ras gene, and the HPV-16 E6 ORF introduced by gene transfection to generate stable transformants. If any of these additional plasmids was omitted from the transfection protocol, no stable control cell lines were ever obtained. In addition, in no case to date have we produced a control cell line (i.e., transfected with expression plasmids for BPV-4 E7, HPV-16 E6, and c-Ha-Ras) capable of growth in low serum or in suspension (Jaggar et al., 1990; Pennie et al., 1993; Cairney and Campo, 1995; Scobie et al., 1997).

Detection of viral protein expression

Total protein extracts from representative clones, which were positive for viral transcripts (by RT-PCR) and which were fully characterised for their transformation status (see below), were subjected to dot blot analysis as described under Materials and Methods. The results demonstrate that E8, its mutants, E5, E5T, and E8–E5
chimeras are all expressed within PalF cells although at somewhat different levels (Fig. 2).

Mutations in E8 affect anchorage-independent cell growth

As previously reported (Pennie et al., 1993), PalF cells expressing E8 can grow in suspension culture. Among the mutants, only cells expressing E8N17S, E8N17A, and E5N–E8C were capable of anchorage-independent growth, with cells containing E8N17A displaying the highest efficiency of colony formation in methocel (Fig. 3) and particularly in agar where the cloning efficiency reached 25% (not shown). E8N17Y, E8T, E8N–E5C, and E5T cells were incapable of colony formation in methocel (Fig. 3) or agar culture (not shown).

These results concur with those obtained by other workers for E5 and point to the importance of the residue at position 17 (Goldstein et al., 1992; Kulke et al., 1992; Sparkowski et al., 1994, 1996) and the requirement of the hydrophilic “tail” for the biological activity of both E5 (Green and Loewenstein, 1987) and E8 proteins. The results also show that, while the C-terminal tail of E8 can substitute for the tail of E5 in conferring anchorage independence, the reciprocal combination abolishes this ability, and therefore the individual C-terminal domains are not functionally interchangeable.

E8 mutants affect cell proliferation in low serum

Although in normal medium with 10% serum (growth medium, GM) there were no differences in growth rates between parental PalF cells and cells with or without E8 (Fig. 4), when the serum was lowered to 0.5% (low-serum medium, LS), only the E8 cells proved capable of continuous growth (Fig. 4). Therefore, E8-expressing PalF cells do not exit the cell cycle when deprived of serum mitogens and behave like E8-3T3 cells (O’Brien and Campo, 1998). Cells expressing BPV-1 E5 can also grow in LS (Fig. 4b) over the 7-day period of the experiment, which, to our knowledge, has not been demonstrated before.

E8 mutant transfectants were assayed for their growth characteristics in GM and in LS. Normal PalFs and all transfectants grew at the same rate in GM, but only E8N17A cells continued to proliferate in LS (Fig. 4a), and there was little or no growth in cells expressing the other mutant forms, including E8N17S (Fig. 4a) and E5N–E8C (Fig. 4b), which could grow in suspension culture. Some cell lines therefore are both capable of anchorage-independent growth and capable of proliferation in low serum, while others are capable of growing in suspension but cannot proliferate in the absence of serum mitogens. Thus the transformation phenotypes of anchorage-independent growth and of growth in low serum can be segregated in E8N17S and E5N–E8C.

Mutations in E8 affect focus formation

PalF cells expressing E8 were able to form foci on a lawn of normal PalF cells (Fig. 5), showing that they can overcome contact inhibition. Cells expressing E8N17A were capable of forming foci in LS (Fig. 4a), and there was little or no growth in cells expressing the other mutant forms, including E8N17S (Fig. 4a) and E5N–E8C (Fig. 4b), which could grow in suspension culture. Some cell lines therefore are both capable of anchorage-independent growth and capable of proliferation in low serum, while others are capable of growing in suspension but cannot proliferate in the absence of serum mitogens. Thus the transformation phenotypes of anchorage-independent growth and of growth in low serum can be segregated in E8N17S and E5N–E8C.

The ability to transactivate the cyclin A promoter correlates with the ability to induce anchorage-independent growth

We have proposed that deregulated expression of cyclin A and its associated kinase activities may underlie
the phenotype of E8 transformed NIH-3T3 cells (O'Brien and Campo, 1998). In the current study, cyclin A promoter activity was determined in cell lines expressing wild-type or mutant forms of E8 to test for a correlation between promoter activation and cell transformation brought about by the different E8 forms. Stable cell lines were transfected with a luciferase reporter gene under the transcriptional control of the human cyclin A promoter (Kramer et al., 1996). Under normal growth conditions expression of wild-type E8 led to at least a 40-fold increase in cyclin A promoter activity in two independent clones when compared with control cells with no E8 (Fig. 6a). A similar pattern of promoter activity was observed in cells expressing wild-type E5 (Fig. 6a). Of the E8 mutants, only E8N17A had comparable promoter transactivation activity; E8N17S and E5N–E8C transactivated the cyclin A promoter to a lesser degree than E8 wild-type, but significantly above the levels for control cells (P

FIG. 3. Anchorage-independent growth. Cells expressing E8wt, E8N17S, E8N17A, or E5N–E8C are capable of anchorage-independent growth in methocel culture. Control cells were transfected with pZipneoE7, pT24, and p4416-E6, while E8 cells were transfected additionally with an expression vector containing wild-type or mutant sequences of E8 as described in the legend to Fig. 1. After 21 days in suspension culture, colony formation was scored by estimating the total number of colonies with a diameter greater than 0.1 mm present in duplicate wells for each cell type. The number in the bottom right-hand corner of each panel represents the number of colonies expressed as a percentage of the total number of cells seeded into each well (1 × 10^5) on day 1. The experiment was performed for at least two independent clones for each cell type and a representative result is presented in each case (final magnification, X40).
values for N17S and E5N–E8C < 0.01); all the other mutants were incapable of promoter transactivation (Fig. 6b).

When the cells were maintained under LS conditions for the 24-h period following transfection, promoter transactivation was sustained in the case of wild-type E8, E8N17A (Fig. 6a), E8N17S, and E5N–E8C (Fig. 6b) but not for the other mutants. Wild-type E5 could still transactivate the promoter in low serum, but reproducibly to a lesser extent than E8, suggesting a greater degree of growth factor dependence (Fig. 6a).

Interestingly, cells expressing E8N17S and the E5N–E8C chimera transactivate the cyclin A promoter to a lesser extent than E8, suggesting a greater degree of growth factor dependence (Fig. 6a).

These results extend those obtained previously with E8-3T3 cells in which the cyclin A promoter is transactivated by E8 and confirm that cyclin A promoter transactivation correlates with anchorage-independent growth. They also show that strong cyclin A promoter transactivation correlates with population growth in low serum.

The characteristics of the transformed cells are summarised in Table 1.

FIG. 4. Cell proliferation in high- and low-serum medium. Growth characteristics of cells maintained in growth medium (GM, 10% serum) and low serum (LS, 0.5% serum). (a) Transformation by E8wt did not alter the growth characteristics of PalF cells in GM and only cells expressing E8N17A continued to proliferate in LS. (b) Cells expressing truncated forms of E8 (E8T) or E5 (E5T) or E8–E5 chimera (E8N–E5C, E5N–E8C) did not proliferate in LS. Two independent clones for each cell type were maintained in LS for 7 days. Cell growth was determined by dye staining (crystal violet) as described under Materials and Methods and is expressed as a growth index representing the fold change in cell population from day 1 (harvested 5 h after the cells were seeded) values. For normal PalF cells: ■, 10% serum; ●, 0.5% serum. For all clones: X, △, 10% serum; ■, ●, 0.5% serum.
DISCUSSION

We have extended our analysis of the functions of BPV-4 E8 in primary bovine cells to test the transforming ability of mutants with alterations in the hydrophobic domain of the molecule and a truncated form of E8 consisting of only the first 32 residues (E8T) as well as chimeras consisting of reciprocal swaps of the two domains of E5 and E8 (Fig. 1).

We show that cells expressing wild-type E8 do not exit the cell cycle after serum withdrawal, lose contact inhibition, and overcome the block in late G1 imposed when anchorage-dependent cells are maintained in suspension. PalF cells, which do not harbour E8, consistently do not show any of these phenotypes, despite the presence of BPV-4 E7, HPV-16 E6, and activated ras (this study and Jaggar et al., 1990; Pennie et al., 1993; Cairney and Campo, 1995; Scobie et al., 1997). Thus, three of the parameters of transformation, i.e., growth in suspension, growth in low serum, and lack of contact inhibition, are conferred to primary cells by the E8 peptide. Despite their capability of growing in semisolid media, these transformed cells are not tumourigenic in nude mice (results not shown).

The altered phenotypes observed for E8 mutants com-

FIG. 5. Focus formation. Only cells expressing E8wt and E8N17A were capable of forming foci on a monolayer of normal PalFs. Two hundred control cells or E8 transfectants were seeded in 6-well plates with 25 × 10³ normal PalF cells. Foci were fixed and stained with a Giemsa solution 21 days after the cells were seeded (final magnification, X40).
pared with wild-type E8 cannot be ascribed to different amounts or differential stability of the mutant forms of E8 as there is no correlation between amount of protein (Fig. 2) and cell transformation or promoter transactivation (Table 1). For instance the nontransforming mutants E8N17Y, E8T, and E8N–E5C are expressed at higher levels than the hypertransforming mutant E8N17A (Fig. 2). This identifies mutant protein function and not differences in expression level as the cause of the different phenotypes observed in this study (Table 1).

In BPV-1 E5, the glutamine residue at position 17 has been shown to be critical for cell transformation (Kulke et al., 1992; Sparkowski et al., 1994, 1996). This appears to be the case also for the asparagine residue in the same position in BPV-4 E8. Mutation of this single amino acid brings about profound changes in the activity of the protein. Thus mutation to a tyrosine residue completely abolishes the transforming capability of E8, while mutation to an alanine residue creates a "hypertransforming" molecule with increased ability to induce anchorage-

**FIG. 6.** Transactivation of the human cyclin A promoter. (a) The transcriptional activity of the cyclin A promoter was determined after transfection of the reporter plasmid pwt929 into independent clones of stable transfectants for each cell type. Luciferase activity is given after normalization on cellular protein content. Values represent the mean of duplicate samples from experiments performed at least twice. Bar represents standard error. (b) Scaled-up representation of control, N17S, N17Y, E8T, E8N–E5C, and E5N–E8C to illustrate statistical significance of promoter transactivation values. *Statistically significant differences versus control cells in both high and low serum. \( P < 0.01 \) for N17S, E5N–E8C; others \( P > 0.1 \). \( P \) was calculated by Student’s \( t \) test.

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<th>TABLE 1</th>
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<td>Transformation Characteristics of Cells Expressing E8 or Its Mutants</td>
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<td>Control (no E8)</td>
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<td>E8 wild type</td>
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<td>BPV-1 E5</td>
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Note. AI, anchorage-independent; LS, growth in low serum. The plus signs represent the extent of expression of the characteristic. nd, not determined. E5T was tested in transient transfection experiments where it failed to transactivate the cyclin A promoter in either high or low serum.
independent growth, and mutation to a serine residue leads to an intermediate state that allows only the maintenance of the anchorage-independent phenotype. Clearly, although the role of the residue at position 17 is unknown, this residue is critical for the integrity of E8 biological functions. Interestingly, a E6 mutant containing, like E8, an asparagine residue at position 17 is not transforming in mouse fibroblast lines (Sparkowski et al., 1994). In addition, in contrast to BPV-1 E5, this residue does not appear to be crucial for E8–ductin interactions, at least in vitro (manuscript in preparation).

Another critical domain of E8 is its C-terminal hydrophilic tail. The C-terminus truncated mutant, containing only the putative transmembrane portion of the molecule, is transformation defective. Confirmation that the C-terminal tail of E8 is essential for PalF transformation is provided by the chimera E8N–E5C, consisting of the hydrophobic portion of E8 and the hydrophilic tail of E5, which is incapable of inducing a transformed phenotype in these cells. Both the membrane domain of E8T and the E8N–E5C chimera may adopt an altered nonfunctional conformation or have a different cellular distribution than wild-type E8. These points still await elucidation. The functional failure of E8N–E5C also points to the non-equivalence of the domains of E8 and E5; while the tail of E5 cannot substitute for the tail of E8, the reciprocal exchange leads to a chimeric molecule still retaining the ability to induce anchorage-independent growth. Indeed, despite the overall amino acid and structural homology of E8 and E5, there are differences between the two transforming proteins in the PalF system: E8, but not E5, allows cells to escape from contact inhibition and E5 appears to be more dependent on serum mitogens for transactivation of the cyclin A promoter. This suggests that these small papillomavirus proteins may have overlapping but different mechanisms of achieving cell transformation.

The use of the mutant forms of E8 has allowed the segregation of its several transforming functions. Thus the ability to induce substrate-independent growth can be separated from the ability to form foci or to prevent cell cycle exit in low serum. Focus formation and growth in low serum still cosegregate in the E8 mutants, but, interestingly, not in wild-type E5, which, as noted above, cannot induce escape from contact inhibition, while allowing growth in low serum. This was an unexpected observation. However, E5, but not E8, can induce foci in NIH-3T3 cells (V. O’Brien, unpublished observations), thus illustrating cell-type-specific differences in the transforming potential of these small papillomavirus proteins.

In PalF cells, as in NIH-3T3 cells (O’Brien and Campo, 1998), E8 expression promotes transcriptional transactivation of a heterologous cyclin A promoter under conditions where cyclin A transcription is normally diminished or is not detectable in control cells. E8 mutants that can transactivate the cyclin A promoter confer to PalF cells the ability to grow in suspension, and mutants that strongly transactivate the promoter permit population growth in low serum (Table 1). These observations support our hypothesis that E8-mediated cell transformation is, at least in part, due to the ability of the viral protein to deregulate cyclin A expression and associated kinase activities (O’Brien and Campo, 1998). This hypothesis can be refined in light of the results described here to suggest that a threshold of cyclin A promoter transactivation correlates with cell transformation; this threshold is well exceeded by E8wt, E8N17A, and E5wt (Fig. 6a) and just exceeded by E8N17S and E5N–E8C (Fig. 6b). Further work to test this hypothesis is under way in NIH-3T3 cells, a system that requires only the introduction of E8 for cell transformation and where the reagents required to carry out these studies are readily available.

Overall, the results demonstrate that the multiple transforming functions of E8 can be segregated, which likely reflects the complex interactions of the domains of E8 with different cellular targets.

MATERIALS AND METHODS

Plasmids

pZipneoHAE8, encoding G418 resistance and carrying the E8 ORF containing a sequence encoding the 11-residue HA-1 epitope of the influenza virus 5’ to the E8 sequence, has been reported previously (Faccini et al., 1996). pZipneoE7, expressing the E7 ORF, and pT24, a pUC13-derived plasmid containing the 6.6-kb activated human c-Ha-ras oncogene, have also been described previously (Pennie et al., 1993). pZipneoHAE8 served as the template for mutant construction. E8 mutants containing single amino acid substitutions at residue 17, a C-terminus truncated form of E8 (E8T; N1-32), in which a premature stop codon has been introduced at residue 33, and a C-terminus truncated form of BPV-1 E5 (E5T; N1-31), with a premature stop codon introduced at position 32, were produced by site-directed mutagenesis using the Altered Sites kit (Promega), following the manufacturer’s instructions. For the construction of E8–E5 chimeras, site-directed mutagenesis was used to create an Accl site in BPV-4 E8 at a position similar to that occurring in BPV-1 E5. Exchange of the 3’ ends of the E5 and E8 ORFs was achieved using standard cloning procedures to give two chimeras: E8N–E5C, containing an ORF encoding residues 1–29 of E8 and residues 30–44 of E5, and E5N–E8C, encoding residues 1–31 of E5 and residues 32–42 of E8.

p4O16-E6 was a gift from Dr. L. Crawford (Department of Pathology, University of Cambridge). This plasmid construct is a pBR322 derivative containing the HPV-16 E6 ORF.
pwt929 is a luciferase reporter plasmid for the human cyclin A promoter. This plasmid construct was a gift from Dr. William Fahl, University of Wisconsin (Kramer et al., 1996).

Cell culture and stable transfection

Primary bovine fibroblasts were derived from the foetal palate (PalFs) as described previously (Jarrett et al., 1990) and used at passages 2–8. Cells were routinely cultured in Dulbecco’s modified Eagle's medium (DMEM) containing 10% foetal calf serum (growth medium) and subcultured to maintain subconfluent monolayers. Cells (5 × 10⁵) were transfected with a range of plasmids using a cationic lipid (DOTAP; Boehringer Mannheim) following the manufacturer’s instructions. Each reaction contained 5 μg of the indicated expression plasmids and sonicated salmon sperm DNA (Sigma) to bring the total amount of DNA to 20 μg. After 16–18 h cells were washed twice with warm medium, transfecants were selected in medium containing 500 μg/ml G418 for 21–28 days, and G418-resistant colonies were then picked and expanded. Two independent clones were used for each cell type in the transformation experiments. All transfectants were routinely maintained in GM supplemented with 500 μg/ml G418.

Detection of viral protein expression in PalF cell lines

Cells were lysed in near-boiling SDS–PAGE sample buffer lacking bromophenol blue and dithiothreitol, sonicated, and clarified by centrifugation at 13,000 × g at 4°C then adjusted to 2 mg/ml total protein in each case. Equal amounts of protein (2 μg) were blotted onto a nitrocellulose membrane (ECL Hybond, Amersham) in a Bio-Dot microfiltration apparatus (Bio-Rad). The membrane was then blocked in PBS containing 5% nonfat milk (Marvel) for 1 h at room temperature and incubated with a dilution (1:1000) of a monoclonal antibody that recognises the HA epitope (12CA5, Boehringer Mannheim) or was washed twice with 2 ml PBS and incubated in LS or GM for a further 24 h before harvesting. Luciferase activity in the supernatants was determined as described previously (O’Brien and Campo, 1998) and activity was normalised for protein content determined using the BCA assay (Pierce Chemical Co.).

Anchorage-independent growth

Cells (1 × 10⁵) were added to 15 ml of methocel medium (Pennie et al., 1993) and the mix was plated in bacterial petri dishes. Cells, tested in duplicate, were left at 37°C for 12 days before being scored and photographed.

Focus formation assay

Two hundred cells of each clone were mixed with 25,000 parental PalF cells. The cell suspension was plated in 6-well plates, in triplicate. Medium was replenished twice weekly and after 20–25 days the cells were washed with PBS, fixed in methanol, stained with 10% Giemsa solution, and photographed.

Determination of cell growth rate in high- and low-serum cultures

PalF cells and derived transfecant cells were seeded in 96-well tissue culture plates, in triplicate, at 3000 cells/well in 200 μl of GM. After 5 h of incubation, during which time the cells attached to the bottom of each well, triplicate wells for each cell type were left undisturbed or were switched to DMEM with 0.5% serum (LS). Cells were washed with prewarmed (37°C) PBS before the addition of 100 μl of a solution of 0.1% crystal violet in 20% methanol. The plate was placed overnight at room temperature. Crystal violet solution was removed and wells were washed extensively in water and then air-dried. Dye was solubilised in 100 μl of 1% SDS, 0.2 M NaOH solution and the absorbance of the solubilised dye was determined immediately at 590 nm using an automated microplate reader (Dynatech MR7000).

Cyclin A promoter reporter assays

Cells were seeded at 20,000 cells/ml in GM in each well of 6-well plates, 5 ml/well, the day before transfection with the luciferase reporter plasmid pwt929 by the standard calcium phosphate method. After 16–18 h, cells were washed twice with 2 ml PBS and incubated in LS or GM for a further 24 h before harvesting. Luciferase activity in the supernatants was determined as described previously (O’Brien and Campo, 1998) and activity was normalised for protein content determined using the BCA assay (Pierce Chemical Co.).

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