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Continued expression of HPV-16 E7 protein is required for maintenance of the transformed phenotype of cells co-transformed by HPV-16 plus EJ-*ras*

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The close association between HPV-16 and cervical cancer implies some role for the virus in development of this cancer. Recent studies have shown that the HPV-16 E7 gene encodes the major transforming activity of the virus in baby rat kidney (BRK) cell transformation assays. To investigate the requirement for continued E7 expression in BRK cells transformed by HPV-16 E7 plus EJ-*ras*, we have developed a system for inducible expression of the E7 gene. The studies reported here show that continued expression of the HPV-16 E7 gene is required for maintenance of the transformed phenotype in these cells. The implications these observations bear on the role of the E7 gene in cervical carcinoma are discussed.

Key words: E7/human papillomavirus/*ras*/transformation

Introduction

The close association between human papillomaviruses (HPVs) and cervical cancer suggests a role for this virus in the development of cervical neoplasia. Over 90% of cervical cancer biopsies contain HPV DNA, of which type 16 is the most common (Durst *et al.*, 1983; Gissmann *et al.*, 1983). In most cases the viral DNA is integrated into the cellular genome, and integration is often accompanied by deletion of large regions of the viral genome (Schwarz *et al.*, 1985; Schneider-Gadicke and Schwarz, 1986; Spence *et al.*, 1988). However, the early viral gene region corresponding to E6 and E7 is retained both in cervical cancer biopsies and in derived cell lines (Schneider-Gadicke and Schwarz, 1986; Spence *et al.*, 1988). In addition, there is also continued expression of the E6 and E7 genes, with E6 and E7 specific mRNA (Schwarz *et al.*, 1985; Yee *et al.*, 1985) and protein (Smotkin and Wettstein, 1986; Androphy *et al.*, 1987; Banks *et al.*, 1987) present within cell lines derived from cervical cancers.

The transforming activity of HPV-16 was first observed in mouse 3T3 cells (Tsunokawa *et al.*, 1986; Yasumoto *et al.*, 1986; Matlashewski *et al.*, 1987a). It was initially thought to be similar to the activity of bovine papillomavirus (Nakabayashi *et al.*, 1983; Sarver *et al.*, 1984; Schiller *et al.*, 1984; Yang *et al.*, 1985) and a role for the E6 gene was therefore suspected. Several groups have shown that HPV DNA is capable of causing transformation of much more recalcitrant human cells, both of fibroblast and epithelial cell origin (Pirisi *et al.*, 1987; Kaur and McDougall, 1988; Matlashewski *et al.*, 1988). It has also been shown that the E6/E7 region of the HPV-16 genome could cooperate with EJ-*ras* in the transformation of primary baby rat kidney (BRK) epithelial cells (Matlashewski *et al.*, 1987b), demonstrating that factors additional to HPV will obviously be important in development of cervical cancer, as appears to be the case from current epidemiological data (de Villiers *et al.*, 1987). This co-transforming activity has been further localized to the E7 region of the HPV-16 genome (Storey *et al.*, 1988) and clearly places E7 into the establishment or immortalizing class of oncogenes. In addition it is also clear that E7 has similarities with the adenovirus Ela gene with a degree of amino acid sequence homology between the proteins. More importantly, there is functional similarity between Ela and E7 with cooperation with EJ-*ras* in transformation of BRK cells and ability to *trans*-activate the adenovirus E2 promoter (Phelps *et al.*, 1988).

From these studies it is clear that the E7 gene encodes the major transforming activity of HPV-16. In all HPV-16 DNA-containing transformed cells analysed there is continued expression of the HPV-16 E7 protein (Smotkin and Wettstein, 1986; Banks and Crawford, 1988). It is therefore crucial to determine whether there is a continued requirement for E7 expression in HPV-16-transformed cells or whether E7 behaves in a 'hit and run' fashion and, after the initial transforming event, is no longer required. The answer to this question would clearly influence our perception of the role of E7 in cell transformation and affect future studies directed towards prevention and treatment of cervical cancer.

To investigate further the mode of action of HPV-16 E7 in cell transformation, we developed a system of inducible expression of the E7 protein in eukaryotic cells based on the ability of the mouse mammary tumour virus long terminal repeat (MMTV-LTR) to respond to glucocorticoid hormones (Huang *et al.*, 1981). This provides a mechanism for hormone-inducible transformation of primary BRK cells. Our results show conclusively that there is an absolute requirement for continued E7 expression for maintenance of the transformed phenotype in cells which have been transformed by HPV-16 E7 plus EJ-*ras*.

Results

Inducible transformation by HPV-16 E7

Transfection experiments were performed using HPV-16 E7 with MMTV-LTR hormone-inducible transcriptional units regulating the expression of the HPV sequences. As shown in Figure 1, the HPV-16 1.9-kb *Nsi*I fragment containing the E7 open reading frame (ORF) was transferred from pJ4Ω16.E7, the construction of which has been described previously (Storey *et al.*, 1988), and ligated into the *Bam*HI sites of the pJ5Ω and pJ5ΩE vectors, the resulting constructs being termed pJ5Ω.16E7 and pJ5ΩE.16E7 respectively. The

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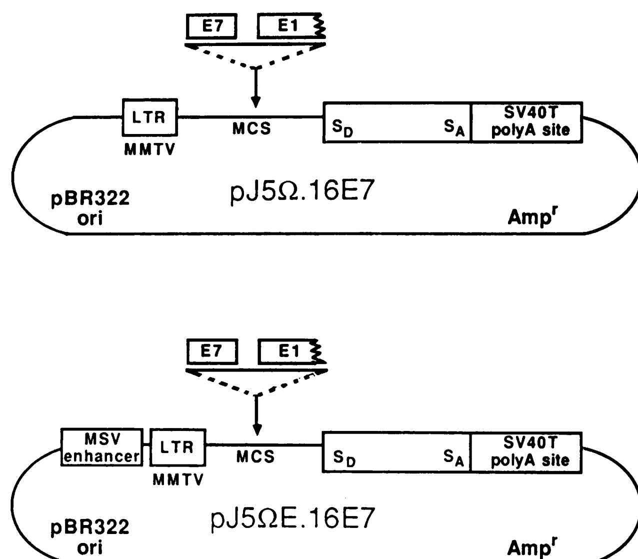

Fig. 1. Structure of HPV-16 E7 expression plasmids. HPV ORFs present are indicated by open bars. MMTV-LTR, MSV enhancer, ampicillin resistance marker, multiple cloning sites (MCS) and splice donor (SD) and splice acceptor (SA) sites are shown. The diagram is not to scale.

Table I. Glucocorticoid-dependent transformation of primary BRK cells

Transfected plasmid	No. of foci with G418 selection						No. of foci without selection			
	−Dex			+Dex			−Dex		+Dex	
	1	2	3	1	2	3	1	2	1	2
pJ5Ω	0	0	0	0	0	0	0	0	0	0
pJ5ΩE	0	0	0	0	0	0	0	0	0	0
pJ5Ω.16E7	0	1	0	31	94	26	0	0	9	13
pJ5ΩE.16E7	8	13	ND	100+	100+	ND	2	4	24	28

Each experiment contained, in addition to the indicated plasmid, pSV2-*neo* and pEJ6.6 containing the activated Ha-*ras* oncogene isolated from the EJ/T24 human bladder carcinoma cell line. The number of foci indicated are those obtained from a single 90 mm dish of primary sub-confluent BRK cells transfected with 5 μg of each plasmid and grown in the presence (+Dex) or absence (−Dex) of dexamethasone. No foci were observed if pEJ6.6 was omitted from the experiment. ND indicates not done.

E7-containing plasmids were transfected into BRK cells alone or together with activated EJ-*ras* and pSV2-*neo* plasmid (Southern and Berg, 1982). Following transfection cells were placed under G418 selection and grown either in the presence or absence of 10^{-6} M dexamethasone. Transformation was monitored by the appearance of dense colonies 3–4 weeks post-transfection and the typical appearance of the plates is shown in Figure 2. The results of several repeated experiments are summarized in Table I. Although numbers vary between experiments, efficient co-transformation with E7 and EJ-*ras* was observed when cells were grown in the presence of hormone. No colonies were ever seen when E7 or EJ-*ras* were transfected alone. In contrast, co-transfections of E7 and EJ-*ras* where cells were grown in the absence of hormone gave drastically reduced levels of transformation, with pJ5Ω.16E7 plus EJ-*ras* routinely failing to produce transformed colonies. Transfection experiments performed without G418 selection gave equivalent results, although with a reduced number of transformed foci. When co-transfected with EJ-*ras* plasmid pJ5ΩE.16E7 gave a greater number of transformed colonies in the presence of dexamethasone, although generally a few colonies were also observed in the absence of dexamethasone. This background level with pJ5ΩE.16E7 is probably due to the MSV enhancer in the pJ5ΩE vector which, although enhancing the expression from the MMTV-LTR, increases the level of transcription in the absence of hormone. For this reason all further experiments were done on cells which were derived using the pJ5Ω.16E7 construct. At this stage four transformed colonies were cloned from the plates and grown out in the presence of hormone. One of these clones, termed 14/2, was then characterized in more detail.

Requirement for continued E7 expression in transformed cells

To investigate the requirement for continued E7 expression in the transformed BRK cells the growth rate of the 14/2 cell line in the presence and absence of dexamethasone was determined. Cells were grown to confluency in the presence of 10^{-6} M dexamethasone and then placed in hormone-free medium for 48 h. The cells were then plated out with 10^{-6} M dexamethasone or without dexamethasone. The growth of the 14/2 cells was then measured over a period of 5 days (Figure 3A). Clearly cells grown in the presence of hormone doubled in number every ~24 h, but the same cells grown in the absence of hormone showed no sign of growth over the 5-day time period. Similar results were also obtained on the remaining three pJ5Ω.16E7/EJ-*ras*-transformed BRK cell clones analysed (data not shown). In comparison, BRK cells transformed by adenovirus Ela gene and EJ-*ras* grew well in both the presence and absence of hormone (Figure 3B). This experiment was repeated on 14/2 cells after 5 months continual passage and the same hormone dependency had been retained (data not shown).

Further analysis was then made of the ability to recover of the cells starved of hormone. After 2 or 10 days in hormone-free medium, 14/2 cells were trypsinized and replated in medium containing 10^{-6} M dexamethasone and their ability to resume growth monitored for a further 5 days. After 2 days of hormone withdrawal the 14/2 cells grew well on re-addition of dexamethasone. Analysis of cells at later times revealed a progressive decrease in the ability of cells to grow upon addition of hormone. After 10 days the cells did not recover and showed no sign of growth after re-addition of hormone (Figure 3C). Since E7 is under the control of the hormone-inducible promoter this implies that there is a continued requirement for E7 expression for maintenance of the transformed phenotype of 14/2 cells.

To further investigate the requirement of E7 for maintenance of the transformed phenotype, the ability of 14/2 cells to grow in different concentrations of dexamethasone was analysed. As before, cells were starved of hormone for 48 h and growth monitored following re-addition of dexamethasone at different concentrations. The results obtained (Figure 3D) show the dose dependence of the cells to dexamethasone. Normal growth is observed at 10^{-6} and 10^{-7} M dexamethasone, but at lower concentrations there is a marked decrease in the ability of the cells to grow. This demonstrates that there is a threshold of E7 expression below which the growth rate of the cells is markedly reduced.

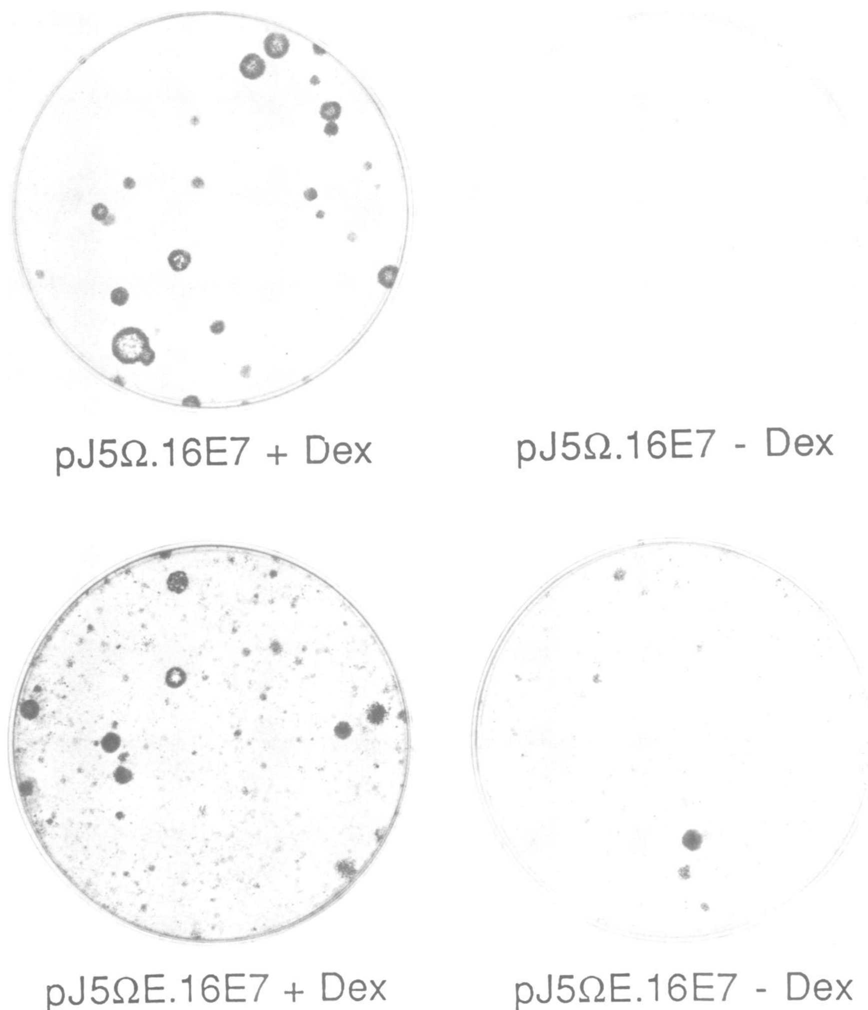

Fig. 2. Transformation of primary BRK cells by HPV-16 E7 DNA. All dishes were transfected with pSV2-*neo*, pEJ6.6 (containing the Ha-*ras* oncogene isolated from the EJ/T24 human bladder carcinoma cell line) and the indicated plasmid in each case. Cultures were grown in the presence of 200 μg/ml G418 and with (+Dex) or without (−Dex) 10^{-6} M dexamethasone as shown. Dishes were fixed and stained 3 weeks after transfection.

Analysis of HPV-16 E7 expression in transformed BRK cells

To correlate E7 expression levels with the 14/2 phenotype, a series of Western blots were done on 14/2 cells grown in the presence or absence of hormone. Cells were grown to confluency and then starved of hormone. After 48 h 10^{-6} M dexamethasone was returned to half of the cells which were then harvested over a range of time points. Cells were extracted with E7 extraction buffer and then immunoprecipitated with a rabbit anti-E7 polyclonal antibody (Smotkin and Wettstein, 1986). The precipitates were then run on PAGE gels, Western blotted onto nitrocellulose and E7 protein detected using a mouse monoclonal antibody specific for HPV-16 E7 (Oltersdorf *et al.*, 1987). The results obtained are shown in Figure 4A. Clearly E7 protein expression is strictly controlled from the pJ5Ω.16E7 construct. No protein is detectable in the absence of hormone but E7 protein is apparent 2 h after the addition of hormone. The level of E7 protein in the presence of hormone rises steadily up to 4−6 h and then reaches a plateau.

From the results in Figure 3D it is apparent that 14/2 cells have increased growth rates with increasing concentrations of hormone and we wished to see if this could be correlated with E7 expression levels. Cells were grown to subconfluency and then starved of hormone for 48 h, after which time different concentrations of dexamethasone were added to the cells for 24 h. The cells were then harvested and analysed for E7 protein by Western blotting as described above. The results obtained are shown in Figure 4B. No E7 protein is detectable with cells grown in 10^{-10} or 10^{-9} M dexamethasone, although E7 does become detectable at 10^{-8} M dexamethasone. Increased levels of E7 protein are observed at 10^{-7} and 10^{-6} M dexamethasone. Clearly the levels of E7 protein expressed correlate well with the observed growth rates of 14/2 cells shown in Figure 3D.

The above results show that E7 protein does not accumulate to a detectable level within 14/2 cells in the absence of hormone. We wished to address the possibility that E7 protein may still be being expressed albeit at low levels. Thus a series of immunoprecipitations were done on 14/2 cells radiolabelled with [^{35}S]cysteine using anti-E7 antibody. The results obtained (Figure 5A) confirmed the results obtained by Western blotting. HPV-16 E7 is clearly being synthesized in cells grown in the presence of hormone but no radiolabelled E7 protein is detectable in cells grown in the absence of hormone. Thus in addition to not being

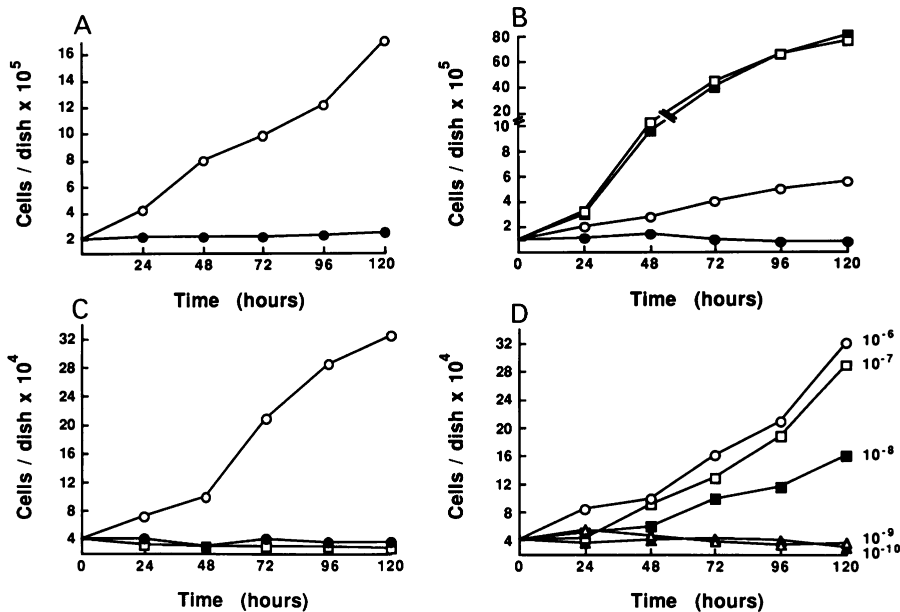

Fig. 3. Growth response of 14/2 cells in dexamethasone. Cells were placed in hormone-free medium for 48 h and then further growth was monitored. **Panel A**, growth rate of cells in presence of 10^{-6} M dexamethasone (○ – ○); growth rate of cells in dexamethasone-free medium (● – ●). **Panel B**, growth rate of BRK cells transformed by adenovirus Ela and EJ-*ras* in 10^{-6} M dexamethasone (□ – □) or dexamethasone-free medium (■ – ■). 14/2 cells in 10^{-6} M dexamethasone (○ – ○) or dexamethasone-free medium (● – ●). **Panel C**, growth rate of 14/2 cells placed in dexamethasone-free medium for 10 days after re-addition of 10^{-6} M dexamethasone (□ – □). 14/2 cells in 10^{-6} M dexamethasone (○ – ○) or dexamethasone-free medium (● – ●). **Panel D**, growth response of 14/2 cells to different concentrations of dexamethasone: 10^{-6} M (○–○), 10^{-7} M (□–□), 10^{-8} M (■–■), 10^{-9} M (Δ–Δ) and 10^{-10} M (▲–▲).

able to accumulate high levels of E7 protein, 14/2 cells in the absence of hormone show no detectable synthesis of the E7 protein.

It could be argued that removal of hormone may affect EJ-*ras* expression in the 14/2 cells and this may have been responsible for the effects upon the transformed state of the cells. To investigate this further, a series of immunoprecipitations was carried out on transformed BRK cells grown in the presence or absence of hormone using a rat monclonal antibody specific for v-H-*ras* protein (Oncogene Science Inc.). As for E7, confluent cells were starved of hormone for 48 h and then 10^{-6} M dexamethasone added to half of the cells as described above for E7. After 12 h methionine-free medium containing 500 μCi [^{35}S]methionine was added to the plates and then left for 18 h. Cells were harvested and extracted in *ras* extraction buffer and immunoprecipitated with anti-*ras* monoclonal antibody. The results shown in Figure 5B demonstrate that the hormone has no significant effect upon *ras* protein expression in 14/2 cells and this is also true for cells transformed by Ela plus EJ-*ras*.

Discussion

The data presented here argue strongly that continued expression of HPV-16 E7 is needed in BRK cells transformed by HPV-16 E7 plus EJ-*ras* for the maintenance of the transformed phenotype. This shows that the HPV-16 E7 gene is required for both the establishment and maintenance of the transformed phenotype. Clearly epidemiological data on cervical carcinomas all indicate that exposure to other agents is needed before papillomavirus induced lesions can progress to become carcinomas. The demonstration that E7 can cooperate with EJ-*ras* in the transformation of primary BRK cells (Phelps *et al.*, 1988; Storey *et al.*, 1988) suggests a route by which oncogenic transformation could occur *in*

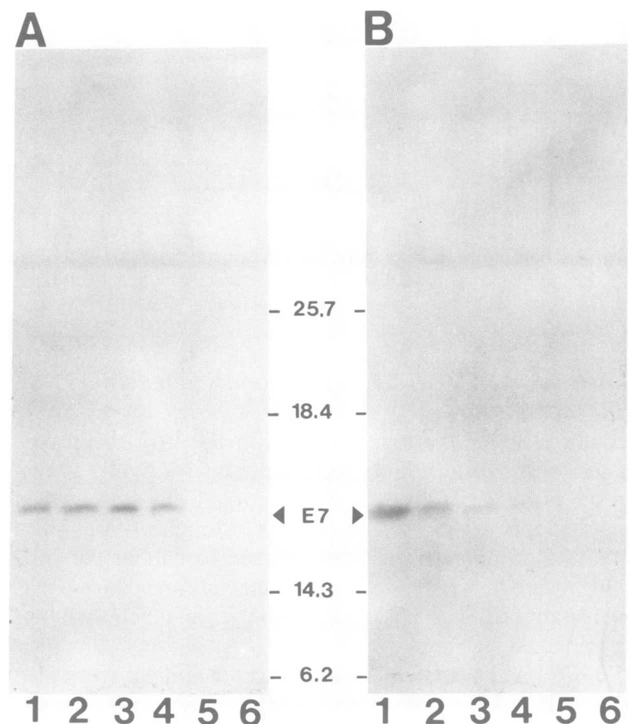

Fig. 4. HPV-16 E7 protein expression in 14/2 cells. **Panel A**, cells were placed in dexamethasone-free medium for 48 h and expression of E7 protein monitored at different times after addition of 10^{-6} M dexamethasone using E7IV monoclonal antibody (Oltersdorf *et al.*, 1987) followed by immunoperoxidase detection. Times following 10^{-6} M dexamethasone addition are: 0 h (**lane 6**), 1 h (**lane 5**), 2 h (**lane 4**), 4 h (**lane 3**), 6 h (**lane 2**) and 8 h (**lane 1**). **Panel B**, levels of E7 protein expression in 14/2 cells at different concentrations of dexamethasone: 10^{-10} M (**lane 6**), 10^{-9} M (**lane 5**), 10^{-8} M (**lane 4**), 10^{-7} M (**lane 3**) and 10^{-6} M (**lane 2**); control CaSki cells (**lane 1**). Arrows indicate position of the E7 protein. Molecular weight markers are; bovine trypsin inhibitor (6.2 kd), lysozyme (14.3 kd), β-lactoglobulin (18.4 kd) and α-chymotrypsinogen (25.7 kd).

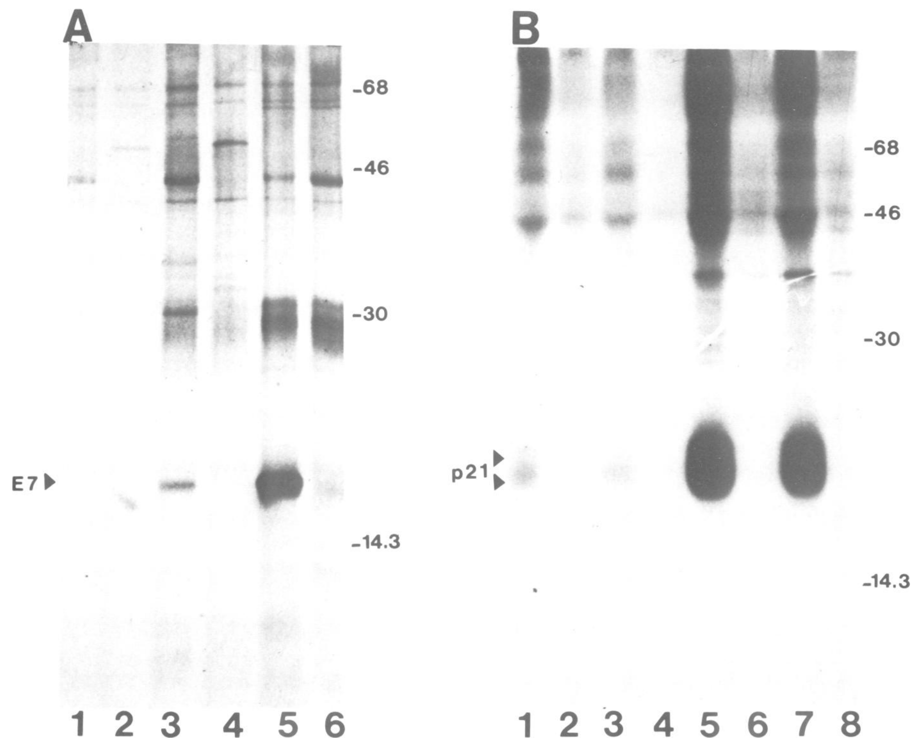

Fig. 5. Immunoprecipitation analysis of E7 and EJ-*ras* expression. **Panel A**, cells (14/2, **lanes 1–4**; CaSki, **lanes 5** and **6**) were labelled with [^{35}S]cysteine as described in Materials and methods and proteins were immunoprecipitated with a rabbit anti-E7 polyclonal serum (**lanes 1, 3** and **5**) or a rabbit pre-immune serum (**lanes 2, 4** and **6**). 14/2 cells were grown in either 10^{-6} M dexamethasone (**lanes 3** and **4**) or dexamethasone-free medium (**lanes 1** and **2**). **Panel B**, cells (E1a/EJ-*ras* transformed BRK,. **lanes 1–4**; 14/2, **lanes 5–8**) were labelled with [^{35}S]methionine as described in Materials and methods and proteins immunoprecipitated with a rat anti-H-*ras* monoclonal antibody (**lanes 1, 3, 5** and **7**) or a rat pre-immune serum (**lanes 2, 4, 6** and **8**). Cells were grown in either 10^{-6} M dexamethasone (**lanes 1, 2, 5** and **6**) or dexamethasone free medium (**lanes 3, 4, 7** and **8**). E7 protein and *ras* p21 proteins are shown by arrows. Molecular weight markers are: lysozyme (14.3 kd), carbonic anhydrase (30 kd), ovalbumin (46 kd) and bovine serum albumin (69 kd).

vivo. The use of this co-transformation system, which must be more similar to cells transformed *in vivo* by HPV than assays using established cell lines, to show the continued requirement for the E7 gene lends further weight to its relevance *in vivo*.

We have now developed a system for the inducible expression of the HPV-16 E7 protein in eukaryotic cells. This makes use of the hormone inducibility of the MMTV-LTR regulatory element (Huang *et al.*, 1981) and clearly pJ5Ω and pJ5ΩE have considerable potential for further studies on the function of HPV gene products and also in many other systems. The use of these vectors for inducible expression of the HPV-16 E7 protein has enabled us to obtain hormone-inducible transformation of primary BRK cells. These cells share the same characteristics as BRK cells transformed with HPV-16 E7 plus EJ-*ras* where the E7 gene is constitutively expressed, except that with pJ5Ω.16E7 we have the ability to turn off the E7 expression. This means that we can obtain rapid clearance of E7 from the cells once expression is turned off due to its relatively short half-life of ~1 h (Smotkin and Wettstein, 1986). Clearly equivalent studies performed in human keratinocytes would be useful; however, this would tend to preclude further analysis on the tumorigenicity of these cells.

Having developed a system for the inducible expression of HPV-16 E7 gene, we could then study the function of this transforming gene. Of crucial importance from these studies is the observation that the E7 gene needs to be continually expressed in the transformed BRK cells for those cells to remain transformed. Clearly in cells transformed by HPV-16 E7 plus EJ-*ras* loss of the E7 protein results in a cessation of cell growth. Throughout these studies we have been able to show hormone responsiveness at the level of E7 protein expression. On removal of hormone, no E7 was detectable in either immunoprecipitations or Western blots. In contrast, assay of *ras* protein levels at the same time failed to demonstrate any significant change in expression of this oncogene. This further supports the conclusion that it is the removal of E7 which results in the reversal of the transformed phenotype.

A further interesting point from these studies is the extent to which the transformed phenotype can be recovered after removal of hormone. After 2 days without E7 expression the cells can still respond to re-addition of hormone, but after 10 days without E7 expression re-addition of hormone has no effect and the cells remain quiescent. Keeping the cells without hormone for periods >14 days results in cells dying and lifting off the plates.

The mechanism by which E7 exerts its action remains unclear, but the observation that E7 can *trans*-activate the adenovirus E2 promoter (Phelps *et al.*, 1988) suggests that one effect of E7 may be at the level of transcription. The results presented here would support the proposition that E7 is required for activation of a cellular gene product needed for cell division. Removal of E7 protein would result in a reduced level of *trans*-activation of this cellular product with a concomitant reduction in cell growth. Alternatively, since the E7 protein possesses homology with the E1a gene product

in conserved regions 1 and 2 but not conserved region 3 (Lillie *et al.*, 1986; Moran *et al.*, 1986; Schneider *et al.*, 1987), this suggests that a mechanism of action of the E7 protein may be via repression rather than *trans*-activation of transcription of cellular genes, since the former phenotypic effect maps to CR1 and CR2. The ability of Ela to cooperate with an activated *ras* gene in the transformation of primary cultures also maps within CR1 and CR2. These conserved sequences, also found in other viral and cellular oncogenes, have recently been implicated in the interaction of oncogene proteins with the retinoblastoma 'anti-oncogene' protein (Whyte *et al.*, 1988). It is possible that E7 has convergently evolved to exploit the same mechanism of inducing cellular proliferation employed by other viral oncogenes. It now remains to be determined which cellular genes are affected by E7 and studies are now currently underway using the inducible system described here to investigate this further.

The importance of the data presented here with reference to the association between HPV and cervical cancer is very significant. In all cell lines derived from cervical tumours which have been shown to contain HPV DNA the E7 gene is retained (Schwarz *et al.*, 1985; Yee *et al.*, 1985; Spence *et al.*, 1988), and where assays have been possible protein is also expressed (Smotkin and Wettstein, 1986; Oltersdorf *et al.*, 1987). The presence of the HPV-16 E7-transforming protein within these cells many years after the initial transforming event suggests that at least for some of this time the E7 protein was required to confer a growth advantage upon the transformed cells. Our results here show that the E7 protein is required to maintain the transformed phenotype of cells transformed *in vitro* by HPV-16 E7 plus EJ-*ras*. Thus, in addition to being able to place E7 in the establishment class of oncogenes, we can also say E7 is required for maintenance. This offers the exciting prospect that inhibition of E7 function *in vivo* may provide a means of reducing the transforming potential of HPV-16 and reverse the phenotype of cells transformed by this protein.

Materials and methods

Construction of the HPV-16 E7 expression plasmids

The HPV-16 DNA was kindly provided for us by Prof. H.zur Hausen. The HPV-16 1.9 kb *Nsi*I fragment containing the E7 ORF was cloned as described previously (Storey *et al.*, 1988) and ligated into the *Bam*HI sites of pJ5Ω and pJ5ΩE.

Transfection and selection

Cultures of primary BRK cells were prepared and transfected by the DNA calcium phosphate co-precipitation method (Wigler *et al.*, 1979). Aliquots of DNA calcium phosphate precipitate (0.4 ml) containing 5 μg of each of the indicated plasmids was added to 90 mm dishes of sub-confluent primary BRK cells. After glycerol treatment the cells were cultured in Dulbecco's modified Eagle's medium containing 10% foetal calf serum and 200 μg/ml G418. Three weeks after transfection colonies were either isolated with cloning rings and propagated as cell lines, or the dishes were fixed in normal saline and stained with Giemsa stain.

Immunoblotting

For analysis of E7 protein synthesized in cell lines a 90 mm plate of confluent cells was lysed using 200 μl of E7 extraction buffer (250 mM NaCl, 0.1% NP40, 50 mM Hepes, pH 7.0, 1% Aprotinin). After 30 min on ice the cells were scraped off the plates and debris removed by centrifugation at 5000 r.p.m. for 2 min. Supernatant was removed and E7 protein precipitated with an anti-E7 rabbit polyclonal serum (Smotkin and Wettstein, 1986). Protein A-Sepharose (Pharmacia) was added and incubated at 4°C for 1 h with rocking. The proteins bound to the beads were then separated on 15% PAGE gels and then electrophoretically transferred to nitrocellulose. E7 was then visualized with a mouse monoclonal antibody specific for HPV-16 E7 protein (Oltersdorf *et al.*, 1987) as described previously (Storey *et al.*, 1988).

Immunoprecipitation

For E7 protein detection cells were labelled with 1 mCi [^{35}S]cysteine per 90 mm dish for 1 h. Cells were lysed as above, then the proteins were immunoprecipitated with anti-E7 antisera and analysed by PAGE followed by fluorography (Bonner and Laskey, 1974).

For H-*ras* protein detection cells were labelled with 500 μCi [^{35}S]methionine per 90 mm dish for 18 h. Cells were lysed in 1 ml *ras* extraction buffer (20 mM Tris−HCl, pH 7.4, 10 mM $MgCl_2$, 100 mM NaCl, 1% Triton X−100, 0.5% sodium deoxycholate, 1% Aprotinin). Protein was immunoprecipitated using AB-2 rat monoclonal antibody (Oncogene Science, Inc.). Proteins were analysed by PAGE as described above.

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