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Comparison of the *in vitro* transforming activities of human papillomavirus types

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The association of certain human papillomavirus (HPV) types with the majority of human cervical carcinomas suggests a role for the virus in the development of this type of cancer. In this paper, we have examined the transforming properties of several HPV types where the early region genes of the virus are under the control of a strong heterologous promoter and show that major differences exist between the HPV types in their ability to transform primary rat kidney epithelial cells in conjunction with an activated *ras* oncogene. Those HPV types most commonly found in carcinomas—types 16, 18, 31 and 33—are capable of co-operating with *ras* to transform primary cells, but those types most commonly found in benign lesions—types 6 and 11—are not. We further demonstrate that the E7 gene of HPV16 by itself is sufficient to co-operate with activated *ras* to produce transformed cells which are tumorigenic in immunocompetent animals.

Key words: transformation/human papillomavirus/E6/E7

Introduction

One of the most striking differences in the natural history of the human genital papillomavirus infection is in the prognosis of lesions containing human papillomavirus (HPV) type 6 or 11 DNA on the one hand and those containing HPV16, 18, 31 and 33 DNA on the other. HPV6 and HPV11 DNAs are frequently found in genital warts and mild dysplasias of the uterine cervix, but rarely in severe dysplasias or malignant carcinomas. The other types—16, 18, 31 and 33—are rarely found in condylomas or mild dysplasias, but their DNAs are found in most severe dysplasias and malignant carcinomas (Dürst *et al.*, 1983; Gissman *et al.*, 1983).

This suggests very strongly that there must be some significant differences between the genes of the various types in the way that infected cells respond to them. HPV6 and HPV11 are relatively closely related, showing >60% homology throughout their genomes (Broker and Chow, 1986). HPV6 is less closely related to HPV18 and types 16 and 18 are not closely related to each other (Boshart *et al.*, 1984). The closest homology between HPV types is found in the L1 open reading frame, corresponding to the major capsid protein (Broker and Chow, 1986) but this is unlikely to be involved in cellular transformation. The different probabilities that HPV types exhibit of inducing malignant transformation and carcinomas, as opposed to the production of benign papillomas, suggests that DNA sequence differences exist between the various HPV types which are important in determining the transforming potential of an HPV type.

In this paper we have used an *in vitro* co-transformation assay (Matlashewski *et al.*, 1987a) to examine the biological activities of HPV16, HPV18, HPV31, HPV33, HPV6 and HPV11. The advantages of this co-transformation assay using primary baby rat kidney (BRK) epithelial cells are threefold. Firstly, the cells are epithelial rather than fibroblastic and, since true papillomaviruses *in vivo* grow only in epithelial cells, this is a much more appropriate cell type. Secondly, the cultures are primary cells rather than established cell lines and are therefore presumed to be normal when placed in culture. Thirdly, the system shows a requirement for two oncogenes or transforming agents, one from the establishment class and one from the transforming class. It is similar in this respect to the primary rat cell system which has been studied extensively (Land *et al.*, 1983; Rassoulzadegan *et al.*, 1983; Ruley, 1983). The requirement for more than one transforming event parallels the situation *in vivo*, where it is clear that papillomavirus infection alone is not sufficient to generate malignantly transformed cells. A second agent such as smoking, X-rays or chemical carcinogen is probably needed to allow the development of a high-grade lesion into a carcinoma. In our previous study we showed that, in conjunction with an activated *ras* oncogene, the DNA of HPV16 under control of a strong heterologous promoter transformed primary rat kidney epithelial cells. This activity requires a protein or proteins from the E6/E7 region of the HPV16 genome. We have now refined this localization and show that the relevant protein is likely to be E7. We then went on to examine other papillomavirus DNAs and show that there are striking differences between the various HPV types in their transforming activity.

Results

E7 is the transforming gene of HPV16

Transfection experiments using HPV16 DNA cloned into a pZIP-NeoSV(X)1 vector (Cepko *et al.*, 1984) forming the plasmids HZIP-16 and HPV16K (Matlashewski *et al.*, 1987a), demonstrated that HPV16 contains one or more oncogenes, derived from the E6/E7 region of the genome, which could co-operate with an activated *ras* gene to transform primary cells (Matlashewski *et al.*, 1987a). We first set up experiments to determine whether the transforming activity resides in E6 or E7 or whether both were required. Using an expression vector similar to pZIP-Neo, pJ4Ω (Wilkinson *et al.*, 1988), we cloned the E6 or E7 open reading frames (ORFs), either separately or together, and tested their transforming ability relative to pJ4Ω16 which contains most of the HPV16 genome (Figure 1). The plasmid pJ4Ω16K contains both the E6 and E7 genes on a DNA fragment derived from the deletion mutant HPVK-16 (Matlashewski *et al.*, 1987a). A plasmid containing the E6

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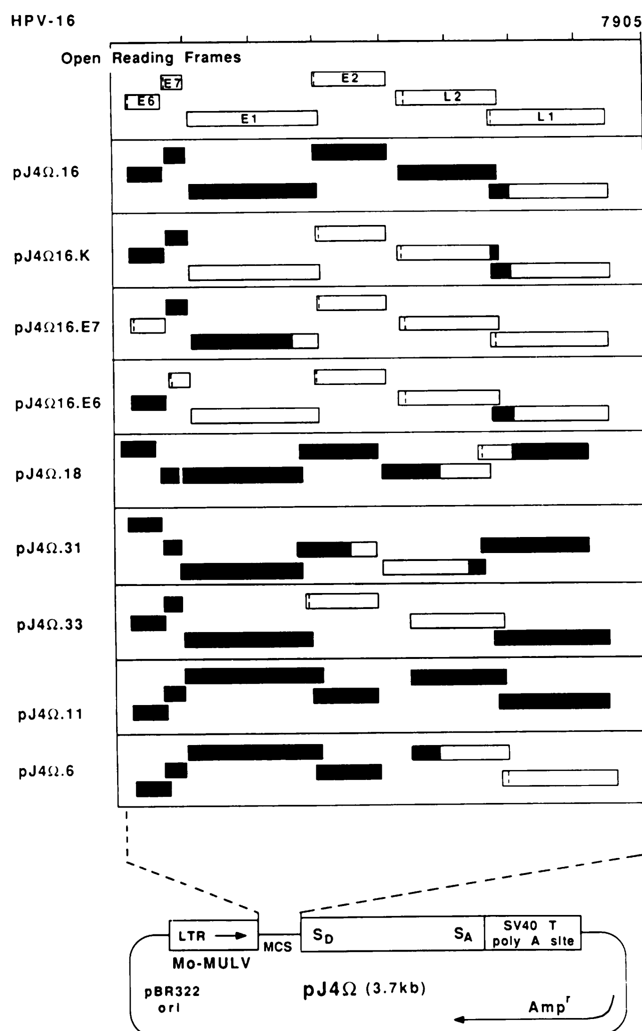

Fig. 1. Structure of recombinant HPV DNA containing plasmids. HPV ORFs present are indicated by solid bars. Mo-MuLV LTR, ampicillin resistance marker, multiple cloning sites (MCS) and splice donor (SD) and splice acceptor (SA) sites are indicated. The diagram is not to scale. The locations of the HPV ORFs are taken from Schwarz *et al.* (1983), (HPV6); Dartmann *et al.* (1986), (HPV11); Seedorf *et al.* (1985), (HPV16); Cole and Danos (1987), (HPV18); Lorincz *et al.* (1986), (HPV31); Beaudenon *et al.* (1986), (HPV33).

gene alone was constructed from a pAT153 vector (Twigg and Sherratt, 1980) containing HPV16 DNA inserted at the *Bam*HI site. This plasmid was deleted between the *Pvu*II site at bp 551 and the *Nco*I site at bp 863, and also between the two *Kpn*I sites at bp 880 and 5377. This deleted plasmid was restricted with *Eco*RI and *Bam*HI and a 1.77-kb fragment was inserted into pJ4Ω. This plasmid, however, contained the E6 ORF which was in the wrong orientation with respect to expression from the LTR promoter. The fragment was correctly orientated by cutting with *Bam*HI, end repairing, then cutting with *Eco*RI. The resulting E6 ORF containing fragment was inserted into a *Bgl*II-cut, end-repaired, *Eco*RI-cut pJ4Ω to form the fragment pJ4Ω16.E6 (Figure 1). This plasmid codes for the entire E6 ORF, except for the C-terminal amino acid leucine which is replaced by His-Gly followed by a stop codon. To generate the E7 vector, a 1.9-kb *Nsi*I fragment containing the E7 ORF was linker adapted such that both *Nsi*I sites were regenerated and both *Bam*HI and *Eco*RI sites were added at either end. This fragment was inserted into the *Eco*RI site of pAT153. This plasmid was restricted with *Bam*HI and the E7 fragment inserted into pJ4Ω to form the plasmid pJ4Ω16.E7. It contains the E7 ORF with virtually no upstream sequence and with 1.6 kb of 3′ sequence beyond the E7 stop codon (Figure 1).

These HPV16 DNA containing plasmids were transfected into BRK cells with or without an activated EJ-*ras* gene. The plasmid pSV2-neo (Southern and Berg, 1982) was included in all transfection experiments. After 24 h the cells were placed under G418 selection. Transformation was monitored by the appearance of foci after 3 weeks (Figure 2). Neither pSV2-neo nor EJ-*ras* by themselves formed foci.

As shown in Table I, the activity of the plasmid pJ4Ω16K localizes the transforming activity of HPV16 to the E6/E7 region, confirming the previous result with HPVK-16 which contains the same sequences in the pZIP-NeoSV(X)1 vector. No foci were ever observed in experiments using pJ4Ω16.E6. However, when pJ4Ω16.E7 was co-transfected with EJ-*ras* the number of foci formed was about the same as that formed with pJ4Ω16. This shows that the E7 gene alone is capable of co-transforming primary cells. In conjunction with our previous results it shows that E7 is both necessary and sufficient for transformation in this assay. The cells produced by transfection with pJ4Ω16.E7 are morphologically altered, growing quickly to high density and showing no signs of senescence after >4 months. The numbers of foci obtained using pJ4Ω16K were always lower than those obtained when either pJ4Ω16 or pJ4Ω16.E7 are used (Table I). However, when pJ4Ω16.E6 and pJ4Ω16.E7 were transfected together, similar numbers of foci to those generated by pJ4Ω16 were observed. This suggests that the lower number of foci formed by pJ4Ω16K is a function of that particular construct, and that the E6 gene does not inhibit transformation by E7. It is possible that a spliced E6−E7 product from the pJ4Ω16K insert has a negative effect on transformation and that this negative effect is lacking where E6 and E7 are on separate plasmids. To test the transforming activities of E7 genes from HPVs 18, 31 and 33, E7 genes from these types were isolated and cloned into pJ4Ω. For HPV18, a 1.4-kb *Xba*I fragment (bp 321−1735), for HPV31, a 2.0-kb *Hpa*I−*Eco*RV fragment and for HPV33 a 644-bp *Dra*I fragment (bp 470− 1114) were used. Each of these E7 alone constructs co-operated with *ras* as efficiently as constructs containing the entire early region.

The transformed cells from colonies of the pJ4Ω16 and pJ4Ω16.E7 transfections were expanded and analysed for HPV16 DNA by Southern blot analysis (Southern, 1975). Total cellular DNA from these cell lines was digested with *Bam*HI and probed with nick-translated HPV16 DNA. Figure 3 shows that integrated HPV16 DNA was present at high copy number in the co-transfected cell lines. Northern blot analysis of total RNA isolated from transiently infected BRK cells shows that HPV16 early region RNA species were expressed (Figure 4a). These BRK cells express RNA species which range in size from ~2 kb to ~5 kb in length. Western blot analysis of a pJ4Ω16-transformed monoclonal cell line demonstrated the presence of E7 protein in these cells (Figure 4b). Immunoperoxidase staining of either pJ4Ω16- or pJ4Ω16.E7-transformed cells using a monoclonal antibody directed against v-H-*ras* showed overexpression of *ras* in these cells (data not shown).

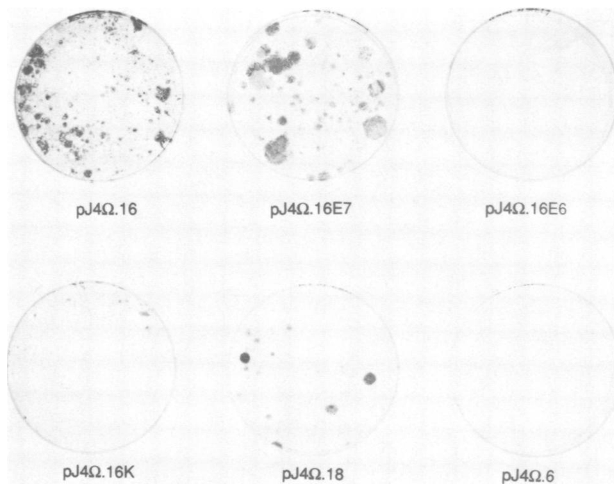

Fig. 2. Transformation of primary BRK cells by HPV DNA. All dishes were transfected with pSV2-neo, pEJ6.6 (containing an activated *ras* oncogene) and the indicated plasmid in each case. Cultures were grown in the presence of 200 μg/ml G418 and stained 3 weeks after transfection.

Table I. Number of foci obtained from plasmid treatment of BRK cells

Plasmid	Experiment		
	1	2	3
pJ4Ω	34	116	38
pJ4Ω16K	7	62	11
pJ4Ω16.E7	15	130	63
pJ4Ω16.E6	0	0	0
pJ4Ω16.E7+pJ4Ω16.E6	24	133	37
pJ4Ω18	17	51	44
pJ4Ω31	16	42	52
pJ4Ω33	27	40	31
pJ4Ω16+pJ4Ω6	30	141	35
pJ4Ω16+pJ4Ω11	27	156	77
pJ4Ω6	0	0	0
pJ4Ω11	0	0	0
pJ4Ω vector alone	0	0	0

Each experiment contained, in addition to the indicated plasmid, pSV2-neo encoding resistance to G418 and pEJ6.6, a plasmid containing the Ha-*ras* oncogene derived from the human bladder carcinoma cell line. The number of foci are those obtained when 5 μg of each plasmid was added to a 90-mm dish of primary sub-confluent BRK cells. No foci were observed if either pSV2-neo or pEJ6.6 were omitted from the experiment.

Co-transformation with other HPV types

To investigate the co-transforming potential of other HPV types, DNA fragments encoding early region ORFs were ligated into pJ4Ω (Figure 1). Constructs were modelled on HZIP-16 as far as possible and contained analogous sequences. HPV6 inserted at the *Bam*HI site of pAT153 was cut with *Bam*HI and *Mlu*I. A 5.2-kb fragment encoding early region genes was isolated and end repaired. This fragment was *Bam*HI linked and cloned into *Bam*HI-cut pJ4Ω. The plasmid pJ4Ω11 was constructed by excising the HPV11 genome with *Bam*HI from the pSV2-neo vector and inserting this fragment into pJ4Ω. The HPV18 genome in pBR322 was cut out with *Eco*RI and recircularized using T4 DNA ligase. The circularized genome was digested with *Kpn*I and a 6.4-kb fragment inserted into pJ4Ω. HPV31 DNA, inserted

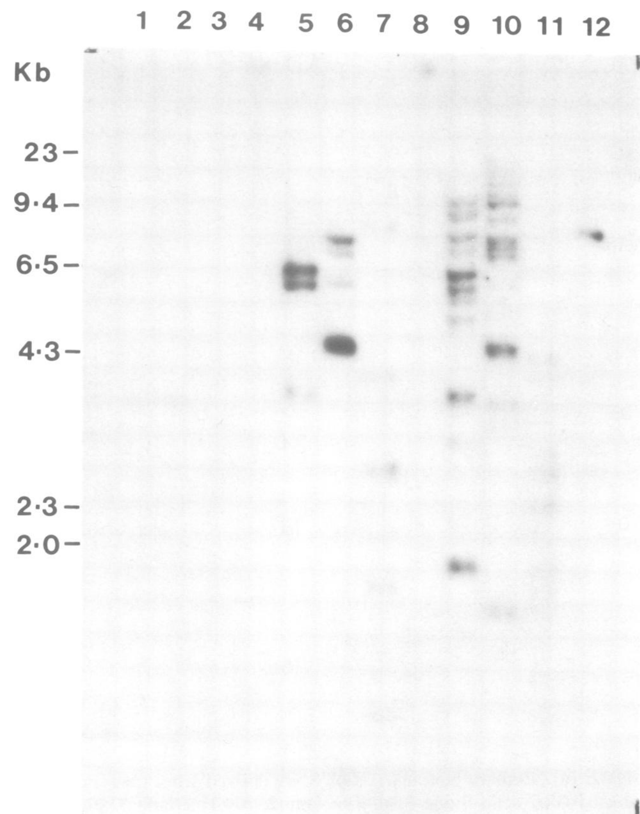

Fig. 3. Southern blot analysis of HPV16 DNA sequences in BRK cells which had been co-transfected with HPV16 DNA, pSV2-neo and the EJ-*ras* oncogene. Each lane contains 10 μg genomic DNA. **Lanes 1–3** contain pJ4Ω digested with *Bam*HI, *Kpn*I and *Pst*I. **Lanes 4–7** contain DNA obtained from a monoclonal cell line derived from a single transformed foci containing pJ4Ω16 (lane 4, uncut DNA; lanes 5–7 are digested with *Bam*HI, *Kpn*I and *Pst*I). **Lanes 8–11** contain DNA isolated from a monoclonal cell line transformed with pJ4Ω16.E7 (lane 8, uncut DNA; lanes 9–11 contain DNA cut with *Bam*HI, *Kpn*I and *Pst*I). **Lane 12** contains 30 pg *Bam*HI cut HPV16 DNA, equivalent to one copy of HPV DNA per cell.

at the *Eco*RI site of pBR322, was cut with *Eco*RI and *Sal*I, yielding a 5.8-kb fragment which was ligated into *Eco*RI/*Sal*I-cut pJ4Ω. HPV33 DNA present in a *Bgl*II site of a modified pBR322 vector was cut with *Hin*dIII and *Bgl*II, giving a 5.6-kb fragment which was cloned into *Hin*dIII/*Bgl*II-cut pJ4Ω. These fragments were transfected into BRK cells as described and placed under G418 selection. The results of these experiments are summarized in Table I. The vectors containing DNA from HPV types 16, 18, 31 and 33 produced similar numbers of transformed foci, whereas no foci were observed with HPV6 or HPV11 constructs. This could have been either because they do not express their early regions or because the proteins expressed lacked transforming activity. To verify that the HPV6 and 11 constructs were expressing early region RNA, BRK cells were transiently infected with pJ4Ω6 and pJ4Ω11. Total cellular RNA was probed with virus-specific early region DNA and compared to RNA isolated from pJ4Ω16-, pJ4Ω16.E7- and pJ4Ω18-transformed BRK cells (Figure 4a). Similar levels of early region transcript were seen in the cells transfected with HPV6, 11 or 16, suggesting that the lack of transforming activity in HPV6 and 11 is not simply due to the absence of early region expression. Furthermore, isolated E7 ORFs

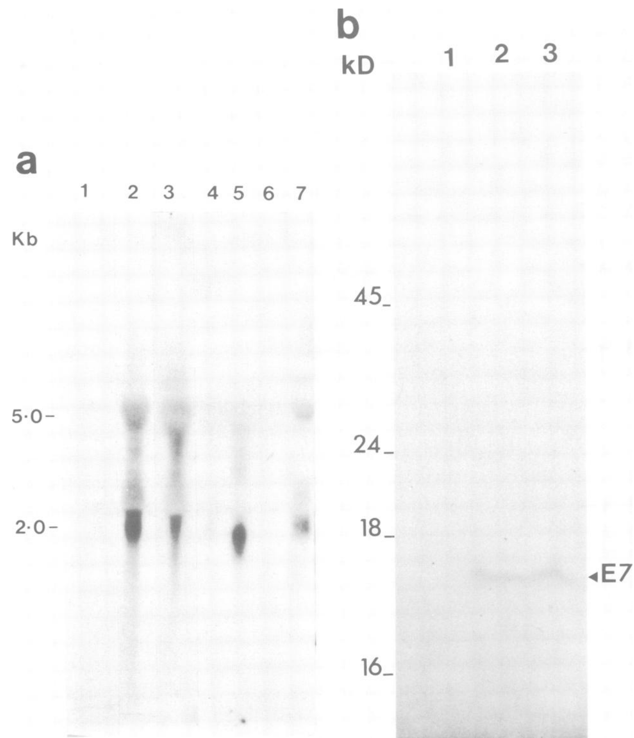

Fig. 4. (**a**) Northern blot analysis of RNA derived from transiently infected BRK cells. **Lane 1**, pJ4Ω6; **lane 2**, pJ4Ω11; **lane 3**, pJ4Ω18; **lane 4**, pJ4Ω16; **lane 5**, pJ4Ω16.E7; **lane 6**, uninfected BRK cells; **lane 7**, HT156, a tumour cell line containing HPV16 DNA. Each lane was probed with a DNA probe corresponding to the regulatory and E6/E7 regions specific for the corresponding HPV type. The probes were: HPV6, a 2.75-kb *Hind*III fragment derived from pJ4Ω6; HPV11, a 2.25-kb *Bam*HI–*Sph*I fragment of genome DNA; HPV16, the 2.1-kb *Bam*HI insert of pJ4Ω16K (nucleotides 5377–6150 and 7454–880); HPV18, a 2.4-kb *Kpn*I–*Taq*I fragment of genome DNA. (**b**) Western blot analysis. Cell extracts of: **lane 1**, untransfected BRK cells; **lane 2**, CaSki cells; **lane 3**, pJ4Ω16 cells were run on a 12% polyacrylamide gel and the proteins transferred to a nitrocellulose filter. The filter was then reacted with the anti-E7 monoclonal antibody E7IV (Oltersdorf *et al.*, 1987) followed by immunoperoxidase detection.

from HPV types 18, 31 and 33 are able to co-operate with *ras* as efficiently as the entire early region of the respective genomes to transform primary BRK cells.

Tumour production

Acquisition of a transformed phenotype *in vitro* is by no means always accompanied by increase in tumorigenic potential. We therefore cloned and tested the cell lines generated by transformation with HPV DNA plus *ras* for their ability to produce tumours in syngeneic rats. When 2×10^6 cells transformed by either pJ4Ω16 or pJ4Ω16.E7 were injected subcutaneously into each flank of three syngeneic rats tumours formed at the site of injection 2–3 weeks later. These tumours were found to be poorly differentiated adenocarcinomas.

Cytokeratin staining

Since the tumours with which HPV types are associated are usually carcinomas, derived from epithelial cells, it was important to show that the cells or cell lines generated by *in vitro* transformation were epithelial in origin rather than fibroblastic. By using specific monoclonal antibodies directed against the cytokeratins characteristic of epithelial cells we were able to demonstrate that the transformed cells were, in all cases, simple epithelial cells. They were stained with monoclonal antibody LE61, specific for keratin 18 (Figure 5). No staining was observed with LP34, which in rat cells stains keratins 5 and 14 but not simple keratins such as keratin 18 (Figure 5) (Lane *et al.*, 1985).

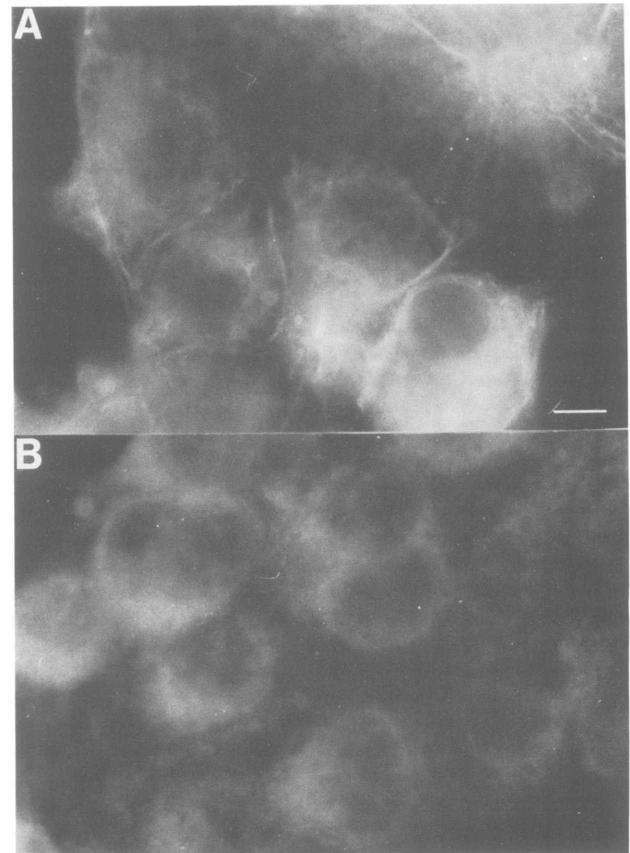

Fig. 5. Immunofluorescence patterns of monoclonal antibody (mAB) staining on methanol fixed BRK cells transformed with pJ4Ω16.E7. **Panel A** was stained with mAB LE61 (keratin 18) and **B** with mAB LP34 (keratins 5 and 14). Scale bar = 2.5 μm.

Discussion

The best-characterized *in vitro* transformation system is the one developed for bovine papillomavirus type 1 (BPV-1). The BPV-1 genome contains at least two separate transforming genes, E5 and E6 (Nakabayashi *et al.*, 1983; Sarver *et al.*, 1984; Schiller *et al.*, 1984; Yang *et al.*, 1985; DiMaio *et al.*, 1986). Transformation of mouse fibroblast cell lines by HPV16 was obtained more recently, using assay systems similar to those used for BPV-1 (Tsunokawa *et al.*, 1986; Yasumoto *et al.*, 1986; Matlashewski *et al.*, 1987b). Subsequently transformation of primary rat epithelial cells was achieved by using HPV16 in conjunction with an activated *ras* oncogene (Matlashewski *et al.*, 1987a). Here deletion analysis indicated that the transforming protein or proteins encoded by HPV16 came from the E6/E7 region of the genome and that none of the other major ORFs was required (Matlashewski *et al.*, 1987a).

We have now shown that the E7 gene by itself co-operates with an activated *ras* gene to transform primary BRK cells. Our conclusion, that HPV16-E7 is both necessary and sufficient for transformation in this assay, is surprising in view

of the results previously obtained with BPV-1. The analysis of BPV-1 transformation which defined E5 and E6 as transforming proteins did not seem to implicate E7. Its function would appear to be related to control of copy number when BPV-1 DNA is replicating episomally in mouse cells, although cells transformed by an E7 mutant were sometimes unstable (Lusky and Botchan, 1985; Berg *et al.*, 1986).

We had expected to find that HPV16-E6 had transforming activity analogous to that of BPV1-E6 and our results with the E6/E7-containing construct HPVK-16 were consistent with a role for E6 (Matlashewski *et al.*, 1987a). However, repeated efforts to obtain transformation with HPV16-E6 in pJ4Ω were uniformly negative, whereas HPV16-E7 always gave transformation. The E7 transforming activity was not antagonized by E6, nor did adding E6 cause a significant increase in E7 transformation. Complex splicing patterns have been observed for the E6/E7 region, generating RNAs which could code for polypeptides made up of parts of E6 and E7 ORFs (Schwarz *et al.*, 1985; Schneider-Gädicke and Schwarz, 1986). We do not have any evidence for a role of any protein derived from, or including part of, the E6 region in our transformation assay, but clearly in other circumstances, e.g. in the absence of a strong heterologous promoter, it may be important to influence the amount of E7 production. The difference between BPV-1 and HPV16 transformation may be related to the use of fibroblasts and epithelial cells respectively in the two assays, or more likely to the fact that co-transformation by HPV16 plus *ras* may relate to carcinomatous transformation as discussed later. Monoclonal cell lines transformed by pJ4Ω16.E7 plus *ras* or by pJ4Ω16 plus *ras* have been growing in culture for >4 months with no sign of senescence. These cells were clearly epithelial by their cytokeratin staining patterns and formed tumours in immunocompetent syngeneic rats within 2−3 weeks.

Having established that transfection with HPV16 plus *ras* generated malignantly transformed cells, we went on to ask whether other genital papillomavirus types were also active. The virus types tested fell into two distinct groups: types 18, 31 and 33, which were as active as HPV16; and types 6 and 11, which were inactive. HPV6 and 11 are usually found in benign genital and laryngeal lesions and generate papillomas. They appear rarely to progress to carcinomas. HPV18, 31 and 33, on the other hand, are found in advanced dysplasias, intra-epithelial neoplasias and carcinomas *in situ*. Our analyses of virus-specific RNA in the HPV6- and HPV11-transfected cells indicated that the E6/E7 region of the DNA was being transcribed, showing that viral genes are being expressed by these types, although transformation is not occurring. Transfection of HPV6 or HPV11 together with HPV16 caused no reduction in transformation by the active type, indicating that HPV6 and HPV11 do not have a negative effect in blocking transformation or killing transformed cells. The DNA sequences of HPV6, 11, 18, 31 and 33 are quite similar overall and in the E7 region in particular. It will be interesting to compare the properties of the viral E7 polypeptides to determine whether this is where critical differences reside and, if so, where within the E7 polypeptide they are located.

There is a striking parallel between the *in vivo* malignancy of HPV-associated lesions and the activity of the corresponding HPV type in our co-transformation assay. This strongly supports the idea that we are studying the carcinomatous type of transformation rather than simply the benign papillomatous type of transformation. This correlation provides a valuable means of investigating the role of papillomaviruses in the development of cervical cancer.

Materials and methods

Construction of HPV expression plasmids

HPV DNAs were kindly provided by Professor H.zur Hausen (HPVs 6, 11, 16, 18), Dr A.T.Lorincz (HPV31) and Dr G.Orth (HPV33). The plasmid pJ4Ω was a gift from J.Morgenstern. DNA fragments of the different HPV types were cloned into the multiple cloning site of pJ4Ω as described earlier.

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% fetal calf serum and 200 μg/ml G418. Cells were fixed in formal saline and stained with Giemsa stain.

Nucleic acid hybridization

Total genomic DNA was isolated from transformed BRK colonies which had been transfected with HPV DNA and the EJ-*ras* oncogene. DNA samples (10 μg) were digested with *Bam*HI, *Pst*I and *Kpn*I and electrophoresed in 1% agarose gels and subjected to Southern blot analysis (Southern, 1975) using Hybond-N filters (Amersham International). Hybridizations were performed using ^{32}P nick-translated HPV genomic DNA for 20 h. Filters were washed in 2 × SSC containing 0.1% SDS (1 × SSC is 150 mM NaCl, 15 mM trisodium citrate, pH 7.0) at room temperature, and then in 0.2 × SSC containing 0.1% SDS at 55°C and exposed to Fuji RX X-ray film with screens.

Total RNA was isolated from transiently infected BRK cells using guanidium thiocyanate (Chirgwin *et al.*, 1979). Glyoxylated RNA (20 μg) was electrophoresed on 1% agarose gels and subjected to Northern blot analysis (Thomas, 1980). Hybridizations were performed as described above.

Immunoblotting

For analysis of E7 protein synthesized in cell lines a 90-mm plate of confluent cells was lysed using 200 μl of lysis buffer (250 mM NaCl, 0.1% NP-40, 50 mM Hepes, pH 7.0, 1% aprotinin).

SDS−PAGE, immunoblotting and protein detection were as previously described (Banks *et al.*, 1987) using an anti-E7 monoclonal antibody E7IV (Oltersdorf *et al.*, 1987).

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References

Banks,L., Spence,P., Androphy,E., Hubbert,N., Matlashewski,G., Murray, A. and Crawford,L. (1987) *J. Gen. Virol.*, **68**, 1351−1359.

Beaudenon,S., Kremsdorf,D., Croissant,O., Jablonska,S., Wain-Hobson,S. and Orth,G. (1986) *Nature*, **321**, 246−249.

Berg,L.J., Singh,K. and Botchan,M. (1986) *Mol. Cell. Biol.*, **6**, 859−869.

Boshart,M., Gissmann,L., Ikenberg,A., Kleinheinz,W., Scheurlen,W. and zur Hausen,H. (1984) *EMBO J.*, **3**, 1151−1157.

Broker,T.R. and Chow,L.T. (1986) *Cancer Cells*, **4**, 589−594.

Cepko,C.K., Roberts,B.E. and Mulligan,R.C. (1984) *Cell*, **37**, 1053−1062.

Chirgwin,J., Przybyla,A.E., MacDonald,R.J. and Rutter,W.J. (1979) *Biochemistry* (*Wash.*), **18**, 5294−5299.

Cole,S.T. and Danos,O. (1987) *J. Mol. Biol.*, **193**, 599−608.

Dartmann,K., Schwarz,E., Gissmann,L. and zur Hausen,H. (1986) *Virology*, **151**, 124−130.

DiMaio,D., Guralski,D. and Schiller,J.T. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 1797−1801.

Dürst,M., Gissmann,L., Ikenberg,H. and zur Hausen,H. (1983) *Proc. Natl. Acad. Sci. USA.*, **80**, 3812−3815.

Gissmann,L., Wolnik,L., Ikenberg,H., Koldovsky,V., Schnurch,H. and zur Hausen,H. (1983) *Proc. Natl. Acad. Sci. USA.*, **80**, 560–563.
Land,H., Parada,L.F. and Weinberg,R.A. (1983) *Nature*, **304**, 596–602.
Lane,E.B., Bartek,J., Purkis,P.E. and Leigh,I.M. (1984) *Ann. N.Y. Acad. Sci.*, **455**, 241–248.
Lorincz,A.T., Lancaster,W.D. and Temple,G.F. (1986) *J. Virol.*, **58**, 225–229.
Lusky,M. and Botchan,M. (1985) *J. Virol.*, **53**, 955–965.
Matlashewski,G., Osborn,K., Murray,A., Banks,L. and Crawford,L. (1987b) In Steinberg,B.M., Brandsma,J.L. and Taichman,L.B. (eds), *Cancer Cells. Vol. 5. Papillomaviruses.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 195–199.
Matlashewski,G., Schneider,J., Banks,L., Jones,N., Murray,A. and Crawford,L. (1987a) *EMBO J.*, **6**, 1741–1746.
Nakabayashi,Y., Chattopadhyay,S.K. and Lowy,D.R. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 5832–5836.
Oltersdorf,T., Seedorf,K., Rowekamp.W. and Gissmann,L., (1987) *J. Gen. Virol.*, **68**, 2933–2938.
Rassoulzadegan,M., Naghashfar,Z., Cowie,A., Carr,A., Grisoni,M., Kamen,R. and Cuzin,F. (1983) *Proc. Natl. Acad. Sci. USA.*, **80**, 4354–4358.
Ruley,H.E. (1983) *Nature*, **304**, 602–606.
Sarver,N., Rabson,M.S., Yang,Y.C., Byrne,J.C. and Howley,P.M. (1984) *J. Virol.*, **52**, 377–388.
Schiller,J.T., Vass,W.C. and Lowy,D. (1984) *Proc. Natl. Acad. Sci. USA.*, **81**, 7880–7884.
Schneider-Gädicke,A. and Schwarz,E. (1986) *EMBO J.*, **5**, 2285–2292.
Schwarz,E., Durst,M., Demarkowski,C., Lattermann,O., Zech,R., Wolfsperger,E., Suhai,S. and zur Hausen,H. (1983) *EMBO J.*, **3**, 2341–2348.
Schwarz,E., Freese,U.K., Gissmann,L., Mayer,W., Roggenbuck,B., Stremlau,A. and zur Hausen,H. (1985) *Nature*, **314**, 111–113.
Seedorf,K., Krammer,G., Durst,M., Suhai,S. and Rowekamp,W.G. (1985) *Virology*, **45**, 181–185.
Southern,E.M. (1975) *J. Mol. Biol.*, **98**, 503–517.
Southern,P.J. and Berg,P. (1982) *J. Mol. Appl. Genet.*, **1**, 327–341.
Thomas,P.S. (1980) *Proc. Natl. Acad. Sci. USA.*, **77**, 5201–5205.
Tsunokawa,Y., Takebe,N., Kasamatsu,T., Terada,M. and Sugimura,T. (1986) *Proc. Natl. Acad. Sci. USA.*, **83**, 2200–2203.
Twigg,A.J. and Sherratt,D. (1980) *Nature*, **283**, 216–218.
Wigler,M., Pellicer,A., Silverstein,S., Axel,R., Urlaub,G. and Chasin,L. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 1373–1376.
Wilkinson,D., De Vries,R.R.P., Madrigal,J.A., Lock,C.B., Morgenstern, J.P., Trowsdale,J. and Altmann,D.M. (1988) *J. Exp. Med.*, **146**, in press.
Yang,Y.C., Okayama,H. and Howley,P. (1985) *Proc. Natl. Acad. Sci. USA.*, **82**, 1030–1034.
Yasumoto,S., Burkhardt,A., Doniger,J. and DiPaolo,J. (1986) *J. Virol.*, **57**, 572–577.

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