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Quantitative keratinocyte assay detects two biological activities of human papillomavirus DNA and identifies viral types associated with cervical carcinoma

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Keratinocytes electroporated with human papillomavirus (HPV) DNA (HPV-6, 11, 16 and 18) exhibited an increased cellular proliferation which was quantitated as microcolony and macrocolony formation. However, only macrocolonies induced by HPV-16 or HPV-18 DNA (the two viral types most commonly found in human cervical carcinomas) gave rise to proliferating, poorly-stratified colonies when grown in the presence of serum and calcium. Hydrocortisone increased the frequency of these differentiation-resistant colonies, and studies showed that they were immortalized, contained one copy of viral DNA per cell, expressed three discrete species of viral RNA and synthesized the viral E7 protein. HPV-induced cellular proliferation and altered differentiation are therefore separable events and may represent the activity of different viral genes.

Key words: papillomavirus/transformation/keratinocyte/cervical carcinoma

Introduction

Human papillomaviruses (HPV) are the causative agents of papillomas or warts and are implicated in the development of cervical dysplasia and anogenital carcinoma (zur Hausen and Schneider, 1987). There are now recognized to be ~50 different genotypes of HPV, and each type appears to display a preference for the anatomic site which it infects (Pfister, 1984). In addition, while several types of HPV may infect cervical squamous epithelium [e.g. HPV-6 (DeVilliers *et al.*, 1981) HPV-11 (Gissman *et al.*, 1982), HPV-16 (Durst *et al.*, 1983) and HPV-18 (Boshart *et al.*, 1984), only HPV-16 and 18 are highly associated with the progression of cervical dysplasia to carcinoma. Over 70% of cervical carcinomas contain either HPV-16 or 18 DNA and another 20% contain additional types such as HPV-31, 33 and 35 (zur Hausen and Schneider, 1987); only isolated examples of HPV-6 DNA have been detected within cervical carcinomas (Durst *et al.*, 1983; Boshart *et al.*, 1984).

The 'transforming activity' of HPV DNA has been studied using a variety of fibroblast and epithelial cell types. For example, HPV-1, HPV-5, HPV-16 and HPV-18 DNAs induce foci on monolayers of established rodent cells and, in some cases, convert these cells to the tumorigenic state (Watts *et al.*, 1984; Yasumoto *et al.*, 1986; Bedell *et al.*, 1987). HPV-16 DNA can also transform primary rat embryo cells when co-transfected with an activated *ras* oncogene, and genetic studies indicate that the E7 viral gene is responsible for this observed biological activity (Matlashewski *et al.*, 1987; Phelps *et al.*, 1988). More relevant to human tumorigenesis is the finding that HPV-16 DNA can immortalize primary cultures of human foreskin keratinocytes although it does not induce these cells to form tumors in nude mice (Durst *et al.*, 1987; Pirisi *et al.*, 1987); the viral gene (or genes) which mediates the immortalization of human keratinocytes has not yet been identified.

Current methods for detecting cellular immortalization by HPV-16 DNA rely upon repeated *in vitro* passaging of keratinocytes and are therefore not amenable to quantitative analysis or to the isolation of clonally derived keratinocyte cell lines. This study describes a quantitative assay which delineates two dissociable activities of HPV DNA, identifies the HPV types associated with human malignancy and generates clonal keratinocyte lines containing a single copy of HPV DNA which is unrearranged within its non-coding and early regions. The quantitative nature of this assay now makes it possible to define both viral and non-viral elements which modulate the expression of the 'transformed' keratinocyte phenotype.

Results

Keratinocyte electroporation and selection

A synopsis of the keratinocyte assay is presented in Figure 1. Human keratinocytes, which were isolated from newborn foreskin using previously described techniques (Yuspa and Harris, 1974), were cultured in serum-free, 0.15 mM Ca^{2+} KGM medium (see Materials and methods) for 20–30 population doublings prior to use in the transfection experiments. 2×10^6 keratinocytes were electroporated with 10 μg linearized HPV or plasmid DNA. HPV-6, 11 and 16 DNAs were cleaved with *Bam*HI (which linearizes the genome within the L1 ORF), and HPV-18 DNA was cleaved with *Eco*RI (which cuts the viral DNA within the E1 ORF). pBR322 DNA was cleaved with *Bam*HI. The keratinocytes were then replated into KGM medium for 2–4 days and analyzed for microcolony formation (colonies containing four or more cells). Ten to twenty randomly chosen microscopic fields were averaged to obtain an estimate of microcolony formation per field, and the total number of microcolonies per flask was calculated. Cell cultures were maintained in KGM for an additional 12–14 days to allow for the outgrowth of large (>0.5 cm) macrocolonies. Following the quantitation of macrocolony formation, the keratinocyte cultures were fed with medium containing 10% fetal calf serum and 2.0 mM Ca^{2+} for an additional 14 days to select for proliferating, 'dedifferentiated' colonies.

Microcolony formation

The effect of HPV DNA on cellular proliferation was studied by using a microcolony formation assay (Figure 2) which

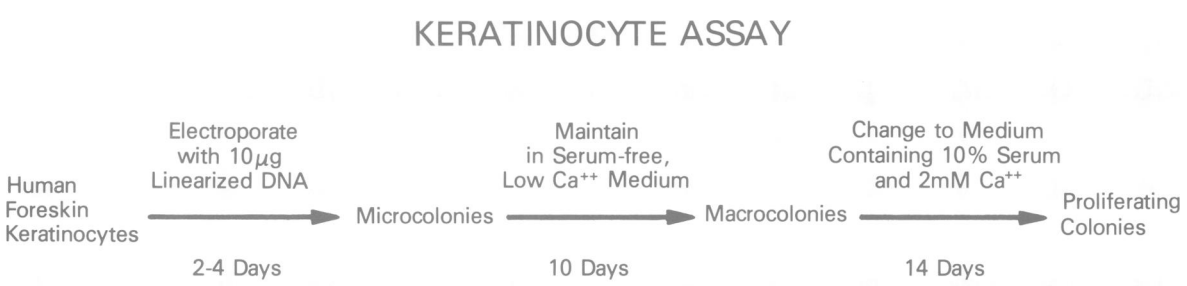


Fig. 1. Schematic representation of the keratinocyte assay.

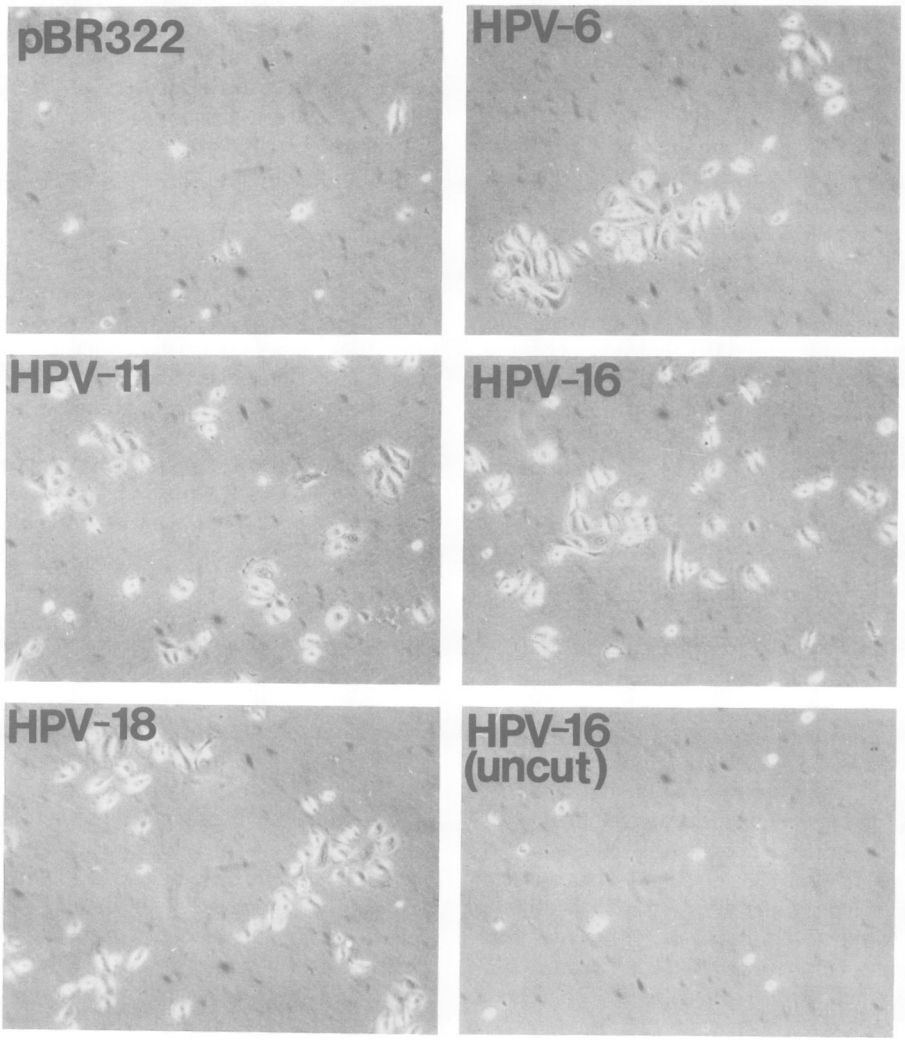


Fig. 2. Phase micrographs of keratinocyte microcolony formation. Keratinocytes were electroporated (2000 V, 25 μF) with each of the designated DNA preparations and plated sparsely ($2-5 \times 10^3$ cells/cm^2 into KGM medium and photographed 3 days later. Colony formation was obvious in each of the cultures receiving linearized HPV DNA but was non-detectable (by random microscopic sampling) in keratinocyte cultures electroporated with non-linearized HPV-16 DNA. Neither linear nor circular pBR322 DNA produced significant microcolony formation.

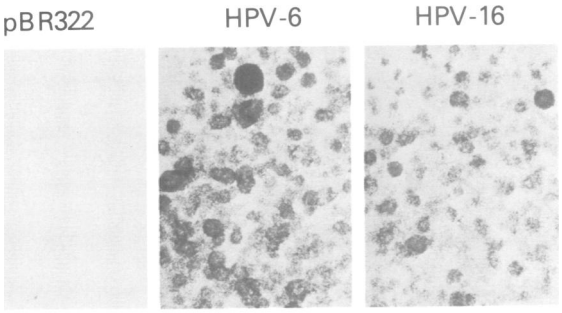
quantitates the proliferation of keratinocytes under growth-restrictive conditions (i.e. growth of late passage cells at low cell density). Microcolony formation was found to be most pronounced in keratinocytes transfected with linearized HPV-6, 11, 16 and 18 DNA and was extremely low (non-detectable by random microscopic sampling) in cells electroporated with circular HPV-16 DNA (Figure 2). The relative inability of non-linearized HPV-16 DNA to stimulate cellular proliferation corroborates previous findings demonstrating that electroporation is much more efficient with linearized DNA (Potter *et al.*, 1984; Schlegel *et al.*, unpublished results).

Quantitation of microcolony formation by HPV DNA is presented in Table I. All HPV DNAs, regardless of viral type, stimulated cellular proliferation to similar levels ($\sim 5 \times 10^4$ microcolonies/10^6 cells). Keratinocytes which were electroporated with no DNA or with circular DNA (either pBR322 or HPV-16) exhibited very low levels of microcolony formation which were usually non-detectable by random microscopic sampling. Keratinocytes transfected

Table I. Quantitation of HPV-induced alterations of keratinocyte proliferation

DNA (10 μg)[a]	Microcolonies per 10^6 cells	Macrocolonies per 10^6 cells	Serum/Ca^{2+}-resistant colonies per 10^7 cells
None	0, 0[b]	0	0, 0
pBR322	1.3×10^3 2.0×10^3	0	0, 0, 0, 0
HPV-6	2.8×10^4 6.2×10^4	135	0, 0, 0, 0 (0)[c]
HPV-11	5.6×10^4	95	0, 0, 0, 0 (0)
HPV-16	5.1×10^4 6.4×10^4	121	3, 6, 7, 9 (32, 35)
HPV-16 (uncut)	0[b]	3	0
HPV-18	4.6×10^4	96	24, 25

[a]All DNA preparations were linearized as described for Figure 1. pBR322 DNA was linearized with *Bam*HI.
[b]The threshold level for detecting microcolonies (by 10 random microscopic fields) is 700 microcolonies/10^6 cells and therefore the experimental observation of 0 microcolonies should be interpreted as <700 microcolonies/10^6 cells.
[c]Numbers in parentheses indicate experiments performed in the presence of 1 μg/ml hydrocortisone.



DNA		COLONIES/FLASK
pBR322	(10μg)	0, 0
HPV-16	0μg	0, 0
	1μg	8, 17
	10μg	91, 95
	50μg	75, 84
	50μg (uncut)	3

Fig. 3. Macrocolony formation and its dependence upon the amount of transfected HPV-16 DNA. Microcolonies induced by each of the indicated HPV DNAs were maintained in KGM medium for 14 days and the number of resultant macrocolonies was quantitated. Representative flasks containing cells transfected with either 10 μg linearized pBR322 DNA, 10 μg linearized HPV-6 DNA or 10 μg linearized HPV-16 DNA were fixed with 3.7% formaldehyde, stained with methylene blue and photographed. Tritration of the amount of HPV-16 DNA used in each transfection demonstrates that 10 μg HPV-16 DNA/ml/2 × 10^6 cells produces maximal macrocolony formation.

with linearized pBR322 DNA showed a low (but detectable) level of microcolony formation which was approximately 2−10% of the values observed for cells transfected with HPV DNA, suggesting that electroporation with 'non-specific' DNA may confer a limited stimulation of keratinocyte proliferation.

Macrocolony formation

Subsequent to the quantitation of microcolony formation, keratinocyte cultures were maintained in KGM medium for an additional 10−14 days to evaluate sustained cellular proliferation (macrocolony formation, Figure 3). Only keratinocytes electroporated with HPV DNA formed macrocolonies, and the number of macrocolonies was dependent upon the amount of input HPV DNA. Maximal macrocolony formation was achieved with 10 μg HPV DNA/ml/2 × 10^6 cells. Data presented in Table I indicate that the efficiency of macrocolony formation for each viral type was similar (~100 macrocolonies/10^6 cells transfected), although repeated experiments showed that HPV-6 DNA was slightly more efficient than HPV-11, 16 or 18. On the average, one microcolony in 600 developed into a macrocolony (Table I). In some experiments macrocolony formation by HPV DNA was as high as 1000 colonies per flask. The ability of pBR322 DNA to induce low levels of microcolony formation, but not macrocolony formation, is consistent with this DNA inducing a transient, non-sustained cellular proliferation. Similarly, circular HPV-16 DNA (which also showed very poor microcolony formation) formed few macrocolonies (3% of linearized HPV-16 DNA). Mock-transfected keratinocytes were also negative for macrocolony formation. The time-dependent decrease in HPV-mediated cell stimulation (i.e. the decrease in the percentage of cells which progress from microcolonies to macrocolonies) is reminiscent of the phenomenon of 'abortive transformation' observed with polyoma and SV40 viruses (Stoker and Dulbecco, 1969), although it is unclear whether the underlying mechanisms are the same.

Serum/Ca^{2+} resistant colonies

A dramatic difference in the growth potential of HPV-transfected cells was observed after transfer to medium containing serum and calcium; these growth conditions induce keratinocyte differentiation and stratification and select for the growth of partially-transformed cells (Yuspa and Morgan, 1981; Kulesz-Martin *et al.*, 1980). In the presence of serum and calcium, macrocolonies induced by HPV-16 or HPV-18 gave rise to proliferating, morphologically identifiable colonies (Figure 4). When viewed in the non-fixed state, these colonies appear as clear, expanding 'plaques' against a background of stratified, differentiated keratinocytes; this macroscopic appearance is due to the altered differentiation and decreased stratification of the HPV-transformed keratinocytes. After fixation with formaldehyde and staining with methylene blue, these HPV-transformed colonies are more basophilic than surrounding differentiated keratinocytes. The microscopic appearance of these colonies is also shown in Figure 4. In contrast to non-transfected keratinocytes, the cells are smaller and display an increased nuclear:cytoplasmic ratio. The proliferative capacity of these colonies is reflected in the mitotic figures present within the colonies as well as the ability of these colonies to expand and push aside the non-proliferating

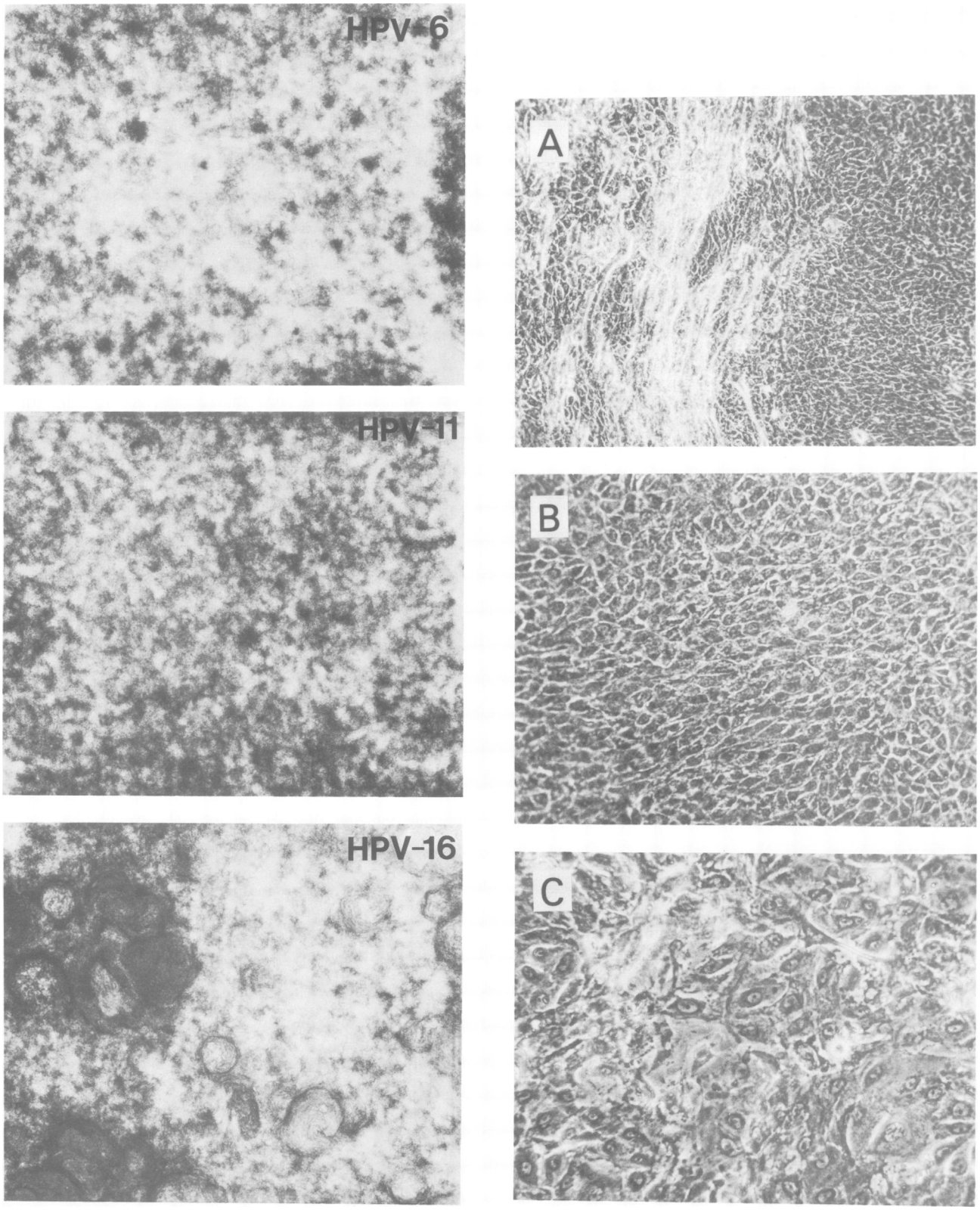


Fig. 4. Development of differentiation-resistant colonies from keratinocyte macrocolonies grown in medium containing 10% serum and 2 mM Ca^{2+}. The macroscopic (**A**) and microscopic (**B**) appearance of serum/calcium-resistant human keratinocyte colonies induced by HPV-16 DNA is illustrated. Proliferating, poorly-stratified colonies develop only from macrocolonies initiated by HPV-16 or HPV-18. The dense border of these colonies represents normal keratinocytes being compressed by the expanding colony (4B-A). The proliferating colonies (4B-B) are composed of small, poorly-stratified cells which grow as a densely-packed monolayer and exhibit a higher mitotic index than the surrounding large, differentiated keratinocytes (4B-C). A keratinocyte in metaphase is present near the center of 4B-B. When fixed and stained, the proliferating cells exhibit an increased basophilia.

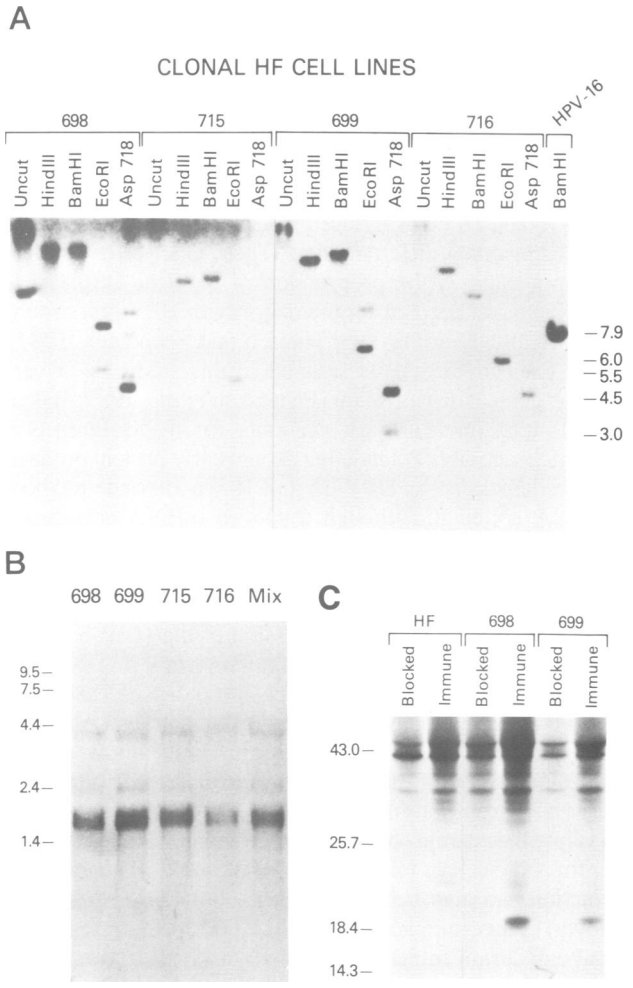


Fig. 5. Presence and expression of HPV-16 DNA in four clonal keratinocyte cell lines. **A.** 10 μg cellular DNA from four keratinocyte cell lines was digested with each of the indicated restriction enzymes, electrophoresed on agarose gels, transferred to nitrocellulose and hybridized with P-32 labeled, purified HPV-16 DNA as previously described (Zhang *et al.*, 1987). The simple pattern and relative signal suggest that each cell line contains a single copy of HPV-16 which is integrated near the *Bam*HI site (5A). Enzyme cleavage patterns also indicate that each cell line differs with respect to the site of integration of HPV-16 DNA into the cell genome. **B.** Total cellular RNA was isolated by standard procedures (Chirgwin *et al.*, 1979) and hybridized with full-length HPV-16 DNA (5C) as described (Baker *et al.*, 1987); three size species of viral RNA were detected in each of the cell lines (1.8, 2.4 and 4.3 kb), including one cell line (MIX) which was derived from a mixture of nine independent serum/Ca^{2+}-resistant colonies. **C.** Two keratinocyte cell lines were labeled with [^{35}S]cysteine and screened for the presence of the HPV-16 E7 protein by immunoprecipitation with an antiserum generated against an E7 synthetic peptide. Cell lines 698 and 699 contained the 19 kd E7 protein (Smotkin and Wettstein, 1986, 1987) (see arrow) which was absent in normal foreskin keratinocytes.

normal keratinocytes. The colonies also show less cell stratification, suggesting that there is probably a pleiotropic effect on cellular differentiation which occurs consequent to the activity of the HPV-16 and HPV-18 genes.

Five of six 'serum/calcium resistant' HPV-16-induced colonies were cloned and established into cell lines. In contrast, none of six HPV-16-induced macrocolonies were established into cell lines, suggesting that only a small number of immortalized cells exist or arise within the total number of macrocolonies. It seems unlikely that exposure to serum and calcium contributes to the development of cellular immortalization since immortalization can occur in the absence of these agents (Pirisi *et al.*, 1987). Rather, resistance to differentiation induced by serum factors or calcium appears to represent a phenotype which accompanies cellular immortalization (Kulesz-Martin *et al.*, 1980) and that growth in medium containing serum and calcium would therefore select for cells with an unlimited growth potential.

In a limited number of experiments, HPV-18 DNA appears to be more efficient (3- to 4-fold) at inducing altered keratinocyte differentiation than HPV-16 DNA. Whether there is any biological correlate to this preliminary finding is not known at present. The keratinocyte assay also demonstrates that hydrocortisone can increase the frequency of serum/calcium resistant colonies by a factor of 3−5 (Table I). Potentially this could either represent the activity of hydrocortisone on the glucocorticoid-responsive element which is present in the non-coding region of HPV-16 DNA (Gloss *et al.*, 1987) or it could simply reflect the dependence of keratinocyte proliferation on hydrocortisone (Rheinwald and Green, 1975).

Four keratinocyte cell lines which were established from single colonies induced by HPV-16 DNA were characterized for the presence of HPV-16 DNA, RNA and protein. Interestingly, all the cell lines contained approximately one copy HPV-16 DNA per cell which was integrated into the cellular genome; as determined by analysis with multiple restriction enzymes, the site of viral DNA integration into the cellular genome was different for each of the cell lines (Figure 5A). In addition, the *Bam*HI restriction site (which was used to linearize the HPV-16 DNA) is lost in each of these cell lines, suggesting that the viral DNA may be integrated into the host chromosome at this site. Further characterization of the integrated viral DNA by cleavage with *Asp*718 (Figure 5B) and *Pst*I (data not shown) indicates that the non-coding region and entire early region of the HPV-16 genome is intact (between bp 7007−5381), suggesting that major rearrangements in the viral genome have not occurred.

Northern blot analysis of the isolated cell lines indicates that each expresses similar quantities and species of viral RNA (Figure 5B). Viral RNAs of 1.8, 2.4 and 4.3 kb size are present, with the predominant species being the 1.8 kb sized RNA. Despite the finding that the HPV-16 DNA is integrated into different sites within the genome of these cell lines, viral RNA expression is virtually identical, suggesting that the viral promoters are responsible for the observed transcriptional activity. It will be important to characterize the viral RNA transcripts and to ascertain the active viral promoters of these integrated viral genomes.

The four HPV-16-containing cell lines also synthesize detectable and similar levels of the E7 early protein (Figure 5C). This protein is the predominant HPV-16 protein present in human cervical carcinoma cell lines (Smotkin and Wettstein, 1986, 1987) and participates in the immortalization/transformation of primary rodent cells (Matlashewski *et al.*, 1987; Phelps *et al.*, 1988).

The presence of this protein in the established keratinocyte cell lines indicates that viral RNA is being efficiently expressed. Since the entire early region of HPV-16 is intact in these cell lines, it is anticipated that additional early proteins such as E6, E2, E4 and E5 are also being

synthesized. Indeed, these cell lines provide a unique cell system for identifying the HPV-16 proteins encoded at the 3′ end of the early region (i.e. E2, 3, 4 and 5) since this region is uniformly deleted or rearranged in several characterized cervical carcinoma cell lines (Schwarz *et al.*, 1985).

Finally, these four keratinocyte cell lines were also injected subcutaneously into nude mice (10^7 cells/0.3 ml/animal) and did not produce tumors when observed for a period of 4 months; this finding agrees well with two previous reports using mass keratinocyte cultures immortalized by HPV-16 (Durst *et al.*, 1987; Pirisi *et al.*, 1988).

Discussion

This study demonstrates that each of the HPV DNAs evaluated can stimulate cellular proliferation, suggesting the activity of a common early viral gene product which has a similar effect on genital keratinocytes. There are several candidate viral genes for this biological effect, including the papillomavirus E5, E6 and E7 genes which have been shown to have demonstrable transforming activity in rodent cell assays (Bedell *et al.*, 1987; Schiller *et al.*, 1985, 1986; Groff and Lancaster, 1986; DiMaio *et al.*, 1986; Yang *et al.*, 1985a,b). Recently, the E5 protein of bovine papillomavirus was shown to exhibit mitogenic activity when microinjected into mouse cells (Green and Loewenstein, 1987) and each of the HPVs used in this study also encodes a similar protein (Bubb and Schlegel, 1988; Halbert and Galloway, 1988). Our results with HPV-18 DNA, however, suggest that the E6/E7 region may encode functions for both cell proliferation and for altered differentiation. HPV-18 DNA, unlike HPV-6, 11 and 16 DNA (which were linearized within the L1 ORF by *Bam*HI) was linearized within the E1 ORF by *Eco*RI. If the HPV-18 DNA integrates into the cellular genome at the site at which it was linearized (as is the case for all of the examined HPV-16 cell lines), this would result in the dissociation of the HPV-18 E5 ORF from the upstream viral promoter but would permit expression of the E6/E7 genes. The demonstrated functional and structural homology of the HPV-16 E7 protein to the adenovirus E1a protein (Phelps *et al.*, 1988) suggests that the viral E7 protein, like the E1a protein, may have a mitogenic activity. It is also possible, however, that more than one viral gene product may be able to stimulate cell proliferation.

The site (within the viral genome) at which HPV DNA integrates into the cellular genome is clearly different for *in vitro* transfected keratinocytes and for naturally occurring human cervical carcinomas. In HPV-16 transfected keratinocytes, HPV DNA is integrated within the L1 ORF whereas, in most cervical carcinomas, it is integrated within the E1/E2 ORFs. An obvious reason for this difference is that the electroporation experiments utilized DNA which was linearized at the *Bam*HI site, and this appears to have forced integration within the L1 ORF. There are several potential explanations for the finding that, in cervical carcinomas, HPV integrates at the E1/E2 region: (i) presented in the context of a circular DNA, the E1/E2 ORFs may represent a 'hot spot' for recombination, and (ii) integration within the E2 ORF may abrogate a repressor of viral gene transcription and thereby increase viral promoter activity and gene expression. Potentially the resultant increase in levels of HPV-transforming proteins could confer a growth advantage to tumor cells. Integrants within the E2 ORF would therefore arise by a process of natural selection in the host for the most aggressive tumor phenotype. Such selective pressures would probably not exist in the *in vitro* keratinocyte assay.

DNA from different types of HPV clearly have different *in vitro* biological activities. For example, despite the fact that HPV-6, 11, 16 and 18 infect genital epithelium, only HPV-16 and 18 DNA induced resistance to serum/calcium-induced terminal differentiation. These latter types of HPV are implicated in the progression of cervical dysplasia to carcinoma and account for the majority of HPV types found in cervical carcinomas (zur Hausen and Schneider, 1987). Since each of the HPVs assayed in this study has a very similar genetic organization (Pettersson *et al.*, 1987; Baker, 1987), it is unclear what accounts for the difference in biological activity. Potentially, variant early protein products may be generated by HPV-16 and 18 via alternate methods of RNA splicing. Although a spliced mRNA encoding a truncated E6 gene product has been described for HPV-16 and HPV-18 (Schneider-Gadicke and Schwarz, 1986), such a splice has not been found in either HPV-6 or HPV-11. It is also possible that the expression of the HPV-6 and 11 genomes is lower in these foreskin keratinocytes than that for HPV-16 or 18 and that the level of a crucial early gene product is below the threshold for cellular 'transformation'. The quantitative keratinocyte assay should allow for the molecular analysis of this important biological difference between the various HPV types.

While the current studies utilized medium containing both serum and calcium for selecting for altered cell phenotypes, selection can also be achieved with only serum (data not shown). Thus, supplementation of KGM medium with 10% fetal calf serum selects for HPV-16 or HPV-18 transfected keratinocytes in the same fashion that Dulbecco's medium containing both Ca^{2+} and serum does (data not shown). This finding also indicates that the selection mechanism is not due to other differences in the components of the KGM and Dulbecco's medium and that Ca^{2+} is not a requisite for selection. The selection of HPV-transformed cells by exposure to serum might reflect the activity of TGF-β which is present in serum and which inhibits keratinocyte proliferation; transformed keratinocytes are resistant to the action of TGF-β (Shipley *et al.*, 1986). However, there are potentially many other factors present in serum which might mediate this selection. Preliminary efforts to select for HPV-transformed keratinocytes in KGM medium supplemented with 1.5 mM Ca^{2+} have not been successful.

Finally, the two separable biological activities of HPVs described in this study may represent *in vitro* correlates of their known *in vivo* biological activities. Four genital-associated HPVs, all of which can stimulate cellular proliferation as manifest by tumor formation in the host, were also able to stimulate cellular proliferation as assayed *in vitro* by micro- and macrocolony formation. Similarly, while only HPV-16 and HPV-18 are associated with the progression to malignancy in humans, only these two viral DNAs mediate altered cellular differentiation.

Materials and methods

Cell culture

Human keratinocytes were isolated from newborn foreskin samples using standard techniques (Yuspa and Harris, 1974). Briefly, the foreskins were

cut into narrow strips (0.5 cm in width) and incubated in 0.125% trypsin overnight at 4°C. The epidermis was stripped from the underlying dermis with forceps, minced with sterile scissors and incubated with 0.125% trypsin at 37°C with agitation in a trypsinization flask. Following three extractions with trypsin, the isolated keratinocytes were suspended in Dulbecco's modified Eagles medium containing 10% fetal calf serum and pelleted by centrifugation. The cells were plated into KGM medium (Clonetics, San Diego, CA) consisting of a modified MCDB 153 medium containing 10 ng/ml epidermal growth factor, 5.0 μg/ml insulin, 0.5 μg/ml ml hydrocortisone, 0.15 mM Ca^{2+} and 50 μg/ml bovine pituitary extract, that contained in addition 1% serum to facilitate cell attachment. On the following day, the keratinocyte cultures were changed to KGM medium without serum. Keratinocytes were passaged using routine trypsinization techniques except that the cells were always pelleted through serum-containing medium to inactivate residual trypsin activity.

Electroporation

Keratinocytes were transfected by electroporation (Potter *et al.*, 1984) since we found that calcium phosphate precipitation methods produced variable amounts of cell death and terminal differentiation. Keratinocytes were harvested by trypsinization, pelleted through medium containing 10% serum and washed twice with phosphate-buffered saline lacking calcium and magnesium (PBS^-, Gibco). The cells were resuspended at a concentration of 2×10^6 cells/ml PBS^- and kept on ice for 15 min. For electroporation, 1.0 ml cell suspension was mixed with 10 μg DNA (in 50 μl) and pulsed with 2000 V, 25 μF). The optimal V and μF settings were determined experimentally by evaluating the formation of macrocolonies and serum/Ca^{2+}-resistant colonies induced by linearized HPV-16 DNA. Following electroporation, the cells were kept at 4°C for 15 min and then plated into KGM medium containing 1% serum and incubated overnight at 37°C. The cultures were then changed to KGM medium and grown as indicated in the experimental protocols.

DNA and RNA hybridization

DNA was extracted and purified from each of the cell lines, electrophoretically separated on 0.6% agarose gels, and blotted onto nitrocellulose paper as described (Zhang *et al.*, 1987). The blots were then hybridized under stringent conditions (60°C, 3 × SSC buffer) using 5×10^6 c.p.m. ^{32}P-labeled nick-translated HPV-16 DNA ($1-3 \times 10^8$ c.p.m./μg DNA) and evaluated by film autoradiography.

Total cellular RNA was isolated by standard procedures (Chirgwin *et al.*, 1979), separated on 1.0% agarose gels containing 2.2 M formaldehyde in the running buffer, blotted onto nitrocellulose and hybridized with the same HPV-16 probe described for the DNA studies (Baker *et al.*, 1987).

Immunoprecipitation

Subconfluent monolayer cultures (10 cm plates) were labeled with 1.0 mCi [^{35}S]cysteine for 4 h at 37°C in 3.0 ml serum-free Dulbecco's modified Eagles medium deficient in cysteine. The cells were washed twice with PBS^-, extracted with 1.0 ml RIPA buffer and the supernatant immunoprecipitated with 20 μl rabbit antiserum generated against a synthetic peptide corresponding to the HPV-16 E7 protein. The protocol used for immunoprecipitation has been described (Burkhardt *et al.*, 1987).

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