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Differential effects of human papillomavirus type 6, 16, and 18 DNAs on immortalization and transformation of human cervical epithelial cells

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ABSTRACT **The human papillomaviruses (HPVs) are associated with specific benign and malignant lesions of the skin and mucosal epithelia. Cloned viral DNAs from HPV types 6b, 16, and 18 associated with different pathological manifestations of genital neoplasia *in vivo* were introduced into primary human cervical epithelial cells by electroporation. Cells transfected with HPV16 or HPV18 DNA acquired indefinite lifespans, distinct morphological alterations, and anchorage-independent growth (HPV18), and contain integrated transcriptionally active viral genomes. HPV6b or plasmid electroporated cells senesced at low passage. The alterations in growth and differentiation of the cells appear to reflect the progressive oncogenic processes that result in cervical carcinoma *in vivo*.**

The human papillomaviruses (HPVs) offer a unique approach to the studies of human epithelial cell carcinogenesis. Clinical and epidemiological studies combined with molecular analysis of the >50 viruses identified to date (1–3) have revealed an apparent anatomical restriction of HPV types with specific benign and malignant lesions of the skin and mucosa (4). Approximately 20% of the HPVs that have been identified were isolated from anogenital tissues (4–9); however, HPV6 and HPV11 predominate in benign exophytic and endophytic condylomas, whereas HPV16 and HPV18 predominate in premalignant and malignant genital tissues. Ninety-five percent of cervical cancers and cervical intraepithelial neoplasias occur within the transformation zone of the uterine cervix (10) and reportedly contain HPV16, HPV18, or related types of HPV. To investigate the oncogenic functions by which these viruses induce these specific types of premalignant and malignant lesions *in vivo*, we have introduced cloned viral DNAs representing HPV6b, -16, and -18 into primary cervical epithelial cells by electroporation and present a characterization of the altered biological properties of the cells, which we attribute to the presence of the different HPV genomes. Parallel cell cultures were also electroporated with cloned simian virus 40 (SV40) DNA, which we and others have shown to partially transform primary human keratinocytes derived from neonatal foreskins (11, 12).

MATERIALS AND METHODS

Establishment of Primary Cervical Epithelial Cultures. Normal cervical epithelial cells were derived from explant cultures of the portio surface and transformation zone of cervix uteri obtained from 35- to 40-year-old women who had undergone hysterectomies for benign uterine fibroids. Primary cultures were initiated and maintained as described (13) in supplemented Dulbecco's modified Eagle's medium containing 10% NuSerum IV (Collaborative Research) in place of fetal calf serum.

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Cloned Viral DNAs and Plasmids. The plasmids used in this work were as follows: pBR322, obtained from the American Type Culture Collection; pGem3, obtained from Promega Biotec; pHPV16 and pHPV18, obtained directly from the laboratory of Harald zur Hausen (6, 8) with the viral inserts cloned into pBR322 at the *Bam*HI and *Eco*RI sites, respectively; pHPV6b containing the full-length viral genome cloned at the *Bam*HI site of pGem3 was constructed from the partial HPV6b fragments in pHPV6b (Amp 2) and pHPV6b (Cl 21), originally cloned in zur Hausen's laboratory (14) and obtained with his permission from Peter Howley; pSV40 was constructed by cloning full-length viral DNA from SV40 (strain Rh911) at the *Bam*HI site in pGem3.

Linearized viral DNAs and vector controls were prepared by cleavage of cesium chloride-purified plasmids by restriction endonuclease digestion with the appropriate enzymes according to the manufacturer's instructions (New England Biolabs).

Electroporation and Subculturing of Cervical Cells. Twenty-one days postinitiation of the primary cultures, when the cells were actively growing and at 75–90% confluency, explants were removed by aspiration and the cell monolayers were disaggregated by exposure to 0.25% trypsin/0.02% EDTA (GIBCO) for 30 s, and incubation at 37°C for 5–10 min. Cells were washed and resuspended ($4–6 \times 10^6$ cells per ml) in Ca^{2+}-, Mg^{2+}-free Dulbecco's phosphate-buffered saline.

Equivalent 0.5-ml cell suspensions were mixed with 20 μg of one of the three HPV DNAs (type 6b, 16, or 18) or 20 μg of SV40 DNA or 20 μg of either pBR322 or pGem3 plasmid DNAs, in sterile polystyrene cuvettes fitted with aluminum electrodes (Bio-Rad). The DNA/cell mixtures were electroporated as described (15, 16) by exposure to a 2-kV electric pulse (5 kV/cm) applied by an ISCO 494 electrophoresis power supply and seeded into 25-cm^2 culture flasks precoated with laminin (4 μg/cm^2; Collaborative Research) in the presence of lethally irradiated NIH 3T3 feeder cells prepared as described (13). The cell cultures were maintained in parallel and subcultured 1:2 every 10–14 days.

Preparation and Analysis of Cellular DNA and Total Cytoplasmic RNA. High molecular weight cellular DNA and total cytoplasmic RNA were prepared from cell lines and analyzed by blot hybridization as described previously (17) and in the figure legends.

Differentiation Assay. To investigate similarities and/or differences in morphogenesis and structural differentiation among the cell lines, they were examined *in vitro* in a modification of the organotypical culture assay (18). In this modified assay, the collagen substratum was replaced by a solubilized basement membrane matrix (Matrigel; Collaborative Research) in culture chambers (Millicell-CM; Millipore), which were incubated in culture plates seeded with NIH 3T3 feeder cells. This assay allows for three-dimensional growth and organization in the presence of mesenchy-

Abbreviations: HPV, human papillomavirus; SV40, simian virus 40.

mal factors providing a growth environment more similar to that *in vivo* than standard tissue culture methods.

The cervical cells were seeded in the Matrigel-coated chambers (1–2 × 10^6 cells per chamber) and allowed to attach and grow while submerged in 1 ml of medium. After 1 week, the medium above the cells was removed and the cells were allowed to grow at the air–medium interface for an additional week. Cultures were fixed at 2 weeks in 0.25% glutaraldehyde and processed for histology as described by the manufacturer (Millipore).

Anchorage-Independent Growth. Cells (2 × 10^5) were seeded into medium containing 0.33% Noble agar (Difco) over prehardened basal layers made with the same growth medium containing 0.42% Noble agar (19). Colonies were counted by light microscopy at 4 weeks.

RESULTS

Immortalization of Primary Cervical Epithelial Cells. In three independent experiments (Table 1), cells electroporated with either pBR322 or pGem3 plasmid DNAs or HPV6b viral DNA senesced at passage 3. In contrast, cells electroporated with HPV16, HPV18, or SV40 continued to proliferate and have been in continuous culture for 10–25 serial passages. Prior to its establishment as a continuous cell line, the SV40 electroporated culture exhibited an intervening "crisis" period (11, 12) marked by the presence of senescent colonies, which were also characteristic of plasmid and HPV6b electroporated cultures at passage 3, and colonies composed of cells that appeared larger and flatter with both types of colonies shedding large numbers of cells into the culture medium. No intervening "crisis" period was observed in the cultures electroporated with either HPV16 or HPV18 DNAs, although the cell lines that have emerged from these cultures are morphologically altered. Unlike cultures of normal cervical epithelial cells or early passage electroporated cells, the HPV16 and -18 established cell lines no longer exhibit a uniform tightly packed cobblestone morphology but are composed of more loosely associated cells that vary in size and shape, are occasionally multinucleate, and are independent of NIH 3T3 feeder cell support for subcultivation as are the postcrisis SV40 electroporated cells.

Presence and Physical State of Viral DNAs. Southern blot analysis of high molecular weight cellular DNAs isolated from early passage cultures (Table 1; Fig. 1*C*, lane b) revealed the presence of the respective viral DNAs (HPV6b, -16, -18, and SV40) in the corresponding electroporated cultures. HPV6b, -16, or -18 sequences were not detected in the plasmid electroporated control culture (Fig. 1*A*, lane a; Fig. 1*B*, lane a) or in the tissues from which the primary cells were derived (data not shown), thus excluding preexisting infection.

Table 1. Properties of electroporated cervical cells

Culture	DNA electroporated	Viral sequences	Extended lifespan	CFE
Z130	pBR322	− (0)	−	NA
Z131	HPV6b	+ (0)	−	NA
Z132	HPV16	+ (1, 7, 9, 17)	+	0
Z133	HPV18	+ (0, 1, 6, 20)	+	1.56
Z134	SV40	+ (1)	+	9.80
Z170	pGem3	ND	−	NA
Z171	HPV6b	+ (0)	−	NA
Z172	HPV16	+ (1, 10)	+	0
Z173	HPV18	+ (1, 9)	+	0
Z180	pGem3	ND	−	NA
Z181	HPV6b	ND	−	NA
Z183A	HPV18	+ (4, 10)	+	2.50
Z183B	HPV18	+ (4, 10)	+	5.20

CFE, colony-forming efficiency (in agar): (no. of colonies/no. of cells seeded) × 100. Numbers in parentheses represent passage number of cells probed for viral sequences. NA, not applicable; ND, not determined.

The HPV16 DNA in the Z132 cell line migrates with high molecular weight cell DNA [>23 kilobases (kb)] when digested with either of two restriction endonucleases, *Hin*dIII or *Eco*RV, that do not cleave the viral genome (Fig. 1*A*, lanes b and c), while digestion with either of two restriction endonucleases, *Sca* I or *Stu* I, that cleave the HPV16 genome at single sites yield patterns suggesting viral DNA integration as a monomer at a single site (Fig. 1*A*, lanes d and e). The integration of the HPV16 genome in the Z132 and Z172 cell lines was further analyzed by digestion with a multicut restriction endonuclease, *Pst* I (Fig. 1*C*, lanes a and b). The absence of the 0.91- and 0.64-kb bands in the *Pst* I digestions indicates integration of the HPV16 genomes within the *Bam*HI/*Pst* I terminal fragments defining the 5′ and 3′ ends of the *Bam*HI linearized viral molecules used for electroporation. In addition, the virus/host junction fragments were detected in the Z132 cell line and are represented by two restriction fragments of altered molecular size (>2.81 kb and 1.58 kb). The presence of the 0.48-kb internal viral fragments in Fig. 1*C* (lanes a and b) was revealed by longer exposure of the autoradiogram.

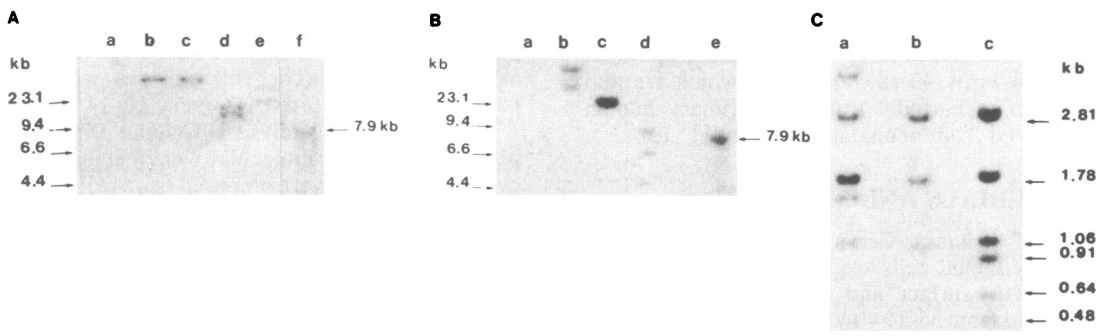

FIG. 1. Detection of HPV sequences in electroporated cervical cell cultures. Genomic DNAs were prepared as described and 10-μg samples were digested with the appropriate restriction endonucleases (indicated below for each lane) as specified by the manufacturer (New England Biolabs) and subjected to electrophoresis on 0.8% agarose gels followed by transfer onto nitrocellulose membranes (Schleicher & Schuell). The resulting blots were baked for 2 hr at 80°C in a vacuum oven and hybridized (as described by Schleicher & Schuell) with ^{32}P-labeled nick-translated DNA probes. (*A*) HPV16 probe. Lanes: a, Z130, *Hin*dIII; b, Z132, *Hin*dIII; c, Z132, *Eco*RV; d, Z132, *Sca* I; e, Z132, *Stu* I; f, 12 pg of linear HPV16. (*B*) HPV18 probe. Lanes: a, Z130, *Hin*dIII; b, Z133, nondigested; c, Z133, *Hin*dIII; d, Z133, *Eco*RV; e, 12 pg of linear HPV18. (*C*) HPV16 probe. Lanes: a, Z132, *Pst* I; b, Z172, *Pst* 1; c, 12 pg of *Bam*HI linear HPV16, *Pst* I. Fragment sizes of coelectrophoresed *Hin*dIII-digested λ phage DNA are indicated for *A* and *B* and fragment sizes of coelectrophoresed *Bam*HI + *Pst* I-digested HPV16 are indicated for *C*.

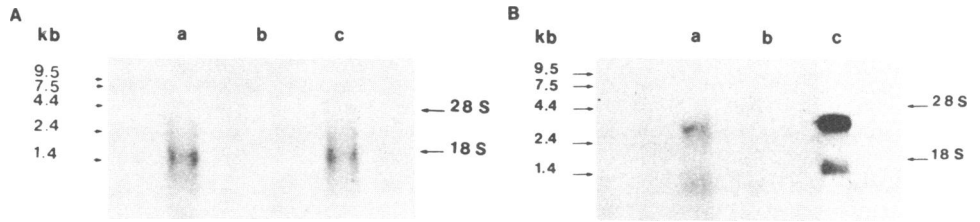

FIG. 2. Expression of HPV mRNAs. Twenty micrograms of total cytoplasmic RNA from each cell line was denatured in 50% formamide/2.2 M formaldehyde for 15 min at 55°C followed by electrophoresis in 1.2% agarose-formaldehyde gels and transfer to nitrocellulose filters (BA85, Schleicher & Schuell). The resulting blots were baked for 2 hr at 80°C in a vacuum oven and hybridized (as described by Schleicher & Schuell). (*A*) ^{32}P-labeled HPV16 RNA probe (20). Lanes: a, Z132; b, normal cervical epithelial cells; c, CaSki cells. (*B*) ^{32}P-labeled nick-translated HPV18 DNA probe. Lanes: a, Z133; b, normal cervical epithelial cells; c, HeLa cells.

The HPV18 viral DNA in Z133 migrated with high molecular weight cell DNA (>23 kb) when uncleaved (Fig. 1*B*, lane b); although the HPV18 signal appears as two bands, the ethidium bromide-stained gel indicated that these bands represent the upper and lower boundaries of a single mass of nondigested DNA that migrated ≈5 mm into the gel. Digestion of the Z133 cell DNA with a restriction endonuclease that does not cleave the HPV18 DNA yielded a single high molecular weight band (Fig. 1*B*, lane c), suggesting integration of the viral genome. Digestion with *Eco*RV (Fig. 1*B*, lane d) did not reveal a 7.9-kb linear viral band characteristic of episomal replication or multimeric tandem integration but produced a restriction pattern suggesting integration of the HPV18 DNA at a single site as a head-to-head dimer. A similar analysis of the physical state of the HPV18 genomes in the Z173, Z183A, and Z183B cell lines also revealed integration of the viral genomes in Z183A and -B and the existence of episomal forms in Z173 (data not shown).

Transcriptional Activity of Viral Genomes. The HPV18 and HPV16 genomes are transcriptionally active in the established cervical cell lines (Fig. 2; data not shown). Northern blot analysis of total cytoplasmic RNA from Z133 (Fig. 2*B*, lane a) revealed the presence of two size classes of HPV18-encoded transcripts, 2.7 and 0.8 kb. These two species are both smaller and less abundant than transcripts present in the HeLa cervical cell line (Fig. 2*B*, lane c), which Schwarz *et al.* (21) have shown to be composed of HPV18 E6, E7, and E1 open reading frames and flanking cellular sequences. Northern blot analysis of total cytoplasmic RNA from Z132 (Fig. 2*A*, lane a) disclosed HPV encoded transcripts mainly represented by a broad band that in some cases could be resolved in two species of 1.5 and 1.1 kb. Longer exposure also revealed a minor species of 3.8 kb (data not shown). These HPV16 transcripts are similar in size and abundance to the HPV16 transcripts isolated from the CaSki cervical cell line (Fig. 2*A*, lane c).

Growth in Organotypical Culture. Primary cervical epithelial cells and pBR322 electroporated cells, Z130, were morphologically distinct from the HPV16 and HPV18 established cell lines when grown in modified organotypical culture and examined by phase-contrast microscopy (Fig. 3 *A–C*). Unlike the HPV established cell lines, both the primary cervical cells and the Z130 cells failed to form confluent monolayers prior to forming multilayered structures as shown for Z130 (Fig. 3*A*). The HPV16 and HPV18 established cell lines shared similarities and exhibited differences in growth behavior (Figs. 3 and 4). The HPV16 established cell lines Z132 (Fig. 3*B*) and Z172 (data not shown) were characterized by the formation of multicellular structures growing above and into the basement membrane matrix, whereas the HPV18 established cell lines formed "dome-like" structures as shown for Z133 (Fig. 3*C*). Observations of the HPV18 cell lines at earlier time points revealed that they also formed multicellular structures growing into the matrix.

Examination of histological cross sections of the cultures revealed further similarities and differences in three-dimensional growth, structural organization, and differentiation among the various cells and cell lines. All of the cultures formed multilayered structures growing above the basement membrane matrix; however, the Z130 culture (Fig. 3*D*)

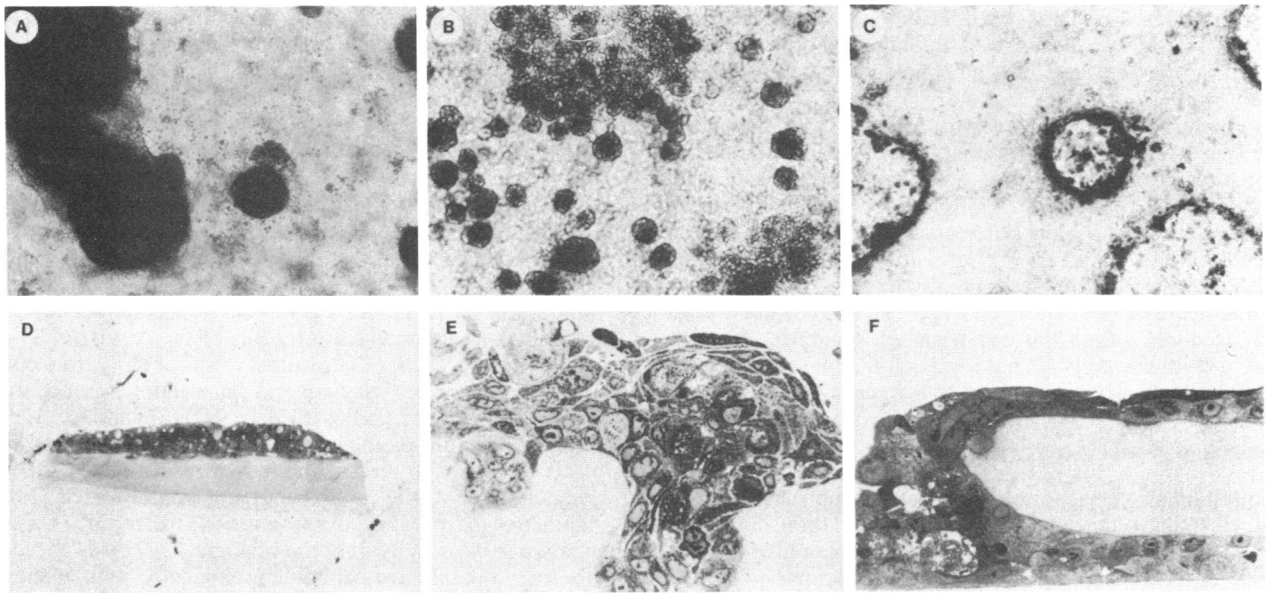

FIG. 3. Morphology of electroporated cervical cells in modified organotypical culture. (*A–C*) Phase-contrast microscopy. (*D–F*) Histological cross section. (*A* and *D*) Z130 at passage 1. (*B* and *E*) Z132 at passage 10. (*C* and *F*) Z133 at passage 10. (*A–C*, ×100; *D*, ×140; *E* and *F*, ×750.)

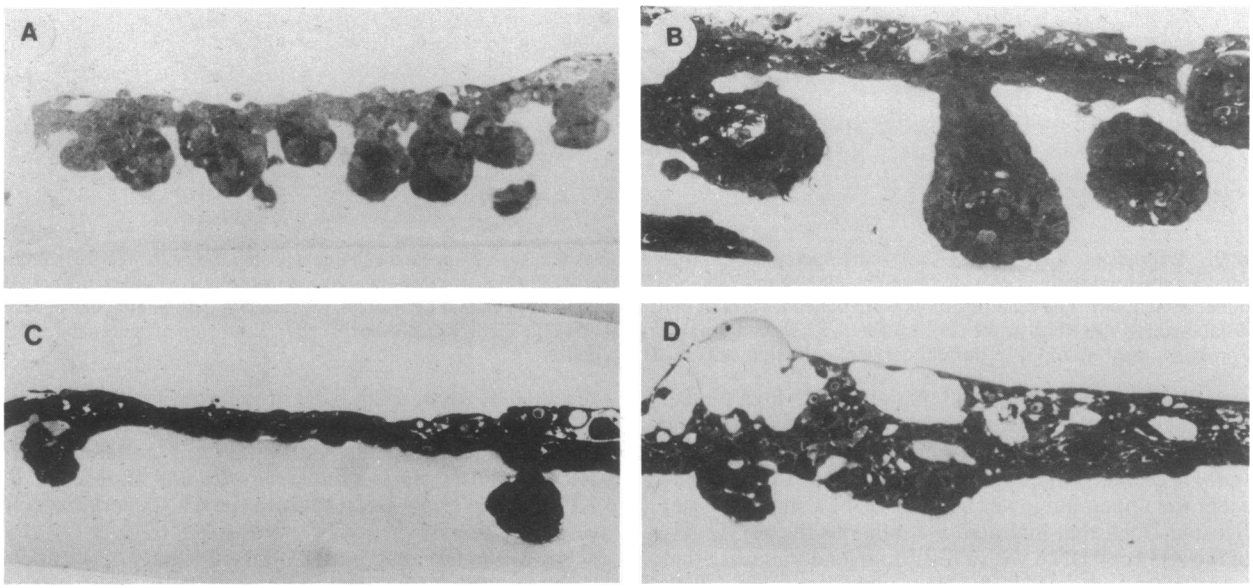

FIG. 4. Histological cross sections of HPV18 electroporated cervical cells in modified organotypical culture. (*A*) Z133 passage 17. (*B*) Z183A passage 6. (*C*) Z173 passage 7. (*D*) Z183B passage 6. (×140.)

showed evidence of cell degeneration at early passage. Z132 cultures (Fig. 3*E*) and Z172 cultures presented features of differentiating squamous epithelia, but unlike normal squamous epithelial cells grown under similar conditions (ref. 18; unpublished observations) the stratified layers were highly disorganized and, in addition, unlike the Z130 culture (Fig. 3*D*), the HPV16 established cell lines exhibited an ability to penetrate the matrix and grow as microtumor-like masses (Fig. 3*E*); characteristics reminiscent of advanced cervical intraepithelial neoplasia and squamous cell carcinoma of the uterine cervix *in vivo* (22). The four HPV18 established cell lines (Figs. 3*F* and 4) share the ability of the HPV16 established cell lines to penetrate and grow within the matrix and are seen completely engulfing the matrix in Fig. 3*F*. These cell lines, however, exhibited the morphological features of nonstratified epithelia and were further characterized by the formation of clear zones within the cell mass, which resembled cysts, most evident in the Z183B culture (Fig. 4*D*). Electron microscopic analysis demonstrated that the cyst-like structures are lined with cells projecting numerous microvilli into these clear zones (unpublished observations), suggesting the presence of a morphological secretory component in these cell lines.

Anchorage-Independent Growth. All of the established cell lines failed to form colonies in soft agar when examined at early passage except for the SV40 transformed cell line. Z132, Z172 (HPV16), and Z173 (HPV18) remained negative for transformation by this criterion after an additional seven subcultivations (Table 1). In contrast, the Z133, Z183A, and Z183B (HPV18) cell lines eventually expressed the anchorage-independent growth phenotype (Table 1), albeit at a lower frequency than the postcrisis Z134 culture. Primary cervical epithelial cells did not form colonies in soft agar after an extended observation period of 8 weeks.

DISCUSSION

Normal human cervical epithelial cells exhibit a limited *in vitro* lifespan, senescing after 22–38 population doublings (13), and their spontaneous escape from senescence has not been reported. Electroporation of these primary cervical epithelial cells with either plasmid DNA or viral DNA from a genital papillomavirus (HPV6b), which usually induces benign lesions *in vivo*, did not alter the *in vitro* lifespan of the cells in three independent experiments. In contrast, two genetically related but distinct papillomaviruses, HPV16 and HPV18, strongly associated with cervical malignancy *in vivo* (5, 7, 23) and with proven oncogenic potential in murine and rat fibroblasts *in vitro* (17, 24–28), confer an extended lifespan on these cells as did SV40, a DNA tumor virus with proven immortalizing potential in a variety of human cells (12).

The differences in the immortalizing potentials of the three HPVs do not appear to be a result of linearization of the viral DNAs at different positions but more likely reflect the intrinsic oncogenic potential of these viruses. Hence, HPV6b, linearized at the *Bam*HI site (nucleotide 4722) interrupting the L2 open reading frame, is equivalent to the *Bam*HI (nucleotide 6150) linearized HPV16 in that the early open reading frames and polyadenylylation signals are intact in both viruses; however, the HPV6b fails to immortalize cervical epithelial cells. Furthermore, disruption of the E1 open reading frame in the HPV18 genome by linearization at the *Eco*RI site (nucleotide 2440) does not affect its ability to immortalize the cervical epithelial cells. The biological effects of HPV16 and -18 on the target cells differs from that of SV40, as demonstrated by the absence of an intervening "crisis period" characteristic of the same cells electroporated with SV40 and previously described for SV40-infected human foreskin-derived keratinocytes (11, 12).

Southern blot analysis established the presence of the specific viral DNAs introduced *in vitro* in the early passage cultures and the established cell lines (Table 1; Fig. 1). The HPV16 and -18 genomes are found exclusively integrated in our cell lines, except Z173, as has been reported for advanced premalignant and malignant lesions of the cervix (6, 29–32), although the presence of episomal forms at early time points, prior to 14 days postelectroporation, cannot be ruled out.

Recent evidence indicates that HPV16 and/or HPV18 produce different biological effects on murine and rat fibroblasts (17, 24–28) versus human foreskin-derived keratinocytes (33–35), the latter becoming immortalized without significant morphological alterations. This report is a comparative *in vitro* analysis of the biological effects of these two viruses, both associated epidemiologically with malignant alterations of the cervical epithelium (6, 29–32) and of a genetically similar virus (HPV6b), which produces benign lesions of the cervical epithelium as well as other genital

epithelia (4, 6, 14, 32, 36, 37), in their natural target cells. Our results support the epidemiological association of cervical malignancy with integrated transcriptionally active HPV16 or HPV18 viral DNAs (6, 29–32). The observed morphological differences between the HPV16 and HPV18 established cell lines, specifically stratified squamous epithelia vs. nonstratified cyst-forming epithelia, suggest that the viruses have induced precursors of the different histological subtypes of cervical carcinoma recently reported to be associated with the two HPV types (38) and support the role of these viruses as potential etiological agents of cervical carcinoma. It is unclear at this time whether the two viruses infect different target cells present in the transformation zone or preferentially alter the differentiation program of a single bipotential target cell.

The established cell lines offer a unique system in which to investigate the role of HPVs in the multistage process of cervical carcinogenesis in human cells as reflected for example in the progressive expression of the transformed phenotype *in vitro* by the Z133, Z183A, and Z183B cell lines. This system will allow us to investigate the regions of the viral genomes responsible for their host-cell specificity and pathogenicity, as well as virus–host-cell cooperative genetic interactions. Furthermore, the correlation between *in vivo* malignancy with the immortalization phenotype and growth properties in modified organotypical cultures may provide a means to evaluate the oncogenic potential of newly identified genital HPVs in their natural target cells.

Note. During the period in which this manuscript was being reviewed for publication, Woodworth *et al.* (39) reported the immortalization of human exocervical epithelial cells with HPV16 and HPV18 DNAs.

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