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Characterization of human cell lines transformed by HSV-2 and HPV-16 or HPV-18

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CHARACTERIZATION OF HUMAN CELL LINES TRANSFORMED BY HSV-2 AND HPV-16 OR HPV-18

by

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LIFE

Kavita Rao Dhanwada is the daughter of Geetha and Vaman Rao. She was born October 12, 1964, in Hyderabad, India. She came to the United States in 1971 and completed her primary education in Columbia, Missouri. Her secondary education was obtained at Macomb High School, Macomb, Illinois, where she graduated in 1982.

She attended the University of Illinois at Urbana-Champaign and received a Bachelor of Science degree in microbiology in May 1986. She was accepted into the doctoral program in the Department of Microbiology of Loyola University of Chicago Medical Center and became a Ph.D candidate in 1988.

In 1989, she married Ramakrishna Dhanwada. She subsequently moved to Lincoln, Nebraska to complete her dissertation project at the University of Nebraska - Lincoln.

Ms. Dhanwada is co-author of the following publications and abstracts:

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INTRODUCTION

This study examines the effect of two human DNA viruses on primary human cells. Herpes simplex virus 2 (HSV-2) and either human papillomavirus type 16 or type 18 (HPV-16, HPV-18) DNA was introduced into human cells to generate transformed cell lines. Cell lines were established with two human cell types: keratinocytes, the naturally infected cell in vivo by HPVs and HSV-2, and fibroblasts. Although fibroblasts are not the target cell of HPVs, they were utilized because of the availability of two cell strains: normal primary gingival fibroblasts (N-16) and a hyperplastic counterpart (R-30). It has been hypothesized that transformation is a multistep phenomenon (reviewed in Bishop, 1985). The primary aim of this investigation was to determine if HSV-2 and HPV-16/18 cooperate to morphologically transform primary human cells.

Transfection of primary cells with HPV types 16 or 18 DNA alone produces an immortalized cell. These cells do not produce tumors when heterotransplanted into immunosuppressed mice (Durst et al., 1987, Pirisi et al., 1987) suggesting progression to the malignant phenotype after HPV infection in vivo requires additional cofactors. HSV-2 is hypothesized to

cooperate with oncogenic HPVs to produce a malignant phenotype (zur Hausen, 1982). Keratinocyte or fibroblast HPV immortalized cell lines were compared with respective HPV/HSV-2 transformed cell lines in four major areas of study. The first area examined was growth characteristics. Saturation density analysis and anchorage independent growth studies demonstrated that transformed cells proliferated more efficiently than their corresponding immortalized counterparts.

The second area of study was to determine if viral DNA was retained in the various cell lines. HPV sequences are present in 80-85% of cervical carcinoma biopsies (Broker and Botchan, 1986, Durst et al., 1983) and are usually integrated into the cellular genome with only a portion of the viral DNA present (Durst et al., 1985). Transformed cell lines were analyzed by Southern blot analysis to determine retention and genomic organization of HPV DNA as well as to study the physical state of HPV DNA. Retention of HSV-2 DNA sequences in transformed cells was examined by polymerase chain reaction (PCR) analysis. Approximately 50% of the cell lines retained HSV-2 DNA sequences. Comparison of HPV immortalized keratinocytes with HPV-18/HSV-2 transformed keratinocytes showed rearrangement of HPV DNA in the transformed cells in contrast to the immortalized cell lines.

Integration of HPV-16 DNA into the cellular genome has been shown to cause alterations of the c-<u>myc</u> protooncogene

(Riou et al., 1987, Durst et al., 1987, Crook et al., 1990) and organization of this gene in transformed cell lines was analyzed. Interactions of the tumor suppressor gene, p53, and subsequent inactivation of this gene are associated with high risk HPVs such as type 16 and 18 (Werness et al., 1990, Scheffner et al., 1990). To understand what role this cellular protooncogene and tumor suppressor gene have in the morphological transformation of primary human cells was the third aim of this study.

The final area of analysis was to examine the tumorigenic potential of the immortalized and transformed cell lines. Nude mice were injected subcutaneously with different cell lines and examined for the production of palpable tumors.

LITERATURE REVIEW

Multistep phenomenon of transformation. Morphological transformation of primary cells is a multistep phenomenon (reviewed in Bishop, 1985, Stanbridge, 1990). Many mechanisms exist in the etiology of human malignancy, however, it is not known what specific factors are required for progression. Malignant tumors are thought to result from a series of events which are cumulative with each subsequent event generating an additional phenotypic alteration. Genetic aberrations, due to endogenous or exogenous stimulation, which extend cellular lifespan, can allow for overall genetic instability within these cells (Meuth, 1990).

Several factors are associated with instability leading to progression. Loss or gain of entire chromosomes can lead to severe aneuploidy (German, 1983), a common characteristic of cancer cells. Large scale deletions within chromosomes (Vogelstein et al., 1989) can also lead to loss or rearrangment of regulatory genes resulting in an increased risk of mutation or gene amplification (reviewed in Meuth, 1990, Giulotto et al., 1987). A specific order of events is not required, rather the summation of these events is hypothesized to be the primary factor in carcinogenesis

(reviewed in Bishop, 1991).

Environmental and chemical mutagens, viral oncoproteins and hereditary factors are associated with carcinogenesis. Several viruses have recently been correlated to the etiology of human tumors: 1) hepatitis B virus associated with cancer of the liver (Wang et al., 1990), 2) Epstein-Barr virus associated with Burkitt's lymphoma and nasopharyangeal carcinomas (Raab-Traub et al., 1983, Fahreus et al., 1988), 3) human papillomaviruses and 4) herpes simplex virus 2 associated with genital carcinomas (Rawls et al., 1969, DiLuca et al., 1989).

Cancer of the cervix is the second leading cancer in women (Parkin et al., 1988). Epidemiological studies of precancerous lesions and invasive cervical carcinomas have demonstrated a correlation between these lesions and sexually transmitted disease (STD). The major risk factors of cervical carcinoma are the early age of intercourse and multiple sexual partners (Rotkin, 1973, Kessler, 1977). The prevalence of viral and viral-associated antigens as well as viral nucleic acid in many anogenital lesions associated with STD has suggested human papillomaviruses (HPVs) are the etiological agents of many of these STD lesions (Becker et al., 1987). With approximately one sixth of all cancer deaths in females due to cervical cancer, there is significant interest in understanding the pathogenesis of HPVs in genital cancer (Parkin et al., 1988).

Classification of PVs. The papillomaviruses (PVs) are a group of small DNA viruses in the Papovaviridae (Ginsberg, 1980). They typically induce proliferative lesions of the skin and mucosal epithelia. PV infections are widespread in mammals affecting cattle, sheep, deer, dogs, monkeys, pigs or man (Sundberg, 1987). Classification is based on host range with type categorization based on nucleic acid hybridization studies. Viral isolates are considered an independent type if there is less than 50% homology with known isolates (Coggin and zur Hausen, 1979).

PVs are the etiological agents of many epithelial disorders. Specific PV types are associated with specific disease processes ranging from warts to human cervical carcinoma. Oncogenic activity of PVs was first described in rabbits where cottontail rabbit papillomavirus (CRPV) induced benign papillomas which later metastisized to lymph nodes and lungs (Rous and Beard, 1935). Over 60 types of human papillomaviruses (HPV) have been identified. Each HPV type has site preferences for either cutaneous or mucosal squamous epithelium where many can cause clinical lesions (Jenson et al., 1988). Benign proliferative lesions of cutaneous epithelium are referred to as warts whereas mucosal lesions are called condylomas.

Approximately two thirds of HPVs infect skin while the remaining types infect keratinizing and nonkeratinizing squamous epithelia of the anogenital tract or the oral mucosa

(Jenson et al., 1988). These mucosotrophic HPVs can be classified with respect to oncogenic potential as either low, intermediate, or high risk. HPV-6 and -11 are found primarily in genital condylomata (Gissman and zur Hausen, 1980, Gissman et al., 1983), rarely detected in cervical carcinomas, and thus are regarded as low risk viruses. HPV-31, -33, and -35 are found in approximately 10% of cervical dysplasias, primarily premalignant lesions (Lorincz et al., 1987, Fuchs et al., 1988), and consequently classified as low or intermediate risk types. HPV-16 and -18 are associated with the majority of invasive cervical carcinomas, the viral DNA can be detected in 85% of cervical carcinoma biopsies (Broker and Botchan, 1986), and thus are regarded as high risk types (Yoshikawa et al., 1985, McCance et al., 1985).

Although the highest incidence of carcinomas associated with HPV infections is in anogenital tissue, specific HPVs can also infect other mucosal surfaces such as the orolaryngeal and bronchial mucosa. HPV-6 and 11 are frequently detected in benign laryngeal and bronchial papillomas (Gissmann et al., 1982, Mounts et al., 1982, Ikenberg et al., 1985) while HPV-16 is also associated with some orolaryngeal tumors, tongue carcinonomas (deVilliers et al., 1985), carcinomas of the buccal mucosa (Loning et al., 1985) and lung carcinomas (Stremlau et al., 1985). However, these cancers are relatively infrequent compared to carcinoma of the cervix.

Growth characteristics of HPVs. Specific phases of the

HPV infection cycle are restricted to differentiated epithelial keratinocytes. The infection cycle begins in the proliferating basal layer of epithelial cells. PV infection appears to require physical contact of virus to basal cells. The cells which are between the squamous and columnar epithelium such as those in the nasal mucosa, the larynx, the cervix, and at borders of healing wounds are called transformation zones (T-zones) and are especially susceptible to HPV infection. These T-zones are a common target for HPV infection (reviewed in Broker and Botchan, 1986). The mechanism of HPV infection and subsequent early gene expression and viral replication is not well understood primarily because a cell culture system is not available. Although it is possible to grow human keratinocytes in vitro, the inability to differentiate these cells does not allow for a complete infection cycle. However, Kreider et al., (1985) have developed a animal model system to produce an active HPV-6 infection. Virus-infected cells are engrafted beneath the renal capsule of mice resulting in the production of putative precancerous lesions. Virus particles can be isolated from these lesions. Viral replication occurs in the basal cells since in squamous epithelium, keratinocytes only proliferate in the basal layer. When cells leave this layer, they undergo a sequence of biochemical and phenotypic changes which constitutes terminal differentiation. Ultimately, these cells are sloughed off as fully differentiated cells (reviewed in

Eckert, 1989). Viral DNA synthesis has been detected by in situ hybridization studies in the stratum spinosum (spiny layer) as well as the stratum granulosum (granular layer) of the epidermis (Wagner et al., 1984). Thus the lifecycle of PVs appears to be closely linked to the state of differentiation of squamous epithelial cells (reviewed in Orth et al., 1977).

Latent HPV infections represent the largest reservoir for mucosotrophic HPV infections (Jenson and Lancaster, 1990). Latently infected tissue does not exhibit any unusual morphology compared to normal squamous epithelium (MacNab et al., 1986) and viral DNA can be detected only by Southern or dot blot analysis of infected tissue. It is not known what induces the latent infection to become active although mechanical or chemical trauma and infection of regenerating squamous cells may lead to recurrence (Ferenczy et al., 1985).

Molecular characteristics of HPV. The genome of papillomaviruses consists of a circular double stranded DNA molecule of approximately 8 kilobase pairs (kbp). The viral DNA is complexed with histones and has an approximate molecular weight of 4.5×10^6 daltons. The viral genome is encapsidated in a 55 nm icosahedral shell and the virion lacks a lipid containing envelope (Pfister and Fuchs, 1987). The genomes of several PVs have been sequenced (Danos et al., 1982, Schwarz et al., 1983, Seedorf et al., 1985, Cole and Danos, 1987) and it is clear that all PVs have a compact and

rather similar genomic organization. All putative open reading frames (ORFs) as well as the long control region (LCR) are on one DNA strand. The early (E) genes encode proteins which are involved with oncogenic transformation, DNA transcription, and replication. The late (L) genes encode the structural proteins carrying type-specific and species specific antigenic determinants (Broker and Botchan, 1986).

The LCR of HPVs is the noncoding 10% region of the viral genome. It contains transcriptional regulatory elements which contribute to the epitheliotrophic nature of HPVs. These elements include a tissue specific enhancer (Gius et al., 1987, Cripe et al., 1987) bound by a cervical cell type specific octamer binding protein (Dent et al., 1991), glucocorticoid response elements (GREs) (Gloss et al., 1987), interferon-responsive elements (Cohen et al., 1988), epidermal growth factor response element (EGFRE) (Yasumoto et al., 1991) as well as transcription factor binding sites for AP-1 (Garcia-Carranca et al., 1988), NFI (Gloss et al., 1989) and keratinocyte specific transcription factors (Mack and Laimins, 1991). DNase I footprinting analysis of the HPV LCRs detected 23 sites specifically bound by nuclear proteins (Gloss et al., 1989). It appears the epithelial specificity of HPV is due to a synergism between cellular factors found at certain concentrations in epithelial cells rather than unique factors present in these cells (Chong et al., 1991).

The majority of the HPV genome has been functionally

mapped. ORF E2 encodes a pleiotropic protein which functions as a transcriptional trans-activator (Phelps and Howley, 1987) and a transcriptional repressor (Cripe et al., 1987). This protein binds as a dimer to conserved palindromic motifs repeated several times within the LCR (Gloss et al., 1987). The E2 protein binds to SP1 sites to transactivate transcription (Li et al., 1991) while a truncated E2 represses transcription (Cripe et al., 1987). ORF E4 is the most abundant gene product in cutaneous HPV-1 infections (Doorbar et al., 1986), although no specific function has been assigned. The functions of ORFs E5 and E1 are not yet known. ORFs E6 and E7 encode two proteins which can: 1) immortalize rodent and human cells (Matlashewski et al., 1987, Kanda et al., 1988, Watanabe et al., 1988, Munger et al., 1989), 2) transactivate heterologous gene expression (Phelps et al., 1988, Grossman et al., 1989), 3) stimulate cellular DNA synthesis (Sato et al., 1989) and 4) play a role in the maintenence of viral DNA (Berg et al., 1986). E6 and E7 are both nuclear proteins (Grossman et al., 1989, Smotkin and Wettstein, 1986). E7, the major protein in cervical carcinoma tissue, is a 21 kd phosphoprotein (Smotkin and Wettstein, 1986). L1 and L2 encode the major and minor structural proteins (Doorbar and Gallimore, 1987).

Pathogenesis of HPV. Integration of HPV DNA into the cellular genome was accepted as a prerequisite for progression to cervical cancer since many carcinomas contained integrated

viral DNA while benign HPV lesions contained episomal HPV DNA (Durst et al., 1985). However, both episomal and integrated forms of complete HPV-16 genomes are present in invasive cervical carcinomas (Matsukura et al., 1989). Thus, integration may not be required for malignant progression but it is the predominant form of HPV DNA in malignant lesions and thought to play an important role.

Viral integration usually results in the disruption of the E1 and/or E2 ORFs and retention of E6, E7 ORFs and the LCR (Durst et al., 1986, Matsukura et al., 1986, Pater and Pater, 1985) with subsequent deletion or rearrangement of cellular sequences (Wagatsuma et al., 1990, Choo et al., 1990). Wagatsuma et al., (1990) analyzed several cervical carcinoma clones containing HPV-16 DNA flanked by cellular DNA sequences to determine if there is specific integration. No specific target sequence was found either in the cellular genome or in the HPV-16 viral genome, however, integration frequently occurred in the E1/E2 ORF (Wagatsuma et al., 1990). Therefore, viral integration frequently disrupts the E1/E2 ORF but not at a specific site within the viral genome.

Although propagation of HPV has not been possible <u>in vitro</u>, the availability of recombinant viral DNAs has allowed for transformation studies of high-risk HPVs specifically, HPV-16 and HPV-18. Early genes, E6 and E7, are intact and expressed in nearly all malignant lesions (Pater and Pater, 1985, Matsukura et al., 1986). Both E6 and E7 of high-risk

HPVs are required to immortalize primary human cells, including keratinocytes (Durst et al., 1987, Kaur and McDougall, 1988, Woodworth et al., 1989) and fibroblasts (Pirisi et al., 1987, Watanbe et al., 1989). E7 alone can transform established rodent cells (Kanda et al., 1988, Bedell et al., 1989), cooperate with an activated ras to transform primary cells (Matlashewski et al., 1987, Phelps et al., 1988) and induce chromosomal abnormalities in murine keratinocytes (Hashida and Yasumoto, 1991). High-risk HPV infection of primary cells does not usually induce tumors in nude mice, however, long term cultivation may result in a malignant clone (Hurlin et al., 1991). The role of E6/E7 in the initiation and maintenence of transformation was demonstrated by von Knebel Doeberitz et al., (1988). Transfection of HPV-18 positive tumor cells (Hela), with hormone-dependent anti-sense E6/E7 plasmid constucts resulted in reduced cell growth and nontumorigenecity of HPV-containing cervical carcinoma cells. A reduction of E6/E7 mRNA and E7 protein levels was detected when compared to transfection with the sense orientation of E6/E7 constructs (von Doeberitz et al., 1988). Thus, E6/E7 ORFs play a role in the maintenence of the malignant phenotype.

Many subtle differences exist between the oncogenic and nononcogenic HPV types. The physical state of viral DNA differs in these two types of infections. In low-risk infections, the DNA remains episomal, whereas in high-risk

infections, the viral DNA is primarily integrated (Durst et al., 1985). The E6/E7 ORFs of the high-risk HPVs can immortalize primary human cells while low risk HPVs cannot (Sato et al., 1989). The generation of E6/E7 mRNAs also differs. The major transcript of mucosotrophic HPVs (both high and low risk) is E7. In high-risk HPVs, E7 is generated from a colinear E6/E7 transcript by splicing out an intron within the E6 ORF. Thus a full length E7 mRNA is synthesized along with two short E6 mRNAs (E6*I and E6*II) (Smotkin et al., 1989). In contrast, no splice site is present in low-risk HPVs and thus only full length E6 and E7 transcripts are generated. E6*I and E6*II were thought to encode type-specific transforming peptides, however no truncated proteins were isolated. The function of the splice within E6 appears to facilitate translation of E7 mRNA and reduce translation of full length E6 rather than to generate active E6* proteins (Sedman et al., 1991).

A common effect of infection with high risk HPVs is growth stimulation of cells (Hawley-Nelson et al., 1989, Sato et al., 1989) leading to chromosomal abnormalities resulting in cell aneuploidy (Durst et al., 1987, Smith et al., 1989). Cytogenetic abnormalities were correlated with high-risk E7 gene functions but not with E6 (Hashida and Yasumoto, 1991). Aneuploidy is seen primarily with high risk HPV infections, while low risk HPVs do not usually cause chromosomal aberrations (Fu et al., 1981). Thus, the ability of E7 to

induce chromosomal abnormalities may be important with respect to its immortalizing function.

Role of cellular factors in progression. Specific cellular proteins also differ in their ability to bind HPV viral oncoproteins. The E7 oncoprotein of high-risk HPVs binds to the retinoblastoma (RB) protein with a 10-fold higher affinity than E7 proteins of low-risk HPVs (Dyson et al., 1989). E6 binds to the tumor suppressor protein, p53 (Werness et al., 1990) and promotes degradation of this cellular protein (Scheffner et al., 1990). Both p53 and RB proteins are thought to play a role in cellular proliferation by negatively regulating cell growth (Sturzbecher et al., 1990, DeCaprio et al., 1989). Mutations of these genes lead to deregulation of the cell cycle resulting in hyperproliferation and chromosomal instability (Hudson et al., 1990, Woodworth et al., 1990) similar to the effect seen with the E6/E7 gene products of high-risk HPVs. Therefore, these cellular proteins may also be involved in the progression of high-risk HPV infections.

Cell specific factors may also play a role in HPV progression. Fusion of HPV positive cervical carcinoma cells (Hela) with active E6/E7 expression and normal human cells (fibroblasts) produced nonmalignant hybrids suggesting cellular factors can override the effect of or downregulate E6/E7 expression (Bosch et al., 1990). The suppressive effect of cellular factors was also demonstrated when the HPV-18 LCR

was placed upstream of the reporter gene chloramphenicol acetyltransferase (CAT). Resulting CAT expression was extinguished in hybrids of cervical carcinoma cells (SiHa) fused with nontumorigenic immortalized keratinocytes. Cycloheximide treatment (4 hrs) of hybrids resulted in the reappearance of CAT expression suggesting suppression was due to a labile protein (Rosl et al., 1991) present in nonmalignant keratinocytes. Chromosomal aberrations may also play a role in HPV pathogenesis. It appears HPV-16 gene expression in primary human fibroblasts is stimulated by a deletion in one copy of chromosome 11 (Saxon et al., 1986).

Several studies have demonstrated that integration of high-risk HPV DNA into the cellular genome results in alterations of the c-myc protooncogene (Riou et al., 1987, Durst et al., 1987, Crook et al., 1990). Amplification of the c-myc gene (Ocadiz et al., 1987) and enhanced c-myc RNA expression (Crook et al., 1990) was observed in several cervical carcinomas. HPV-18 integration results in specific activation of myc genes in genital tumors (Couturier et al., 1991). Taken together this suggests the c-myc oncogene may also play a role in the pathogenesis of cervical cancer.

The long latency period, the relatively low number of high-risk HPV infected individuals who develop progressive cervical cancer as well as the presence of HPV-16 in histologically normal tissue (MacNab et al., 1986, Tidy et al., 1988) suggests that HPV alone is not sufficient for

progression to invasive carcinoma. It has been hypothesized that herpes simplex virus 2 (HSV-2) cooperates with the oncogenic HPVs to produce the malignant phenotype (zur Hausen, 1983).

Growth characteristics of HSV-2. HSV-2 is a large human DNA tumor virus of the Herpesviridae family. Classified as an alphaherpesvirus, along with herpes simplex virus 1 (HSV-1) and varicella-zoster virus (VZV), it can replicate efficiently in a wide host range, spread rapidly in culture due to a short life cycle, and cause complete lysis of host cells. In addition to a lytic cycle, alphaherpesviruses cause latent infections in sensory neurons (Roizman, 1985). The HSV-2 virion consists of an enveloped icosahedral capsid enclosing a DNA containing viral core. The approximate size of the viral genome is 150 kbp. The structure of the HSV genome includes two regions of unique sequences, U_L and U_S, each flanked by a set of inverted repeats (reviewed in Roizman, 1985).

Cytopathic effect (CPE) of replicating virus on host cells is seen by an enlargement of cells and dissolution of cell nuclei. Viral CPE occurs in the parabasal cells of the squamous epithelium. A vesicle filled with cell debris, inflammatory and multinucleated giant cells forms under the cornified epithelium as cell lysis progesses. The lesion can spread into adjacent parabasal cells and rupture, releasing viral antigens and evoking a subsequent host cell response

(reviewed in Rawls, 1985).

HSV-2 is primarily spread by sexual contact and risk factors associated with these infections are similar to those of other sexually transmitted diseases (STD; Stavraky et al., 1983). In fact, many STD patients have concurrent HSV-2 infections (Knox et al., 1982). While most HSV-2 genital infections are not life threatening, an association between HSV-2 and cervical carcinoma seems clear (Rawls et al., 1969, Naib et al., 1973, McDougall et al., 1980, Nahmias and Norrild, 1980, DiLuca et al., 1989, Hildesheim et al., 1991).

Role of HSV-2 in transformation. Duff and Rapp (1971, 1973) demonstrated that HSV-2 can transform mammalian cells. Immortalization of hamster embryo fibroblasts was induced by ultraviolet (UV)-inactivated HSV-1 virus (Duff and Rapp, 1973). Immortalization by inactivated HSV-2, either UV or photodynamic, was also demonstrated in rodent (MacNab, 1974) and human cells (Takahashi and Yamanishi, 1974). Thus, inactivated HSV-2 virus will transform human cells, however, virus is not recovered (Takahashi & Yamanishi, 1974, Kucera and Gusdon, 1976). Early experiments suggested a "hit and run" mechanism to describe HSV transformation since the continued presence of HSV was not required for transformation (Skinner, 1976, Galloway and McDougall, 1983).

Within the HSV genome, three unique morphological transforming regions (mtr) have been identified. Mtr I is contained in the HSV-1 genome and maps to a XbaI-F fragment

(map unit 0.30 - 0.45) (Camacho and Spear, 1978). The HSV-2 genome contains two regions capable of transforming cells, mtr II and mtr III. Mtr II is within the Bgl II-N fragment (map unit 0.585 - 0.63) while mtr III is contained within the Bgl II-C fragment (map unit 0.42 - 0.58) (Galloway and McDougall, 1981, Jariwalla et al., 1983). The minimal transforming region of mtr II is a 730 bp fragment (Galloway et al., 1983). The HSV-2 mtr III contains a minimal transforming fragment of 486 bp (486 TF) which apparently does not encode an oncogenic protein (Jones et al., 1986). Rather, the viral DNA contains multiple regions of alternating purines and pyrimidines, G+C rich sequences, potential SP1 transcription factor binding sites, stem-loop structures and insertion-like elements (Jones et al., 1986). 486 TF also functions as a complex transcriptional promoter element (Jones, 1989). It was hypothesized that the multiple repetitive DNA sequence motifs associated with recombination and gene activation in 486 TF alter cellular gene expression leading to transformation (Jones, 1989).

Function of RR in HSV-2 mediated transformation. The role played by the HSV encoded ribonucleotide reductase (RR) proteins in viral transformation has been examined since mRNAs encoding this dual subunit protein are within the Bgl II-C and Bgl II-N fragments (McLauchlan and Clements, 1982, Swain and Galloway, 1986). HSV RR is composed of a large subunit, RR1, and a small subunit, RR2 (Cohen et al., 1985, Frame et al.,

1985, Bachetti et al., 1986). The two transcripts have 3'-coterminal ends with the 1.2 kb mRNA encoding RR2 contained within Bgl II-N. The 5.0 kb mRNA encoding RR1 has all but the carboxy terminus within Bgl II-C (McLauchlan and Clements, 1982, Swain and Galloway, 1986). HSV-2 RR1 was proposed as a transformation specific protein (Huszar and Bacchetti, 1983), however, 486 TF does not encode the catalytic function of the RR1. This suggests that RR1 expression is not required to induce transformation, at least in rodent cells (Jones et al., 1986). The amino terminus of RR1 contains a 355 hydrophilic amino acid (aa) domain which does not share homology with other herpesvirus RR enzymes (Swain and Galloway, 1986). It was recently demonstrated that a 290 aa polypeptide from this domain was able to induce enhanced malignant transformation of rodent cells (Ali et al., 1991) suggesting the entire RR1 protein is not necessary for transformation. In summary, the exact role RR1 or RR2 plays in neoplastic transformation has not been established.

Mutagenic effect of HSV-2. The specific mechanisms of transformation of HSV are not known. However, certain aspects of HSV resemble chemical mutagens which induce random cellular alterations. Since HSV-2 transformation does not appear to require retention of viral DNA and a HSV encoded oncoprotein has not been identified, it was hypothesized that HSV-2 could induce stable, heritable changes in the cell which ultimately leads to transformation (Clarke and Clements, 1991).

Herpesviruses efficiently induce chromosomal aberrations such as breaks and rearrangments (Hampar and Ellison, 1961, Bejcek and Conley, 1986) and act as a biological mutagens. Damage is followed by cellular DNA repair (zur Hausen,1983). HSV-2 infections of nonpermissive cells have been shown to increase the mutation frequency of the cellular hypoxanthine-guanine phosphoribosyl transferase (HGPRT) gene by 2 to 10 fold (Pilon et al., 1985). The mutation rate of HGPRT was 5 to 10 fold higher than background when human cells were infected with inactivated HSV-1. The effect was comprable to the chemical carcinogen 4-nitroquinoline-1-oxide (4-NQO) (Schlehofer and zur Hausen, 1982). Neither viral replication nor gene expression is required for the mutagenic effect of HSV (Clarke and Clements, 1991). Thus, mutagenesis may be an important mechanism used by HSV in carcinogenesis.

Another mechanism by which HSV is suggested to transform cells is gene amplification. Studies have demonstrated inactivated HSV can amplify genes with similar efficiencies as chemical carcinogens (Lavi, 1981, Schlehofer et al., 1983). HSV-2 DNA secondary structure has been implicated in the transformation process. Both the minimal transforming fragments of Bgl II-N (730 bp) (Galloway et al., 1983) and Bgl II-C (486 TF) (Jones et al., 1986) have unique DNA sequences. Both are able to form stem-loop structures which may promote DNA insertions and transpositions (Jones et al., 1986). Additionally, 486 TF contains sequences able to form Z-DNA

which is implicated in homologous recombination and chromosomal destabilization (Nickol et al., 1982). Recombination between HSV-2 and cellular DNA can lead to deregulation of cellular genes (MacNab et al., 1985, Leavitt et al., 1985). Viral recombination also results in the excision of extrachromosomal plasmids containing viral DNA sequences fused to altered cellular sequences (Jariwalla et al., 1986). Therefore, HSV-2 DNA structure may be an important factor in transformation. Recently, the ability of HSV-2 to induce cervical neoplasia was demonstrated when inactivated whole virus was applied to the mouse cervix and analyzed for the presence of premalignant and malignant lesions (Anthony et al., 1989). Lesions, similar to those seen in women with cervical carcinoma, were detected in 61% of the mice of which 21% were invasive carcinomas. No cancers were detected in control animals treated with uninfected HEp-2 cellular DNA or calf thymus DNA (Anthony et al., 1989). Thus, in an animal model HSV-2 can induce cervical carcinoma.

Epidemiology of HPV/HSV-2 in cervical carcinoma. Studies assessing the possible correlation between HSV-2 DNA and high-risk HPVs in the pathology of cervical carcinomas have looked for the simultaneous presence of both viral DNAs in genital tumor tissues (DiLuca et al., 1987, 1989, Hildesheim et al., 1991). Thirty percent of genital tumor tissue had homology to HSV-2 mtr II and mtr III fragments (DiLuca et al 1989) while 6/8 tumors (DiLuca et al., 1989) and 8/8 tumors (DiLuca et

al., 1987) showing homology to HSV-2 DNA sequences were also positive for HPV-16/18 DNA. Seroepidemiological studies have shown that women with cervical cancer had higher levels of HSV-2 specific antibodies than controls (Kaufman and Adam, 1986). Hildesheim et al., (1991) found a significant interaction between HSV-2 and HPV-16/18. In HPV 16/18 positive women, HSV-2 seropositive women had a 60% increased risk of cervical cancer compared to seronegative women. These results suggest a possible synergism between these two oncogenic viruses. A transformed keratinocyte cell line with HSV-2 mtr II DNA and HPV-16 DNA can produce tumors in nude mice. In contrast, HPV-16 immortalized parental cells do not (DiPaolo et al., 1990) implying Bgl II-N plays a role in the progression to carcinoma. Thus, several lines of evidence, molecular as well as epidemiological, implicate HSV-2 and HPV-16/18 as cofactors in the development of cervical carcinoma.

The frequent incidence of high-risk HPV nucleic acids in cervical carcinoma biopsies has suggested these viruses play a role in the induction of genital cancer. However, the presence of high-risk HPV containing tissue which is histologically normal (MacNab et al., 1986) suggest other factors play a role in progression. HSV-2, with its ability to act as a biological mutagen, is a viable candidate to mediate the progression of high-risk HPV infection to genital cancer.

METHODS AND MATERIALS

Cells. Human fibroblast cells, R-30, N-16, and C-1, were obtained from Dr. Tom Hassell. These cells were split in 1:3 ratios weekly and maintained in Earles modified Eagles medium (Sigma) supplemented with 10% fetal bovine serum (Sigma), penicillin (10 U/ml), and streptomycin (100 ug/ml). Primary human keratinocytes, FE-A, and FEP-L (HPV-18, HPV-16 immortalized cell lines) were obtained from Dr. James McDougall. Keratinocytes were maintained in serum-free keratinocyte medium (Gibco) supplemented with epidermal growth factor (5 ng/ml) and bovine pituitary extract (50 ug/ml), penicillin (10 U/ml) and streptomycin (100 ug/ml). Cells were split 1:4 weekly.

Plasmids. Plasmid HZIP-16 was obtained from Dr. G. Matlashewski and contains 6.6 kb of the HPV-16 genome flanked by the Moloney murine leukemia virus long terminal repeats (Matlashewski et al., 1987). A 1.3 kb fragment of the L1 ORF was deleted for cloning purposes. All other ORFs are undisturbed. Plasmid pEJ6.6 containing the Ha-ras oncogene was obtained from ATCC (#41028, Rockville, MD). Plasmid pHPV18 containing the entire HPV-18 genome (7857 bp) was obtained from Dr. James McDougall. Two c-myc plasmids were

used in the analysis. Plasmid p5-UTR containing a 1.6 kb Sac I fragment of the 5'-untranslated region of the c-myc gene, was obtained from ATCC (#39286, Rockville, MD) and plasmid pEX2, containing a 420 bp Pst I fragment of exon 2 of c-myc DNA, was obtained from Dr. Len Erickson. Plasmid php53 containing a 2.1 kb Bam HI human p53 cDNA insert was obtained from Dr. Moshe Oren. All of the HSV-2 plasmids contained various sequences derived from HSV-2 mtr III and contained the ampicillin resistance gene (Jones et al., 1986).

Calcium phosphate transfection of fibroblasts. Low passage R-30 cells or N-16 cells were transfected with 25 ug DNA using calcium phosphate precipitation (Graham and Van der Eb, 1973). A 25% DMSO shock was used to facilitate uptake of DNA following calcium phosphate precipitation. Seventy two hours after transfection, the antibiotic geneticin (Sigma) was added to the cultures (400 ug/ml) to select for cells containing plasmids. All cultures were cotransfected with pSV2neo (5 ug/60mm dish). Geneticin selection was carried out for 2-3 weeks post transfection or until all the cells in the control dish were dead. When densely growing colonies became macroscopic in size, the cells were trypsinized and subcultured as described above in the absence of geneticin.

Polybrene transfection of keratinocytes. Due to the toxicity of calcium phosphate on keratinocytes, the polybrene transfection method (Rhim et al., 1986) was used for these cells. Polybrene, hexadimethrine bromide (Sigma), is a

cationic compound which disrupts cell membranes and allows for DNA passage into cells. Either normal human keratinocytes (NHK) or immortalized HPV cell lines, FE-A containing HPV-18 or FEP-L containing HPV-16, were transfected with 10 ug plasmid DNA using 10 ug polybrene (1 mg/ml) in 1 ml serum-free medium (SFM). Cultures were incubated at 37° C and rocked every hour. A 30% DMSO shock (4 minutes) was performed after 6 hours to facilitate uptake of DNA. All cultures were cotransfected with pSV2neo (2 ug/60 mm dish). Forty eight to seventy two hours after transfection, geneticin was added to cultures (50 ug/ml for FE-A cells and 500 ug/ml for FEP-L cells) to select for cells with plasmids. Geneticin selection was carried out for 3-5 weeks post transfection or until the control cells were dead. When densely growing geneticin resistant cells became macroscopic, cells were trypsinized and cultured in the absence of geneticin.

Isolation of high molecular weight DNA. This procedure has been previously described by Maniatis et al., (1986). Tissue culture cells were washed and trypsinized. Cells were centrifuged for 10 minutes. The pellet was washed in TKMC solution (10 mM Tris pH 7.5, 10 mM KCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$) and resuspended in 500 ul TKMC solution and 25 ul 10% Triton X-100 and incubated on ice for 5 minutes. The cell suspension was centrifuged and the pellet resuspended in 250 ul TNE solution (50 mM Tris pH 7.5, 100 mM NaCl, 10 mM EDTA), 2.5 ul 10% SDS and 100 ug RNase A. Samples were incubated at 37° C

for 1.5 hours and 7.5 ul Proteinase K (20 mg/ml) was added and samples were incubated overnight at 37° C. The samples are extracted two times with phenol:chloroform:isoamyl alcohol (50:49:1). Two volumes of 95% ethanol was added and samples were shaken for an additional 30 minutes followed by a 5 minute spin. The ethanol precipitation was repeated. The pellet (after a second ethanol wash) was dried and resuspended in TE buffer (10 mM Tris-Hcl, pH 7.4, 1 mM EDTA). The genomic DNAs were digested with the indicated restriction enzyme (10 U/ug). The quantities of DNA was estimated by electrophoresing on a 1% minigel.

Plasmid DNA isolation. Large scale isolation and minipreparations of plasmid DNA were performed by the alkaline lysis method described by Maniatis et al., (1989). For large scale isolation, the bacteria with the respective plasmid were shaken in overnight 5 mls of Luria broth (LB) with 50 ug/ml ampicillin (Sigma). This 5 ml preculture was used to inoculate a one liter LB culture containing 50 ug/ml ampicillin. The culture was incubated at 37° C with vigorous shaking until it reached an $O.D._{595}$ of 0.6-0.7. Fifty ug/ml chloramphenicol (Sigma) was subsequently added to cultures and incubation with shaking was continued overnight. Bacteria were harvested by centrifugation at 8000 rpm for 10 minutes at 4° C in a Beckman JA-10 rotor. The pellet was resuspended in 9 ml of solution I containing 50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0. Bacterial membranes were

disrupted by addition of 1 ml fresh lysozyme solution (5 mg lysozyme in solution I) and incubated for 5 minutes at room temperature. Cells were lysed by addition of 20 ml of solution II containing 0.2 N NaOH, 1% SDS (w/v) and kept on ice for 10 minutes. Twenty ml of an ice-cold solution of 5 M potassium acetate (pH 4.8) was added and tubes were shaken vigorously and subsequently incubated on ice for 10 minutes. The lysates were transferred to two 50 ml Oak Ridge tubes and centrifuged at 20,000 rpm at 4° C in a Beckman JA-20 rotor for 20 minutes. The supernatant was transferred to 2 new Oak Ridge tubes and 0.6 volume of isopropanol added. DNA was precipitated for 15 minutes at room temperature and tubes were centrifuged for 30 minutes at 20° C in the Beckman JA-20 rotor. The pellets were resuspended in 2.4 ml TE buffer.

For purification of supercoiled plasmid DNA, cesium chloride (CsCl)-ethidium bromide equilibrium centrifugation was performed. For each ml of DNA solution, 1 g CsCl and 0.8 ml ethidium bromide (10 mg/ml) was added. The samples were centrifuged in a Beckman VTi70 rotor at 50,000 rpm for 20 hours at 20° C. Closed circular plasmid DNA was removed by side puncture and the CsCl gradient was repeated. Ethidium bromide was removed by repeated extraction with isoamyl alcohol saturated with CsCl. Plasmid DNA was precipitated by adding 8 ml TE buffer (to reduce CsCl concentration), two volumes of ethanol and incubated at -20° C overnight. DNA was pelleted by centrifugation at 12,000 rpm for 30 minutes at 4°

C and resuspended in TE buffer.

For minipreparations of plasmid DNA, a 1.5 ml LB culture with 50 ug/ml ampicillin and the specific bacterial colony was incubated at 37° C with vigorous shaking overnight. Samples were centrifuged for 1 minute and resuspended in 100 ul solution containing 50 mM glucose, 10 mM EDTA pH 8.0, 25 mM Tris-Hcl pH 8.0, 4 mg/ml lysozyme and incubated for 5 minutes at room temperature. To the cell suspensions, 200 ul of a fresh solution of 0.2 N NaOH, 1% SDS was added followed by another 5 minute incubation at room temperature. One hundred fifty ul of an ice-cold solution of 5 M potassium acetate (pH 4.8) was added, samples placed on ice for 5 minutes, and subsequently centrifuged for 5 minutes at 4° C. The supernatant was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous layer was removed. DNA was precipated by the addition of 2 volumes of ethanol for 10 minutes at room temperature and pelleted by centrifugation. Pellets were washed with 70% ethanol, dried in a vacuum, and resuspended in TE buffer.

Electrophoresis. Two types of gel electrophoresis were performed.

a) Agarose gel electrophoresis. Undigested and digested genomic DNA samples were analyzed by electrophoresis on horizontal 1% agarose gels. Gels were prepared in a buffer (1X TBE) containing 89 mM Tris-borate, 2 mM disodium EDTA, pH 8.0 (TBE) and 0.5 ug/ml ethidium bromide (w/v). Samples were

suspended in TE buffer and gel loading dye (250 mM EDTA, 1% SDS, 0.1% bromphenol blue (w/v), 50% glycerol). Electrophoresis was carried out at 5 V/cm for 3 hours and 1X TBE used as the running buffer.

b) Polyacrylamide gel electrophoresis (PAGE). Small DNA fragments digested with restriction endonucleases were separated on 6%, nondenaturing polyacryamide gels. Electrophoresis was carried out for 16 hours at 5 mA in 1X TBE buffer. Gels were stained with 5 ug/ml ethidium bromide and subsequently visualized with an ultraviolet transilluminator (305 nm).

Isolation of DNA fragments from agarose or PAGE gels. Agarose or polyacrylamide gels stained with ethidium bromide were visualized with an ultraviolet transilluminator. Specific DNA bands were excised from the gel with a razor blade and placed into dialysis bags containing 500 ul TE buffer. The DNA was electroeluted from the gel slice in 0.5X TBE running buffer (Maniatis et al., 1986). The DNA was purified by phenol:chloroform:isoamyl alcohol (25:24:1) extraction, an ether extraction, and concentrated by ethanol precipitation.

Labelling of DNA probes. Probes labelled with ^{32}P-dCMP were prepared by random priming from a kit by IBI using [alpha-^{32}P]-dCTP. The labelled probes were separated from unincorporated nucleotides by Sephadex G-50 (Pharmacia) chromatography. The probes were eluted with NETS solution

containing 0.1% SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM disodium EDTA, pH 8.0. Separation of probe from unincorporated nucleotides was monitored with a Geiger counter. Specific activity of the probe was approximately 5-10 x 10^7 cpm/ug DNA.

Southern blot hybridization. Prior to blotting agarose gels, DNA was partially depurinated in 0.25 N HCl for 15 minutes, denatured in 0.5 M NaOH-0.9 M NaCl for 1 hour (two 30 minute incubations) and neutralized in 0.5 M Tris-HCl- 0.9 M NaCl (pH 7.6) for 1 hour (two 30 minute incubations). The gels were subsequently transferred to nitrocellulose (Micron Separations, Inc., Westboro, MA) (Maniatis, 1989) with 20X SSC (1X SSC is 150 mM NaCl, 15 mM Na-citrate, pH 7.0). The membranes were prehybridized in 50% formamide, 5X SSC, 0.1% SDS (w/v), 0.05M disodium phosphate, pH 6.8, 2X Denhardt's (1X Denhardt's is 0.02% ficoll, 0.02% polyvinylpyrrolidone and 0.02% bovine serum albumin) and 50 ug/ml sheared salmon sperm DNA for 2 hours at 42° C. The hybridization solution contained the same components as the prehybridization solution in addition to the heat-denatured probe. Membranes were hybridized at 42° C overnight. The filters were washed 3 times in 2X SSC, 0.2% SDS for 20 minutes each and subsequently washed 3 times in 0.2X SSC, 0.5% SDS for 20 minutes each. All filters were air dried and placed in X-ray cassettes with Cronex Lightning Plus intensifying screens (DuPont) and Kodak X-OMAT AR film at -80° C.

Isolation of total cellular RNA. Total cellular RNA was extracted from cells by the method of Chomczynski and Sacchi (1987). Cells (approximately 1 x 10^6) were washed with phosphate-buffered saline (PBS) and lysed with 1.8 ml of a solution containing 4 M guanidine thiocyante, 25 mM sodium citrate, pH 7.0, 0.5% sodium lauryl sarcosinate, and 0.1 M 2-mercaptoethanol. Lysates were transferred to new tubes to which 180 ul of 2 M sodium acetate, pH 4.0, 1.8 ml of water-saturated phenol and 360 ul chloroform:isoamyl alcohol (49:1) was added to each sample. Samples were incubated on ice for 15 minutes followed by a 30 minute centrifugation at 4000 rpm at 4^0 C. The aqueous phase was transferred to new tubes and RNA was precipitated overnight at -20^0 C with an equal volume of isopropanol. RNA was pelleted by a 30 minute spin at 4000 rpm at 4^0 C. The RNA pellet was resuspended in 200 ul lysis solution and reprecipitated in a microfuge tube. The pellet was resuspended in TE and stored at -70°C.

Cloning HPV-16 DNA fragments into recombinant plasmids. Plasmid HZIP-16 was digested to completion with Bam HI, to remove the HPV-16 DNA insert, followed by digestion with Pst I to obtain vector-free HPV-16 fragments. Plasmid pT7T3 containing dual opposable RNA polymerase promoters, T7 and T3, was also digested with Bam HI and Pst I or with Pst I alone. To prevent self-ligation of the vector, the 5'-phosphate groups were removed with bacterial alkaline phosphatase (IBI). All DNAs were ethanol precipitated and resuspended in TE

buffer. The ligation reaction mixture contained vector and insert DNA at a concentration ratio of 1:1 and 1 unit of T4 ligase. Reactions were incubated overnight at 15° C.

A 5 ml culture of Escherichia coli strain HB101 was grown overnight and used to inoculate 100 ml of LB. The culture was grown until $O.D._{595}$ of 0.6-0.7. Cells were pelleted at 2000 rpm for 10 minutes (JρuAn CR412), resuspended in 20 ml of 100 mM $CaCl_2$, centrifuged again for 10 minutes at 2000 rpm, resuspended in 10 ml 100 mM $CaCl_2$ and incubated on ice for 1 hour. Bacteria was added in either a 200:1 or 20:1 ratio (v/v) to the ligation mixture. Samples were incubated on ice for 40 minutes followed by a 2 minute incubation at 45° C. Subsequently, 1 ml of LB and 3 ml of top agar (0.137 M NaCl, 10% casein hydrolysate, 0.8% agar) was added and transformation mixtures were poured onto LB agar plates containing 50 ug/ml ampicillin. Colonies resistant to ampicillin were analyzed for insert DNA by restriction endonuclease analysis after mini-preps were performed as described above.

Generation of RNA probes. RNA probes were generated by in vitro transcription kits from Ambion. Target DNA was cloned into multipurpose cloning vectors with dual opposable RNA polymerase promoters, pSP6T7, pT7T3. To detect mRNA, complementary RNA or antisense RNA was synthesized. Vector DNA was linearized with a restriction endonuclease on the amino terminus of the encoded gene product to generate

antisense transcripts. Template DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) followed by an ether extraction to remove any RNAses, precipitated and resuspended in TE buffer. For the in vitro transcription, the reaction contained 1 ug of linearized template DNA, 1 ul of 10 mM stock concentrations of ATP, GTP, and CTP, 2 ul of 10X transcription buffer, (supplied by Ambion), 1 ul of 200 mM dithriotol (DTT), 1 ul placental ribonuclease inhibitor, 5 ul of [alpha-^{32}P]-UTP (400-800Ci/mmol), and 1 ul of the specific RNA polymerase needed for generation of the antisense transcripts. The reaction was incubated at room temperature for 30 minutes and the reaction terminated by the addition of 1 ul of DNase I for 15 minutes at 37° C. Subsequently, 20 ul gel loading buffer (80% formamide, 0.1% xylene cyanol (w/v), 0.1% bromphenol blue (w/v), 2 mM disodium EDTA) was added and samples denatured for 5 minutes at 85° C, and electrophored on 8 M urea/5% acrylamide gels at 150 V for 30 minutes. The gel was autoradiographed, the full length RNA transcript excised, the gel slice placed in 350 ul elution buffer (0.5 M ammonium acetate, 1mM disodium EDTA, 0.1% SDS), and eluted overnight at 37° C. The amount of radioactivity present in the eluted probe was determined by liquid scintillation counting.

Northern blot hybridization. Total cellular RNA (10 ug) was electrophoresed through a 1% agarose gel containing 0.66 M formaldehyde and 1X MOPS buffer (200 mM 3-[N-morpholinopropane-sulfonic acid], 5 mM sodium acetate, pH 7.0,

1 mM disodium EDTA). Prior to electrophoresis, 10 ug RNA was precipitated and redissolved in sample buffer containing 0.75 ml deionized formamide, 0.15ml 10X MOPS, 0.24 ml formaldehyde, 0.1 ml RNase free water, 0.1 ml glycerol and 0.08 ml 10% bromphenol blue (w/v). RNA was transferred to nylon filters (Hybond N^+, Amersham) by blotting overnight in 20X SSPE (1X SSPE contains 0.18 M NaCl, 0.01 M sodium phosphate, 1 mM disodium EDTA, pH 7.7). Probes labelled with [alpha-^{32}P]-dCTP (1 x 10^7 cpm/ug DNA) were prepared by random priming and hybridized to filters in a solution containing 5X SSPE , 5X Denhardt's solution, 0.5% SDS, 50% formamide and 50 ug/ml sheared salmon sperm DNA. Hybridization was carried out at 42° C for 16 hours. The filters were washed with 2X SSPE and 0.1% SDS two times for 10 minutes at room temperature followed by 1X SSPE and 0.1% SDS for 15 minutes at 65° C. Filters were air dried and autoradiographed.

Ribonuclease protection analysis. Ribonuclease protection analysis (RPA) was performed using the RPA kit by Ambion. An in vitro generated RNA probe (7 x 10^4 cpm ^{32}P-UTP) was mixed with 10 ug total cellular RNA. Samples were precipitated with 0.5 M ammonium acetate and 2.5 volumes of ethanol and resuspended in a hybridization buffer (80% formamide, 40 mM PIPES pH 6.4, 400 mM sodium acetate, pH 6.4, 1 mM EDTA), denatured for 4 minutes at 85°C and hybridized overnight at 45° C. Unprotected single stranded RNA on hybrids was digested with a solution containing RNase A/RNase

T1 for 30 minutes at 37° C. Equal volumes of 20% SDS and proteinase K were added and samples incubated for 15 minutes at 37° C. Subsequently, samples were extracted with phenol:chloroform:isoamyl alcohol (25:24:1). The RNAs were precipitated with 2.5 volumes of ethanol and run on a 8 M urea/5% acrylamide denaturing gel to separate the RNA species. The gel was dried and subjected to autoradiography.

Anchorage independent growth analysis. To determine to cloning efficiency of transformed cells in soft agar, anchorage independent analysis was done. Base plates were made with equal volumes of 1.2% molten agar and 2X growth medium containing Eagle's modified Essential medium, glutamine, sodium bicarbonate (pH 7.2), 20% fetal bovine serum, penicillin (10 U/ml) and streptomycin (100 ug/ml) resulting in a 0.6% agar mixture. Cells suspensions (1 X 10^5 cells) diluted in 1X growth medium were mixed with equal volumes of 0.6% agar and immediately poured onto base plates. Plates were allowed to solidify at room temperature and transferred to a 37° C CO_2incubator. Colonies were counted after 2-4 weeks.

Polymerase chain reaction (PCR) analysis. High molecular weight DNA was prepared from various cell lines as previously described and digested with either Eco RI or Pst I overnight at 37° C. Oligonucleotide primers (Genosys Biotechnologies) 21 bases in length, were chosen to amplify a target sequence of 366 base pairs located within the HSV-2 mtr III 486 TF

sequence. The primers were synthesized according to the published sequence of 486 TF (Jones et al., 1986). Primer 917 contained nucleotides 917 to 937 of the plus strand (5′-TCACGGAGGACGACTTTGGGC-3′) and primer 1262 contained nucleotides 1262 to 1282 of the minus strand (5′-GCGTGTTCGGGGTGCTGTGGA-3′). The probe used to detect the amplified sequence was the 486 TF sequence (486 bp). PCR was performed in 100 ul volumes in standard commercial buffer (Promega) containing 1.5 mM $MgCl_2$. Cycling parameters for PCR were: 1 minute at 94° C for denaturation, 1 minute of primer annealing at 60° C, and 2 minutes at 72° C for extension. A total of 40 cycles was run. For PCR, 1 ug of template DNA was used. Amplification products were analyzed on 2% Nu-Sieve 3:1 agarose (FMC Bioproducts) gels containing 1X TBE buffer and 0.5 ug/ml ethidium bromide. Subsequently, Southern blot analysis of the gels was done using random primed ^{32}P-labelled 486 TF probes. Prehybridization was carried out for one hour at 61° C in 5X SSPE, 5X Denhardt's and 1% SDS. Blots were hybridized in the same solution to which the denatured probe was added. Membranes were washed in 1X SSPE and 1% SDS three times at room temperature and once at 65° C. Filters were air dried and subjected to autoradiography.

Precautions were taken to avoid DNA carryover. Isolation of pre-target and post-target DNA was done in physically different areas, disposable labware and positive displacement pipettes were used for all DNA manipulations, and protective

covering for clothing, hair and face was used. A positive control for all PCR analysis was a plasmid, p486, containing the 486 TF insert (Jones et al., 1989). Negative controls were DNA from normal cells (either keratinocytes or fibroblasts) containing no virus (neither HPV-16, -18 or HSV-2) and the "no template" reactions which contain all components of the PCR reaction except template. "No template" lanes provided a control on the performance of the PCR.

Tumorigenicity assay in nude mice. To determine the oncogenic potential of transformed cell lines, 5 x 10^6 cells were injected subcutaneously into nu/nu mice (Harlan Sprague Dawley). Mice were observed weekly for the presence of lesions. Seven to eight weeks post-injection, animals were sacrificed and lesions removed. Tumorigenic cells were dislodged from tissue with Dispase (Boeringer Mannheim) overnight and subsequently plated in serum-free keratinocyte medium (Gibco).

RESULTS

To demonstrate that human papillomavirus types 16/18 and human herpes virus-2 can act as cofactors in the development of cervical cancer, the experimental approach taken was to establish human cells containing both viral DNAs. The first half of the Results section will examine and characterize primary fibroblast cell lines generated from two human cell strains. The second half of the Result section will examine cell lines generated from primary genital keratinocytes transfected with transforming domains of both viruses.

Selection of primary human fibroblasts. Two primary human cell types derived from gingival tissue were utilized. Gingival fibroblasts were obtained from 1) a normal individual (N-16) and 2) from an epileptic patient treated with phenytoin (R-30), an anti-seizure drug (Delgado-Escueta et al., 1983). A common side effect of phenytoin is the induction of gingival overgrowth. Approximately 50% of patients taking this drug have gingival hyperplasia and overgrowth can lead to complete obstruction of teeth (Hassell et al., 1983). Pseudolymphoma syndrome and Hodgkin's disease are associated with prolonged use of phenytoin (reviewed in Wolf et al., 1985). Cells were scraped from gingival tissue of normal and epileptic patients

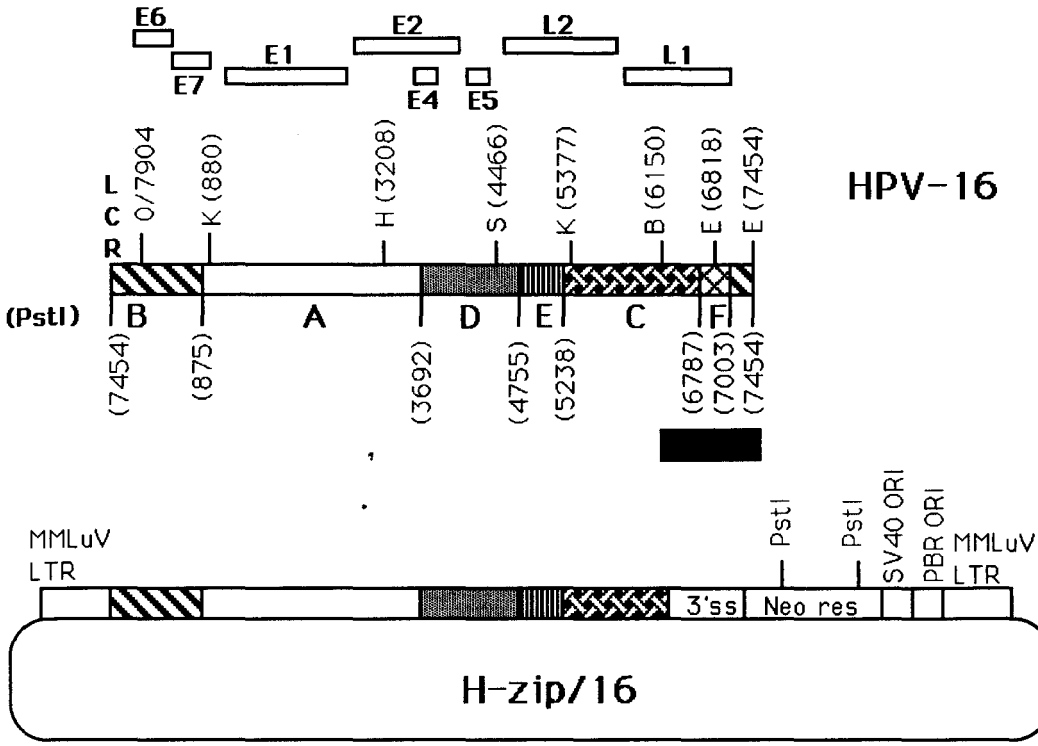
and primary cultures developed from the respective tissue. This aspect of the investigation was designed to determine if R-30 cells (hyperplastic in vivo) and N-16 cells (normal primary cells) differ with respect to morphological transformation by HSV-2 and/or HPV-16.

Transfection analysis of fibroblast cell lines. To test for morphological transformation of R-30 and N-16 cells, a plasmid containing the early genes of HPV-16, HZIP-16 (Matlashewski et al., 1987) (Figure 1), was transfected either alone or in combination with plasmids containing HSV-2 sequences derived from morphological transforming region III, mtr III (Jones et al., 1986), into the respective cell lines. In addition, a plasmid containing an activated H-ras gene, pEJ6.6, was cotransfected with HZIP-16 to serve as a positive control for cooperation with HPV-16. Matlashewski et al., (1987) demonstrated HPV-16 and H-ras could cooperate to transform primary cells. Table 1 shows the results from the transfection experiments using N-16 and R-30 parental cells. Phenytoin-treated cells (R-30) were more susceptible to immortalization by HZIP-16 alone compared to fibroblasts from a normal individual (N-16). These differences were not merely due to higher levels of plasmid DNA uptake in R-30 cells since both cell types contained similar levels of DNA at 48 hours post transfection (data not shown). When H-ras, an activated cellular oncogene, was transfected into R-30 cells or N-16 cells alone, densely growing geneticin resistant colonies were

Figure 1. Schematic map of HPV-16 and HZIP-16.

(I) Restriction enzyme map of HPV-16 genome and location of ORFs. The prototype HPV-16 genome is depicted. To construct HZIP-16, the region between Bam HI and Eco RI was deleted by Matlashewski et al (1987). HZIP-16 contains Moloney murine leukemia virus LTRs, a neomycin resistance gene, origins of replication of SV40 and pBR322 vector sequences. LCR is the long control region of HPV-16. Restriction enzyme sites are K (Kpn I), H (Hinc II), S (Stu I), E (Eco RI) and B (Bam HI). This plasmid was used as source of HPV-16 DNA to generate all fibroblast cell lines.

(II) Restriction map of HSV-2 mtr III region containing 486 TF.


E6
E7
E1
E2
E4
E5
L2
L1
HPV-16
LCR
0/7904
K (880)
H (3208)
S (4466)
K (5377)
B (6150)
E (6818)
E (7454)
(PstI)
B
A
D
E
C
F
(7454)
(875)
(3692)
(4755)
(5238)
(6787)
(7003)
(7454)
MMLuV LTR
PstI
PstI
SV40 ORI
PBR ORI
MMLuV LTR
3'ss
Neo res
H-zip/16

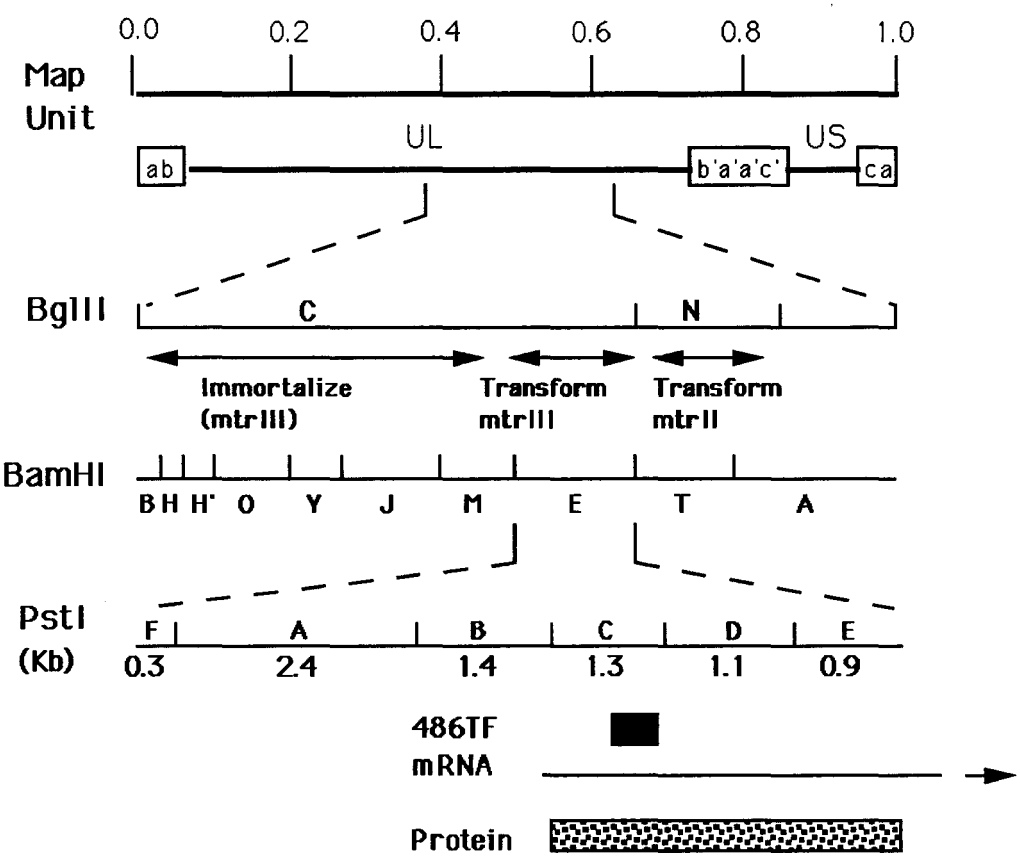

0.0
0.2
0.4
0.6
0.8
1.0
Map Unit
UL
US
ab
b'a'a'c'
ca
BglII
C
N
Immortalize (mtrIII)
Transform mtrIII
Transform mtrII
BamHI
B H H' O Y J M E T A
PstI (Kb)
F
A
B
C
D
E
0.3
2.4
1.4
1.3
1.1
0.9
486TF
mRNA
Protein

Table 1. Transfection analysis of R-30 and N-16 cells.

	R-30			N-16		
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
HZIP-16/H-*ras*	4	7	5	3	6	7
HZIP-16/486 TF	5	4	6	4	3	5
HZIP-16/BglII-C	6	2	7	0	3	0
HZIP-16	5	7	4	0	1	0
H-*ras*	1	0	0	0	0	0
HSV-2 486 TF	0	0	0	0	0	0
HSV-2 BglII-C	0	0	0	0	0	0
pSV2neo	0	0	0	0	0	0

R-30 or N-16 cells (5 x 10^5 cells/60 mm dish) were transfected with 25 ug of plasmid DNA using the calcium phosphate precipitation procedure (Graham and Van der Eb, 1973) followed by geneticin selection (400 ug/ml). Values indicate the number of geneticin resistant colonies per 60 mm dish.

not observed. The geneticin resistant colony observed in H-ras transfected R-30 cells senesced when trypsinized and transferred to a new dish. Furthermore, cells transfected with pSV2neo alone did not yield densely growing geneticin resistant colonies. Since an activated H-ras oncogene will transform immortalized human and rodent cells but usually not primary cells (Newbold and Overell, 1983, Sager et al., 1983), it appears R-30 and N-16 cells are not immortalized. When geneticin resistant colonies from N-16 cells were trypsinized and passaged (three independent experiments and resistant colonies from 12 plates transfected with HZIP-16 alone, HZIP-16/H-ras or HZIP-16/486 TF, HZIP-16/Bgl II-C, HZIP-16/Bam HI-E), stable cell lines were not obtained regardless of the transforming sequences transfected into these cells. Typically these cells senesced after only four or five passages. In contrast, geneticin resistant colonies obtained from R-30 cells transfected with HZIP-16 alone or in combination with H-ras or HSV-2 DNA sequences underwent at least 75-80 population doublings (PD) after trypsinization of the original colony in the absence of geneticin. Table 2 contains a summary of the fibroblast cell lines and the primary cells used in the analysis. In summary, these studies demonstrated that HPV-16 DNA immortalized R-30 cells more efficiently than N-16 cells and neither cell strain was transformed by H-ras alone.

Retention of viral DNA in transformed cell lines. The

Table 2. Fibroblast cell lines.

PRIMARY or PRIMARY-LIKE CELLS:

N-16 - primary gingival fibroblasts

R-30 - hyperplastic gingival fibroblasts (phenytoin treated)

C-1 - cyclosporin treated gingival fibroblasts

CELL LINES:

139 - HZIP-16 / H-ras

140 - HZIP-16 / HSV-2 486 TF

141 - HZIP-16 / HSV-2 Bgl II-C

145 - HZIP-16

181 - HZIP-16

227 - HZIP-16 / {H-ras + 486 TF}

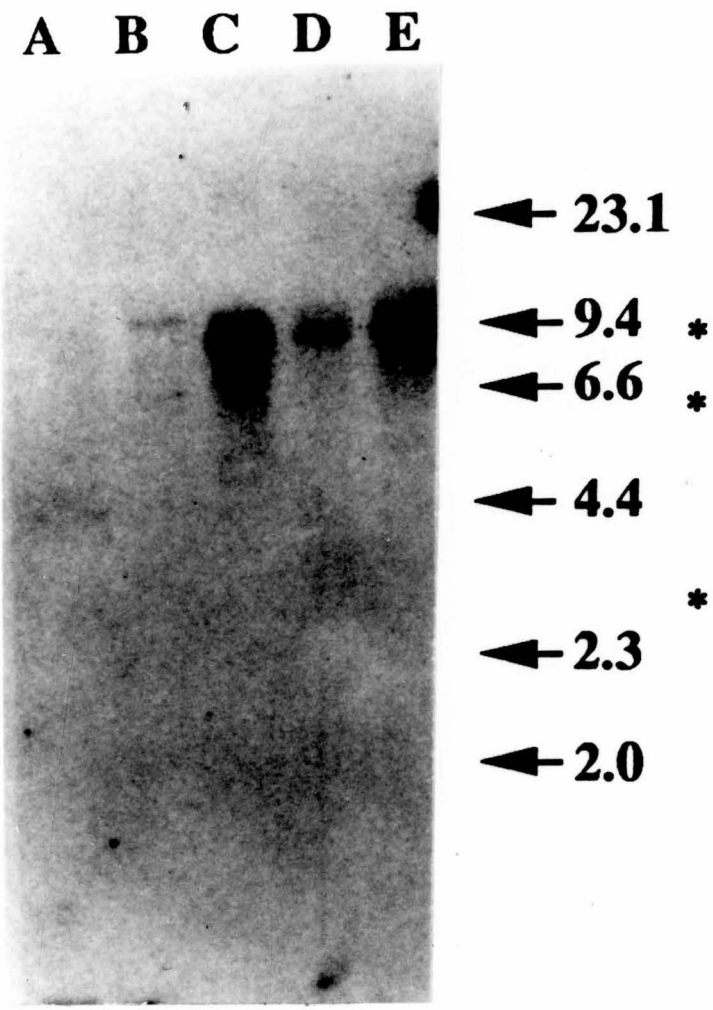
228 - HZIP-16 / H-ras

268 - HZIP-16 / HSV-2 Bam HI-E

Early passage primary and primary-like fibroblast cells used in the analysis. The transformed cell lines generated from calcium phosphate transfection and subsequent geneticin selection.

various cell lines were analyzed to detect retention of transfected DNA. HPV-16 DNA is generally integrated in malignant lesions *in vivo* (Durst et al., 1985) and thus it was of interest to assess the presence of HPV-16 DNA sequences in transformed cell lines. When an EcoRI digest of genomic DNA was probed with HPV-16 DNA, it was evident all transformed cell lines retained HPV-16 DNA (Figure 2). In all cell lines except cell line 139, a 9.4 kb HPV- specific hybridizing band was detected. Minor bands which also hybridized to the HPV probe were detected in cell line 140 (7.5 kb band) and cell line 145 (7.9 kb). In cell line 139, two HPV-specific bands (12.5 kb and 6.4 kb) were detected. Since a recent report demonstrated that HPV-16 can be detected in oral epithelial tissue (Yeudall and Campo, 1991), it was important to determine if HPV-16 sequences were present in R-30 cells. As expected, the HPV probe did not hybridize to R-30 genomic DNA. The intensity of HPV-16 specific hybridization to DNA prepared from the various cell lines differed dramatically. Since the amount of DNA loaded into each lane was similar, these results indicated cell line 145 (R-30 transfected with HZIP-16 alone) and cell line 140 (R-30 cells cotransfected with HZIP-16 and HSV-2 486 TF) had approximately equivalent amounts of HPV-16 specific DNA. Cell line 141 (R-30 cotransfected with HZIP-16 and BglII-C) and cell line 139 (R-30 cotransfected with HZIP-16 and H-*ras*) also had equivalent amounts of HPV-16 specific DNA, however, at lower levels compared to cell lines 140 and

Figure 2. EcoRI digest of DNA from cell lines. Genomic DNA was prepared from the various cell cultures (Maniatis et al, 1982), digested with EcoRI and subjected to Southern blotting. (A) R-30, (B) 139, (C) 140, (D) 141, (E) 145. The probe was derived from the Pst I-A and Pst I-B fragments of HPV-16. Asterisks denote the site of migration of Eco RI digestion products of HZIP-16. Molecular size markers, lambda DNA/Hind III, are shown in kb (23, 9.0, 6.6, 4.4, 2.3, 2.0).


A B C D E
23.1
9.4
6.6
4.4
2.3
2.0
*
*
*

145. When uncut genomic DNA of the respective cell lines was probed with HZIP-16 sequences, the hybridizing fragment migrated with the high molecular weight genomic DNA suggesting integration had occurred (Figure 3). Lane H contains uncut HZIP-16 plasmid. Both relaxed and supercoiled forms of the plasmid hybridize to the HPV-16 probe as well as DNA from transformed and immortalized cell lines while no hybridization is detected with R-30 and N-16 genomic DNA. These studies indicated HPV-16 DNA sequences were retained in all cell lines and the level of HPV-16 specific DNA varied among the respective cell lines.

To map which regions of the HPV-16 genome were retained in the various cell lines, Southern blots were probed with individual PstI fragments of the HPV-16 genome. Since all of the Pst I fragments hybridized to DNA prepared from cells with HZIP-16 but not control cells, it appears the entire genome of HPV-16 was retained (Figure 4). Furthermore, the regions were retained at different copy levels in the various cell lines. In addition to seeing intact Pst I fragments (fragments migrating with control HZIP-16/Pst I/Bam HI), a number of different sized bands also hybridized to the probes. This agrees with our conclusion that HPV-16 DNA has integrated into the cellular genome and may also suggest tandem duplications of the HPV-16 genome occurred. These studies indicated the genome of HPV-16 was retained in all cell lines and the copy level of HPV-16 DNA varied dramatically among the respective

Figure 3. Analysis of undigested high molecular weight DNA from fibroblast cell lines. Genomic DNA prepared from fibroblast cell lines was subjected to Southern blotting. Undigested DNA was used to determine integration of HPV-16 DNA sequences into the high molecular weight DNA species. The probe used was the 6.6 kb HPV-16 Bam HI insert from HZIP-16. Lane (A) N-16, (B) R-30, (C) 139, (D) 140, (E) 141, (F) 145, (G) 181, (H) HZIP-16.

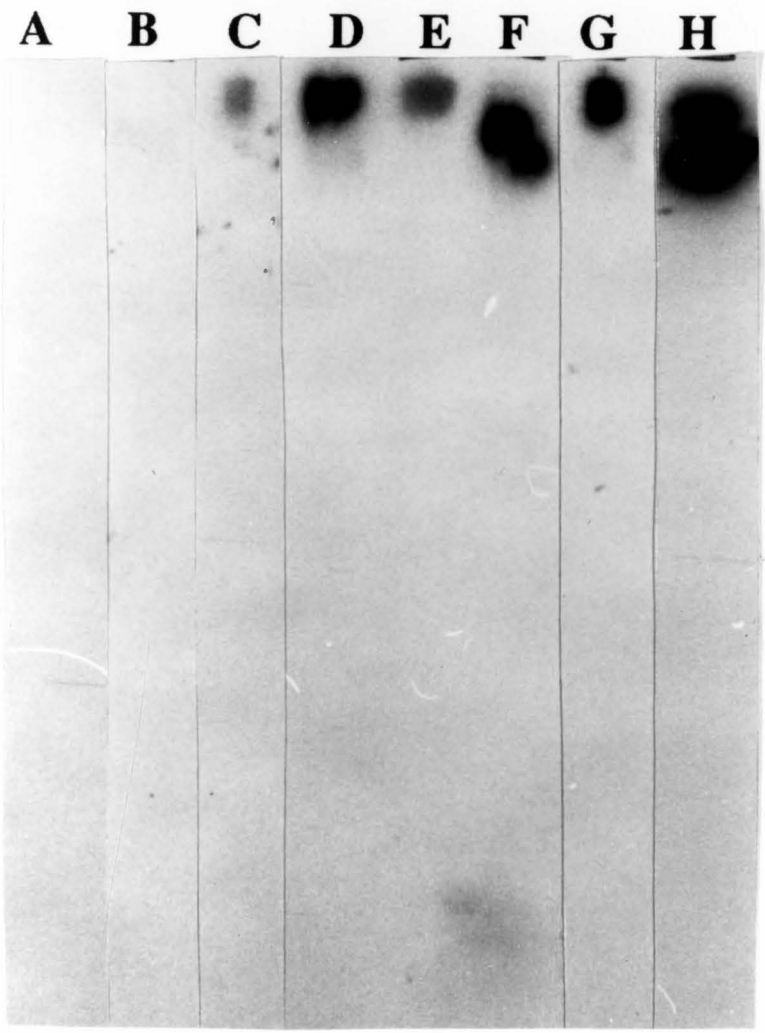

A
B
C
D
E
F
G
H

Figure 4. Analysis of HPV-16 DNA in transformed R-30 cells. (I) High molecular weight DNA was prepared from the various transformed cells, digested with the restriction enzyme PstI, and subjected to Southern blot analysis. Southern blots were probed with the individual PstI or PstI/BamHI fragments of HPV-16. The location of these probes are presented in (II) and are above the respective Southern blot. The bars above the figures are not drawn to scale. However, in (II) the respective Pst I fragments are drawn to scale. DNA was prepared from cell lines:

(A) R-30 (hyperplastic human fibroblasts),

(B) 139 (R-30 cotransfected with HZIP-16 and H-ras,

(C) 140 (R-30 cotransfected with HZIP-16 and HSV-2 486 TF),

(D) 141 (R-30 cotransfected with HZIP-16 and HSV-2 Bgl II-C),

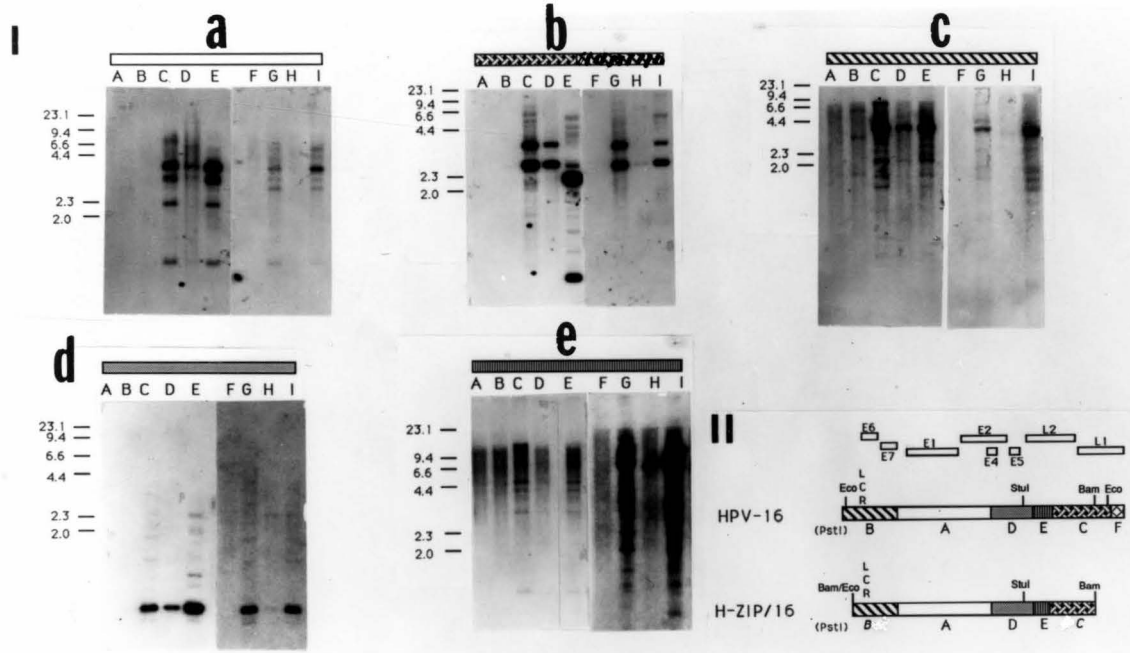
(E) 145 (R-30 transfected with HZIP-16),

(F) C-1 (fibroblasts from a patient treated with cyclosporin),

(G) 181 (R-30 transfected with HZIP-16),

(H) 227 (R-30 cotranfected with HZIP-16, H-ras),

(I) 228 (R-30 cotransfected with HZIP-16 and H-ras).


I
a
b
c
d
e
A B C D E F G H I
23.1
9.4
6.6
4.4
2.3
2.0
II
E6
E7
E1
E2
E4
E5
L2
L1
HPV-16
Eco
L
C
R
Stul
Bam
Eco
(PstI)
B
A
D
E
C
F
H-ZIP/16
Bam/Eco
Bam

cell lines.

HSV-2 DNA retention in fibroblast cell lines. The retention of HSV-2 DNA sequences in transformed cells was examined by polymerase chain reaction (PCR). The stable retention of HSV-2 DNA in transformed cells has not been consistently observed and thus HSV-2 transformation is postulated to be mediated by a "hit and run" mechanism (reviewed by MacNab, 1987). Southern blot analysis using HSV-2 DNA sequences as probes failed to detect HSV-2 DNA (Figure 5). However, when high molecular weight DNA was subjected to PCR analysis using primers derived from 486 TF, retention of HSV-2 DNA was detected in three cell lines (Figure 6). All three had been transfected with HSV-2 DNA sequences: 1) cell line 140 (HZIP-16 + HSV-2 486 TF), 2) cell line 141 (HZIP-16 + HSV-2 Bgl II-C) and 3) cell line 268 (HZIP-16 + HSV-2 Bam HI-E) (Figure 6). The amplified band migrated as a 366 bp fragment and comigrated with the positive control (486 TF). In contrast, cell line 145 (HZIP-16 alone), cell lines 139 and 227 (HZIP-16 + Ha-ras), R-30 cells and C-1 (primary gingival cells treated with cyclosporin) cells did not contain amplified products which hybridized to the 486 TF probe. Furthermore, the "no template" negative control lane also did not contain amplified products which hybridized to 486 TF. These results demonstrated HSV-2 DNA sequences derived from 486 TF were retained in transformed human cells.

Chromosome analysis of transformed cells. To examine the

Figure 5. Retention of HSV-2 DNA in fibroblast cell lines. High molecular weight DNA prepared from fibroblast cell lines, digested with Eco RI, was analyzed by Southern blot analysis to determine retention of HSV-2 mtr III sequences. The HSV-2 mtr III 486 bp Pst I-Sal I fragment was used as the probe. Lane (A) R-30, (B) 139, (C) 140, (D) 141, (E) 145, (F) 268, (G) 486 TF. Size markers, lambda DNA digested with Hind III, are denoted by lines.

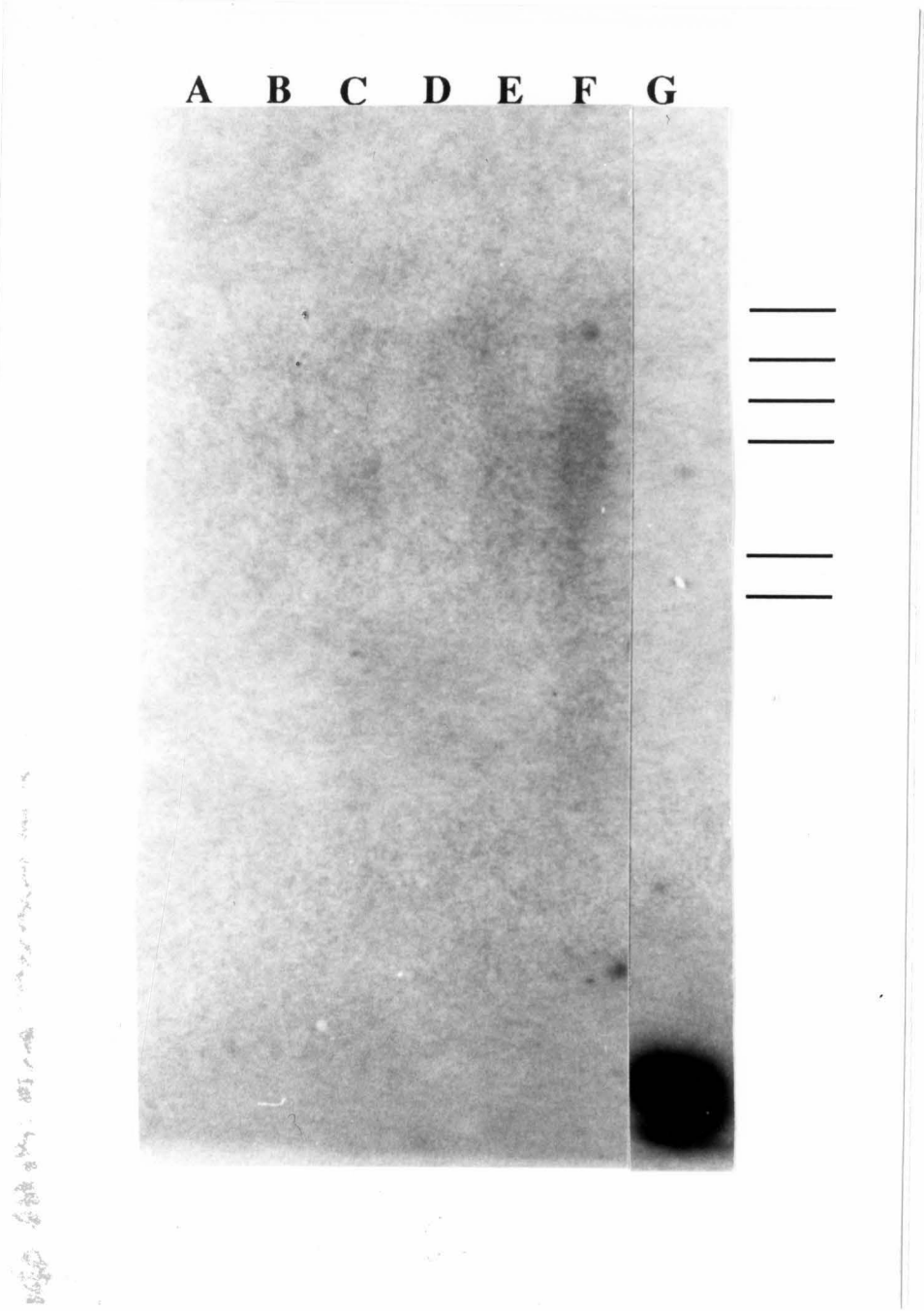
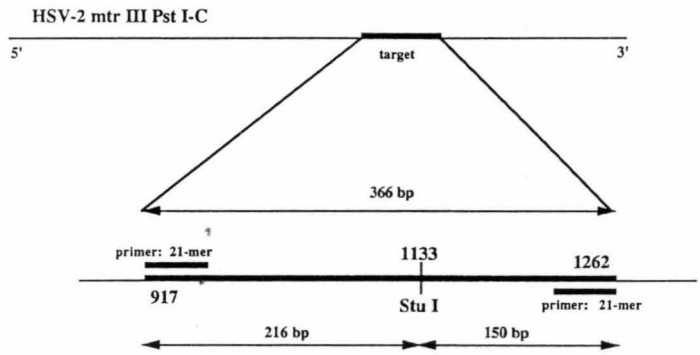

A
B
C
D
E
F
G

Figure 6. Polymerase chain reaction (PCR) analysis of transformed fibroblast cell lines. High molecular weight DNA was prepared from the respective cell lines, digested with Pst I and 1 ug of digested DNA used for the PCR reaction. Primers were derived from HSV-2 mtr III 486 TF.

(I) Map of HSV-2 mtr III derived primers, 917 and 1262.

(II) Southern blot analysis of PCR reaction using 486 TF as the probe. Lane (A) contains 0.1 ug pCPS-1 plasmid (486 TF cloned into Pst I and Sal I sites of pUC19). Lanes B-I contain DNA prepared from cell line 139 (B), cell line 140 (C), cell line 141 (D), cell line 145 (E), cell line 227 (F), cell line 268 (G), R-30 cells (H), and C-1 cells (I). Lane (J) contains the "no template" control. Lane (K) PhiX174 DNA digested with Hae III (bp) and positions of these fragments denoted by lines (1353, 1078, 872, 603, 310, 281, 234, 174, and 118 bp).

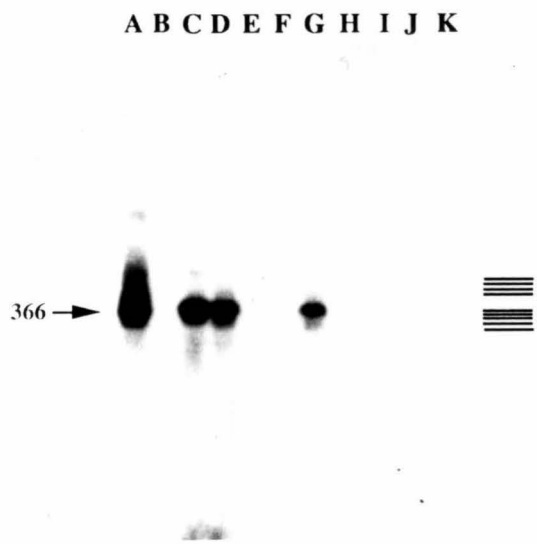
I.



Primer 917: 5' - TCACGGAGGACGACTTTGGGC - 3'

Primer 1262: 5' - GCGTGTTCGGGGTGCTGTGGA - 3'

II



chromosomal composition of the various cell lines, karyotype analysis was performed. Only 50% of the cells transfected with HZIP-16 alone (145) were aneuploid and none of the aneuploid cells contained more than 100 chromosomes. However, 90% of the cells derived from lines cotransfected with HZIP-16 and HSV-2 DNA (140 and 141) were aneuploid (Table 3) and at least 25% of the cells had more than 100 chromosomes/cell. Approximately 50% of R-30 cells cotransfected with HZIP-16 and H-ras (139) were also aneuploid. These studies suggested that aneuploidy correlated with the presence of multiple transforming agents.

Transformation assays. Anchorage independent growth capabilities of the various cell lines were analyzed to confirm abnormal growth characteristics. Growth was assessed by plating cells (PD 30-35 post-geneticin selection) in 0.3% agarose and scored after 10 days. Cells cotransfected with HZIP-16 and regions of HSV-2 mtr III sequences (140, 141 or 268) grew more efficiently in soft agar than cells transfected with only HZIP-16 (145 and 181) (Table 4). Only 3% of cells transfected with HZIP-16 alone (cell lines 145 and 181) formed colonies after 10 days. However, cells cotransfected with HZIP-16/HSV-2 (140, 141, and 268), had more than 10% colony formation after 10 days. As expected, cell line 139 (HZIP-16 and H-ras) also exhibited anchorage independent growth (after 10 days) and R-30 cells did not grow efficiently in soft agar. Furthermore, saturation density of R-30 cells transfected with

Table 3. Chromosome number per cell of the transformed cell lines.

Cell strain	Chromosome number in a cell				
	39-50	51-70	71-100	101-200	X > 200
R-30	17	0	3	0	0
139 (HZIP-16/H-ras)	9	0	5	6	0
140 (HZIP-16/486 TF)	2	0	13	5	0
141 (HZIP-16/Bgl II-C)	2	0	11	4	3
145 (HZIP-16)	10	4	6	0	0

Karyotype analysis of the various cell cultures was performed at PD 20 or 21 following geneticin selection (except the parental cell R-30). The number of chromosomes in a population of cells was determined.

Table 4. Growth characteristics of transformed R-30 cells.

Cell line	0.3% agarose colony formation (%)[1]	Saturation density[2]
R-30	< 1	$1 \times 10^6 \pm 1 \times 10^5$
139 (HZIP-16/H-ras)	30	$4.9 \times 10^6 \pm 1.3 \times 10^5$
140 (HZIP-16/HSV-2 486 TF)	15	$5.2 \times 10^6 \pm 1.8 \times 10^5$
141 (HZIP-16/HSV-2 Bgl II-C)	15	$4.3 \times 10^6 \pm 2.0 \times 10^5$
268 (HZIP-16/HSV-2 Bam HI-E)	15	$5.1 \times 10^6 \pm 1.5 \times 10^5$
145 (HZIP-16)	3	$2.4 \times 10^6 \pm 0.6 \times 10^5$
181 (HZIP-16)	4	$2.7 \times 10^6 \pm 1.1 \times 10^5$

The ability of R-30 cells and the various transformed derivatives of R-30 cells were analyzed for growth in soft agar.

[1] 1×10^5 cells of each line were plated in medium containing 0.3% agarose and scored after 10 days. Numbers indicate the percentage of cells which formed colonies. To be considered a colony, the diameter of the colony was at least 1 mm.

[2] Saturation density was the number of cells/100 mm dish at confluence. Plates were seeded with 3×10^5 cells and the various cultures were fed three times a week. After two weeks in culture, the cells were trypsinized and counted using a hemacytometer.

HZIP-16 alone (145) was lower than cells cotransfected with HZIP-16/HSV-2 or HZIP-16/H-ras. When the individual cell lines were injected subcutaneously into nude mice (5 x 10^6 - 8 x 10^6 cells/animal), tumor formation was not readily observed after 6 weeks (Table 5). Transitory nodules were observed in animals injected with some of the transformed cells. However, these nodules regressed after several weeks. This may imply the cell lines were not fully transformed or the transformed cells were rejected by the mouse. In summary, anchorage independent growth was more efficient when R-30 cells were cotransfected with HZIP-16 and HSV-2 DNA sequences or H-ras.

Comparison of R-30 and N-16 cells. Since R-30 cells were immortalized more efficiently by HZIP-16 than N-16 cells, a comparison of the two cell types was subsequently made. No obvious differences were detected in morphological characteristics (FACS, 90° vs. forward scattering ratios), growth rates, or saturation densities (V. Veerisetty, unpublished data). In contrast, karyotype analysis of the two cell types revealed that all R-30 cells have a stable chromosomal translocation between chromosome 8 and chromosome 18 (Figure 7). Approximately 15% of R-30 cells contained more than 50 chromosomes.

Since the c-myc protooncogene is present on chromosome 8 (Neel et al., 1982) and alterations of this gene have been associated with HPV-16 and transformation (Riou et al., 1987,

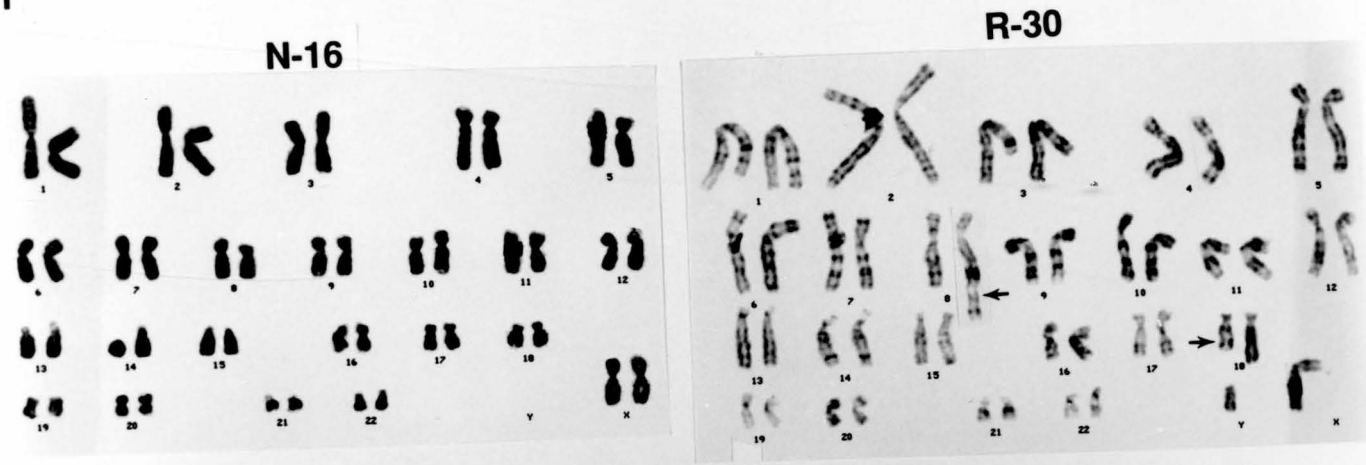
Table 5. Tumorigenicity analysis of fibroblast cell lines.

Cell line	Tumor formation
R-30	0/3
139 (HZIP-16/H-ras)	0/3
140 (HZIP-16/HSV-2 486 TF)	0/3
141 (HZIP-16/HSV-2 Bgl II-C)	0/3
145 (HZIP-16)	0/3
268 (HZIP-16/HSV-2 Bam HI-E)	0/3

Tumorigenic potential of fibroblast cell lines was analyzed for production of palpable tumors. 5×10^6 cells were injected subcutaneously into nu/nu mice. Eight weeks post injection, mice were anesthetized and observed for the presence of lesions.

Figure 7. Karyotype analysis of N-16 and R-30 cells. (I) Arrows indicate aberrations in chromosome 8 and 18 of R-30 cells. All R-30 cells contained the aberrant chromosome 8 and 18. (II) Chromosome number in a population of N-16 and R-30 cells. The modal chromosome number for N-16 cells and R-30 cells was 46.

I



II

Cell Strain Desig.	Chromosome Number in a Cell						
	31-40	41-50	51-60	61-70	71-80	81-90	91-100
N-16	0	19	0	0	0	0	1
R-30	1	16	0	0	0	2	1

Durst et al., 1987, Crook et al., 1990), it was important to examine c-myc expression and organization in R-30 cells. Genomic DNA was prepared from R-30 cells, R-30 derived cell lines or N-16 cells and Southern blot analysis performed. Two c-myc probes, a 1.6 kb Sac I fragment of the 5'-untranslated region and a 420 bp Pst I fragment of exon 2, were used to detect aberrations in gene organization of c-myc DNA. No detectable rearrangements were observed in any of the R-30 cell lines when compared to N-16 cells (Figure 8). To measure steady state levels of c-myc RNA expression, ribonuclease protection analysis was performed. An in vitro transcript was synthesized from the 420 bp Pst I fragment of exon 2 and used as a probe. As expected, a ribonuclease resistant fragment approximately 420 nucleotides was detected when cellular RNA was hybridized to an antisense c-myc transcript (Figure 9). R-30 cells and transformed R-30 cells clearly contained higher steady state levels of c-myc RNA when compared to N-16 cells or C-1 cells (early passage human gingival fibroblasts prepared from a patient treated with cyclosporin). When the radioactivity in the c-myc band was quantified, R-30 cells as well as transformed R-30 cells consistently contained at least three fold higher levels of c-myc RNA compared to either N-16 or C-1 cells. These results demonstrated R-30 cells contained higher steady state levels of c-myc RNA and suggested the translocation of chromosome 8 was related to overexpression.

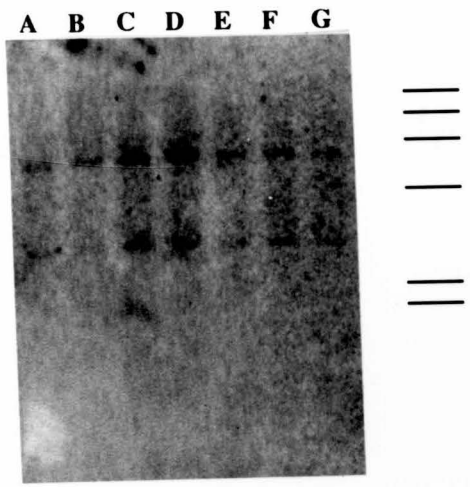
Examination of the human p53 gene in fibroblast cell

Figure 8. C-myc gene organization in fibroblast cell lines. High molecular weight DNA was prepared from fibroblast cell lines, digested with restriction enzymes and Southern blot analysis was performed. Size markers, lambda DNA digested with Hind III, are denoted by lines.

(I) Genomic DNA was digested with Pst I and probed with the 1.6 kb Sac I fragment of the 5'-untranslated region of c-myc. Lane (A) N-16, (B) R-30, (C) 139, (D) 140, (E) 141, (F) 145, (G) 268.

(II) Genomic DNA was digested with Eco RI\Kpn I and probed with the 420 Pst I fragment of exon 2 of c-myc. Lane (A) N-16, (B) R-30, (C) 139, (D) 140, (E) 141, (F) 145, (G) 268.

I



II

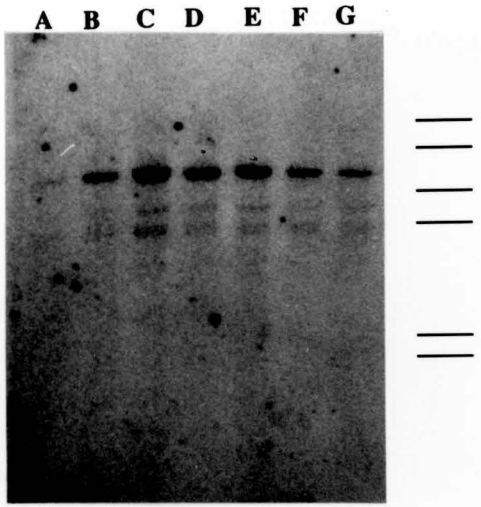
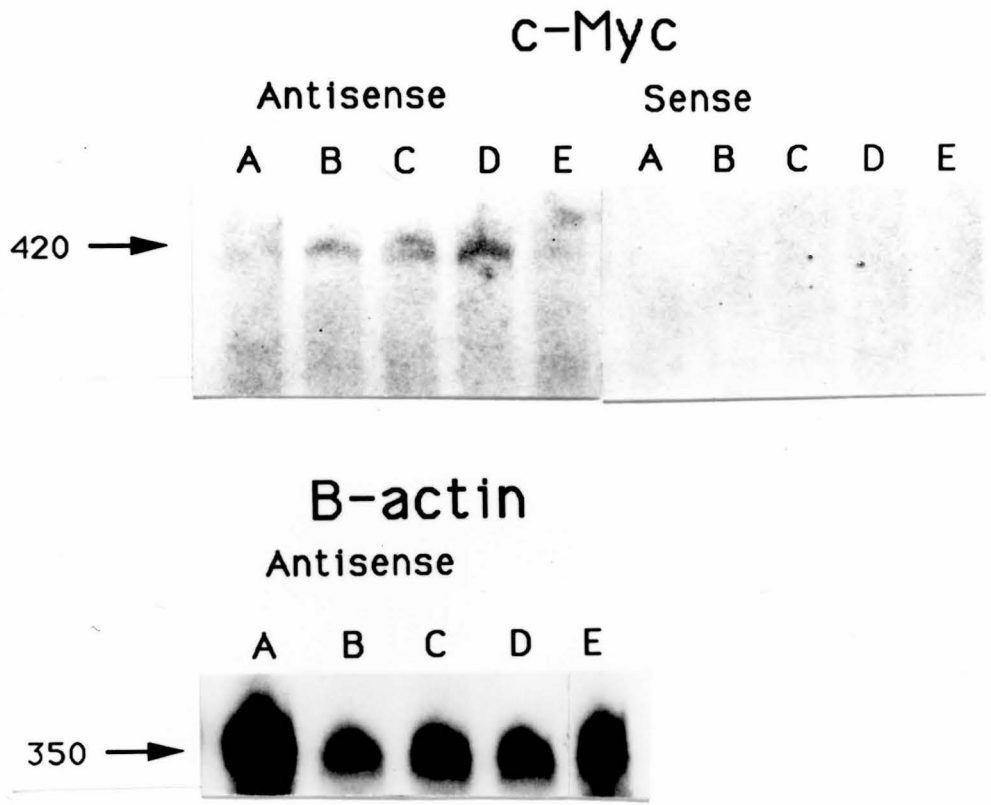


Figure 9. Ribonuclease protection analysis of N-16, C-1 and R-30 derived cell lines. ^{32}P-UTP labelled RNA probes were synthesized from the 420 bp Pst I fragment of c-myc (exon 2). (A) Antisense SP6 probe contained the strand complementary to the c-myc mRNA coding strand while the sense T7 probe was the same as the coding strand. Lane (a) N-16, (b) R-30, (c) 140, (d) 145, (e) C-1. (B) Total cellular RNA of cell lines probed with B-actin. Lane (a) N-16, (b) R-30, (c) 140, (d) 145, (e) C-1.


c-Myc
Antisense
Sense
A B C D E A B C D E
420
B-actin
Antisense
A B C D E
350

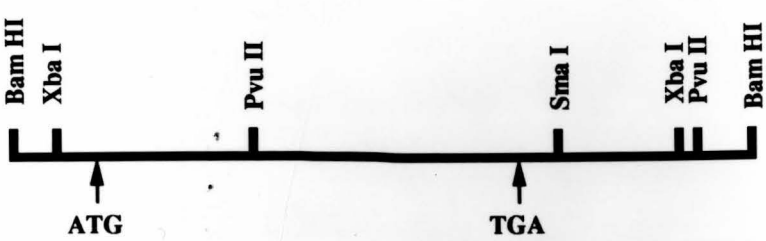
lines. The E6 oncoprotein of HPV-16/18 is able to bind and promote degradation of the tumor suppressor protein, p53 (Werness et al., 1990, Scheffner et al., 1990). p53 is able to act as a suppressor of transformation (Eliyahu et al., 1989, Finley et al., 1989, Hinds et al., 1989, Scheffner et al., 1990). To determine if human p53 (Hp53) is rearranged in transformed fibroblasts, organization of this tumor suppressor gene was examined. A 1.5 kb Xba I-Sma I fragment of a 2.1 kb cDNA clone of hp53 was used as a probe. High molecular weight DNA was prepared from fibroblast cell lines and Southern blot analysis was performed (Figure 10). The Hp53 gene was detected in all cell lines, however, no alterations of this gene were detected when compared to R-30, HPV-16 immortalized and HPV/HSV-2 transformed.

Generation of transformed keratinocyte cell lines. To study interactions between HSV-2 and HPV-16 or HPV-18 in their natural host, human epithelial cells, genital keratinocytes previously immortalized with HPVs, were transfected with plasmids containing HSV-2 mtr III sequences. HPV-16 (FEPL) and HPV-18 (FEA) immortalized cell lines were generated from primary human foreskin keratinocytes (Kaur and McDougall, 1988, Kaur et al., 1989) (Figure 11). To generate HPV-16 immortalized cell lines (FEPL) a plasmid containing the HPV-16 E6, E7 and LCR were used to immortalized normal human keratinocytes (Kaur and McDougall, 1988). FEPL cells were subsequently utilized as parental cells in this transfection

Figure 10. Hp53 gene organization in fibroblast cell lines. (I) Schematic diagram of 2.1 kb Bam HI cDNA hp53 insert. The 1.5 Xba I-Sma I fragment was used as the probe in all hp53 Southern blotting analysis. The 2.1 kb Bam HI cDNA hp53 insert was cloned into pBR322. Translational start and stop codons indicated by arrows.

(II) Genomic DNA prepared from fibroblast cell lines, double digested with Eco RI and Xba I, was subjected to Southern blotting analysis. Lane (A) R-30, (B) 139, (C) 140, (D) 141, (E) 145, (F) C-1, (G) 181, (H) 268, (I) 1.5 kb Xba I/Sma I hp53 fragment. The probe used for hybridization was the 1.5 kb hp53 fragment. Size markers, lambda DNA digested with Hind III are denoted by lines.

I



II

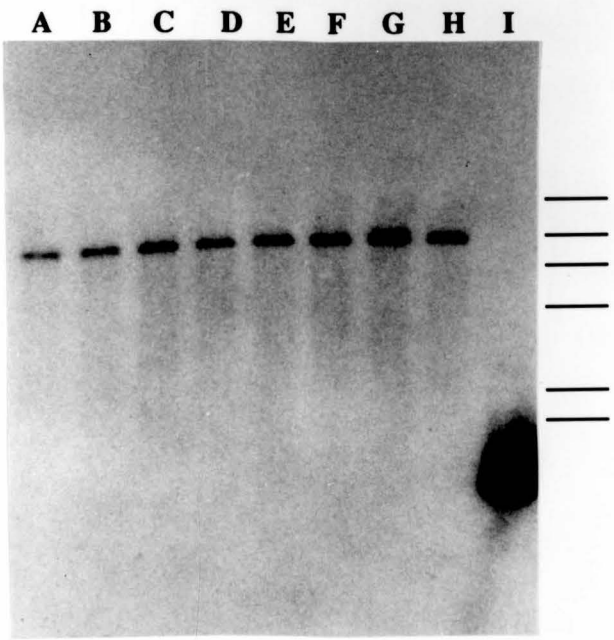
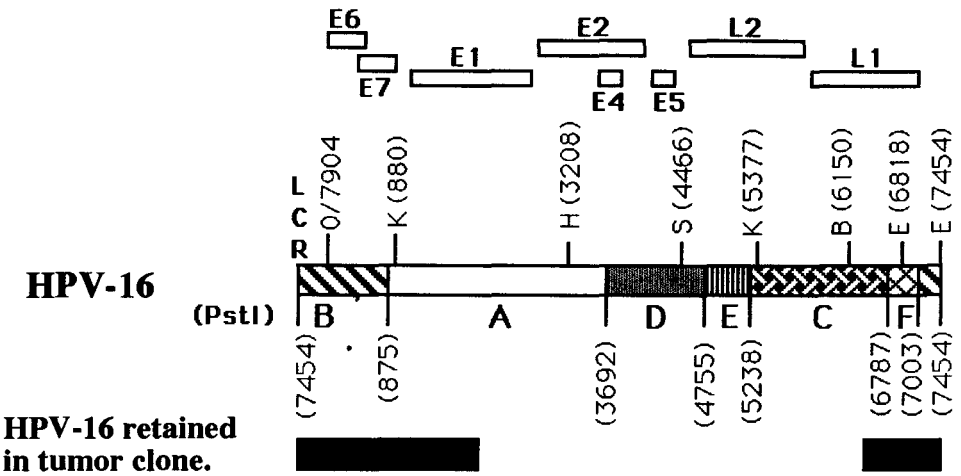


Figure 11. Schematic map of HPV-16 and HPV-18.

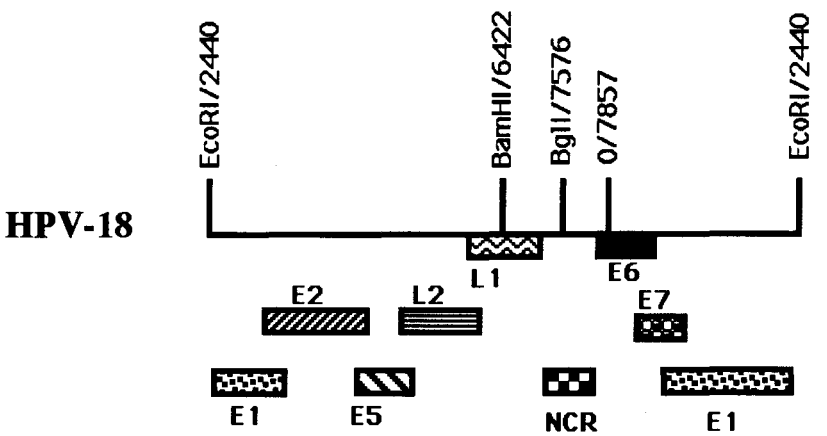
(I) Map of HPV-16 indicating the 3.4 kb fragment of HPV-16 DNA retained in a tumor clone (black boxes) used to immortalize primary genital keratinocytes (Kaur et al, 1989). These HPV-16 immortalized keratinocytes were subsequently used to generate HSV-2/HPV-16 cell lines (designated as FEPL- cells). Restriction enzyme sites are K (Kpn I), H (Hinc II), S (Stu I), E (Eco RI) and B (Bam HI).

(II) Restriction map of HPV-18. pHPV18, containing HPV-18 genome and pBR322 vector sequences, was used to transfect primary genital keratinocytes (Kaur and McDougall, 1988). The resulting HPV-18 immortalized cell lines were used to generate HSV-2/HPV-18 cell lines (designated as FEA- cells).

I


E6
E7
E1
E2
E4
E5
L2
L1
L
C
R
0/7904
K (880)
H (3208)
S (4466)
K (5377)
B (6150)
E (6818)
E (7454)
HPV-16
(PstI)
B
A
D
E
C
F
(7454)
(875)
(3692)
(4755)
(5238)
(6787)
(7003)
(7454)
HPV-16 retained
in tumor clone.

II


HPV-18
EcoRI/2440
BamHI/6422
BglI/7576
0/7857
EcoRI/2440
L1
E6
E2
L2
E7
E1
E5
NCR
E1

analysis. HPV-18 immortalized (FEA) cells were generated by cotransfecting primary human epithelial cells with a plasmid containing the entire HPV-18 genome and pSV2neo and selecting for growth in geneticin (Kaur and McDougall, 1988). FEA cells were also used as parental cells in this analysis. Both cell lines were maintained in culture over one year prior to these studies.

To test for morphological transformation of FEPL and FEA cells, plasmids containing HSV-2 DNA sequences derived from mtr III (Jones et al., 1986) were transfected into parental cell lines. Additionally, a plasmid containing an activated H-ras gene, pEJ6.6, was transfected into immortalized cells to serve as a positive control for cooperation with HPVs (Matlashewski et al., 1987). Table 6 shows the results from the transfection experiments using HPV-16 (FEPL) and HPV-18 (FEA) immortalized cells. Three independent experiments were performed. G418 selection was utilized because parental HPV-immortalized cells have lost their geneticin resistance and thus are sensitive to drug selection. Stable cell lines were obtained with all transfectants. FEPL and FEA cells had similar transfection efficiencies. No single HSV-2 mtr III region was more efficient for transforming either FEPL or FEA cells. The overall efficiency of Experiment 1 from Table 6 was low compared to subsequent experiments. In summary, results demonstrated that geneticin resistant cell lines could be obtained from HPV immortalized cells transfected with HSV-2

Table 6. Transfection analysis of immortalized keratinocytes.

	FEA (HPV-18)			FEPL (HPV-16)		
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
HSV-2 486TF	2	5	8	0	7	10
HSV-2 Pst I-C	1	5	6	1	6	8
HSV-2 Bam HI-E	1	8	6	1	7	12
Ha-ras	3	4	9	0	8	15
pSV2neo	0	2	4	0	5	10

HPV-16 or -18 immortalized genital keratinocytes (5 x 10^5 cells/60 mm dish) were transfected with 10 ug plasmid DNA using the polybrene transfection procedure (Rhim et al, 1986) followed by geneticin selection (50 ug/ml for FEA cells, 500 ug/ml for FEPL cells). Values indicate the number of geneticin resistant colonies.

mtr III derived sequences.

Growth characteristics of transformed cell lines. A comparison of HPV immortalized and HPV/HSV-2 transformed cell lines was undertaken to determine if differences existed. Subtle morphological differences between HPV-16 immortalized and HPV-16/HSV-2 transformed keratinocytes were observed (Figure 12). Transformed cells were irregularly shaped, less organized compared to immortalized cells. Furthermore, morphological differences were seen between primary keratinocytes and an immortalized FEPL clone in Figure 13. Differences were more apparent in transformed FEPL cells than FEA cells. Saturation density analysis indicated that FEPL or FEA cells transformed by mtr III DNA fragments contained two times the number of cells after one week in culture than the immortalized counterpart (Table 7). HPV/H-ras cell lines (FEA or FEPL) behaved similarly to HPV/HSV-2 transformants while HPV immortalized cells with pSV2neo had saturation density values similar to the HPV immortalized parental cells.

To analyze anchorage independent growth capabilities of the various cell lines, growth was assessed by plating cells in 0.3% agarose and scoring after 10 days. All three HPV-16/HSV-2 transformed cell lines grew at similar efficiencies in soft agar. Immortalized FEPL cells were able to grow with low efficiency in soft agar, however, not at the rate of the transformed counterparts. In contrast, the HPV-18/HSV-2 transformed cells did not grow in soft agar. Furthermore, FEA

Figure 12. Photograph of immortalized FEPL cells (I) and FEPL cells transformed with the HSV-2 mtr III Bam HI-E fragment (II).

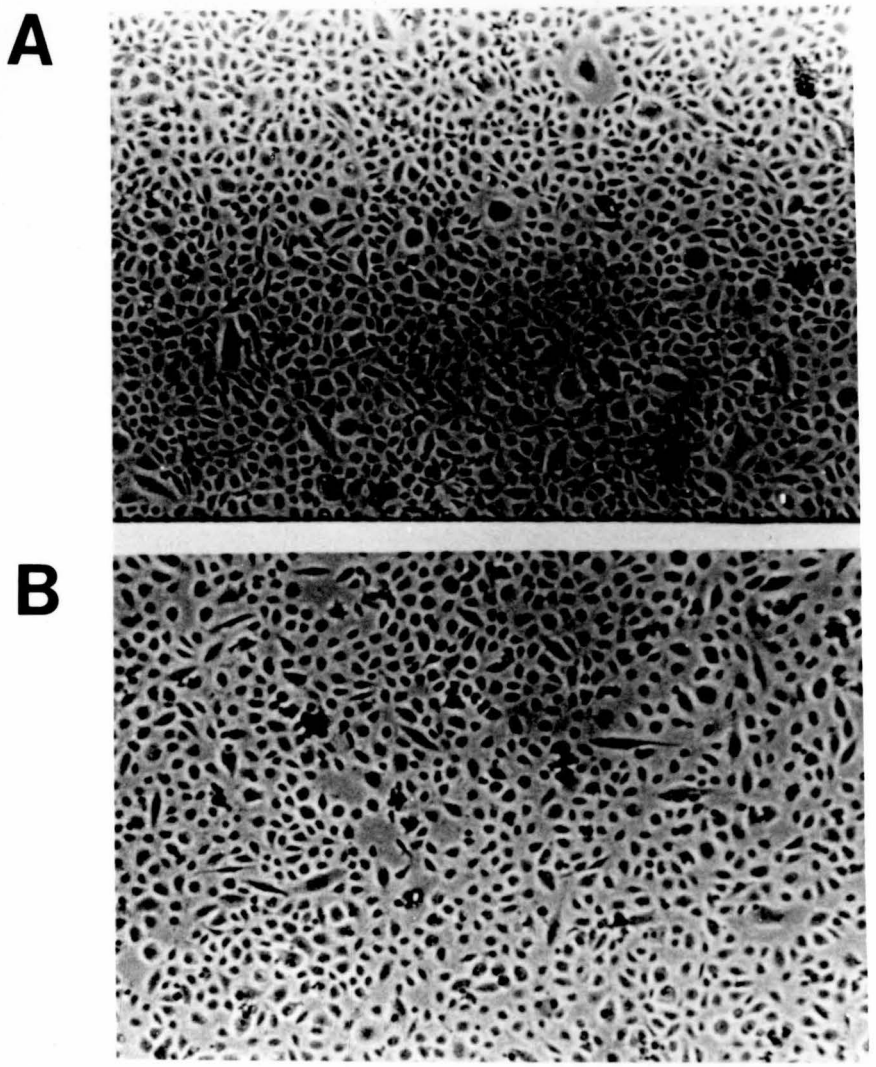

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Figure 13. Primary keratinocytes immortalized with HPV-16. Photograph of immortalized HPV-16 geneticin resistent colony seen along with primary keratinocytes.

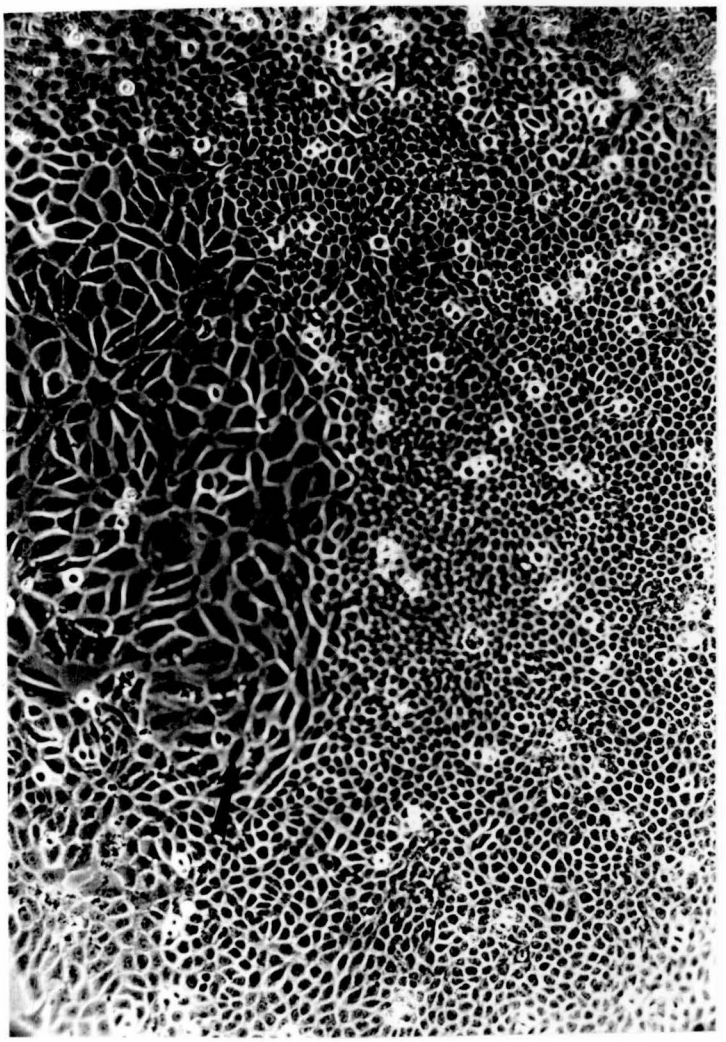

Table 7. Growth characteristics of keratinocyte cell lines.

Cell line	0.3% agarose colony formation[1]	Saturation density[2]
FEA-N (HPV-18)	< 1	$1.2 \times 10^6 \pm 6.5 \times 10^5$
ATF-1 (HPV-18/486 TF)	< 1	$2.8 \times 10^6 \pm 4.4 \times 10^5$
APC-1 (HPV-18/Pst I-C)	< 1	$2.0 \times 10^6 \pm 8.8 \times 10^5$
ABE-1 (HPV-18/Bam HI-E)	< 1	$2.6 \times 10^6 \pm 6.3 \times 10^5$
FEPL-N (HPV-16)	5	$3.2 \times 10^6 \pm 8.7 \times 10^5$
PTF-1 (HPV-16/486 TF)	15	$6.3 \times 10^6 \pm 3.8 \times 10^5$
PPC-1 (HPV-16/Pst I-C)	15	$5.9 \times 10^6 \pm 5.8 \times 10^5$
PBE-1 (HPV-16/Bam HI-E)	15	$6.4 \times 10^6 \pm 4.5 \times 10^5$

Growth characteristics of keratinocyte cell lines were analyzed for anchorage independent growth and saturation density rates.
[1]1×10^5 cells of each cell line were plated in medium containing 0.3% agarose and scored after 10 days. Numbers indicate the approximate percentage of cells which formed colonies. Colonies were counted if size was at least 1 mm.
[2]Saturation density was the number of cells/100 mm dish at confluence. Plates were seeded with 3×10^5 cells and after one week in culture, cells were trypsinized and counted using a hemocytometer.

immortalized cells did not grow efficiently in soft agar (Table 7). In summary, slight differences in morphology were detected in HPV-16/HSV-2 transformed cell lines but these differences were not readily seen in HPV-18/HSV-2 cell lines. Morphology changes were clearly apparent after primary keratinocytes were immortalized with HPVs. Both HPV-16 and HPV-18 transformed cell lines had saturation density values twice as great as their immortalized counterpart. Only HPV-16/HSV-2 transformed cells exhibited anchorage independent growth capabilities.

HPV DNA retention in keratinocyte cell lines. FEA and FEPL cell lines have one to two copies of integrated viral genomes/cell (Kaur and McDougall, 1988, Kaur et al., 1989). This study was conducted to examine the organization of HPV DNA after the cells were transformed by HSV-2 mtr III or H-ras. High molecular weight DNA from various cell lines was analyzed to detect rearrangments of HPV viral DNA by Southern blot analysis.

Genomic DNA from FEPL cell lines, containing only 3.4 kb of HPV-16 sequences, was hybridized to three separate probes: a large 6.6 kb probe spanning the entire HPV-16 early genome (excised from HZIP-16, does not include the late region), a 1.0 kb probe covering the LCR, E6 and E7 ORFs (Pst I-B fragment of HPV-16) and a 2.6 kb probe containing the E1, E2 ORFs (Kpn I/Hinc II fragment of HPV-16). Two HPV-16 specific fragments were detected when Pst I digested genomic DNA was

probed with the 6.6 kb HPV-16 probe (Figure 14). Pst I digestion of 3.4 kb HPV-16 sequences retained in FEPL cells yields 4 fragments: 2 which contain the early HPV-16 regions and 2 which span the late HPV-16 regions. The latter two bands would not be detected with the 6.6 kb probe. These results suggest the early region of the 3.4 kb sequence is intact. Hybridization with the 6.6 kb probe did not show any rearrangments of the HPV-16 genome. Neither the 1.0 kb Pst I-B (Figure 15) nor the 2.8 kb Kpn I-A (Figure 16) revealed any rearrangements of the HPV-16 DNA in transformed cell lines. Malignant lesions which contain HPV DNA integrated into the cellular genome frequently have only a portion of the viral DNA present (Durst et al., 1985). Viral DNA usually consists of an intact E6, E7 and LCR (Matsukura et al., 1986, Pater and Pater, 1985, Seedorf et al., 1987). The Kpn I-A probe was utilized to detect possible rearrangments of viral DNA since HPV integration occurs within the E1/E2 ORFs and alterations generally occur in this region. However, no rearrangments of HPV-16 DNA were detected in the transformed cell lines, although, changes in the E6 and E7 genes of HPV would not be expected to occur.

To determine if rearrangements of the HPV-18 DNA were present in transformed cells, Southern blot analysis was performed. High molecular weight DNA prepared from FEA derived keratinocyte cell lines was hybridized to a 7.9 kb HPV-18 probe, spanning the entire genome of HPV-18. Figure 17

Figure 14. Organization of HPV-16 DNA in FEPL-derived cell lines. High molecular weight DNA was prepared from FEPL-derived cell lines, digested with Pst I and Southern blot analysis was performed. DNA was probed with the 6.6 kb Bam HI HPV-16 insert of HZIP-16. Lane (A) FEPL-N, (B) PTF-2, (C) PPC-1, (D) PPC-2, (E) PBE-2, (F) Pneo, (G) 6.6 kb HPV-16 Bam HI fragment. Size markers, lambda DNA digested with Hind III, are denoted by lines.

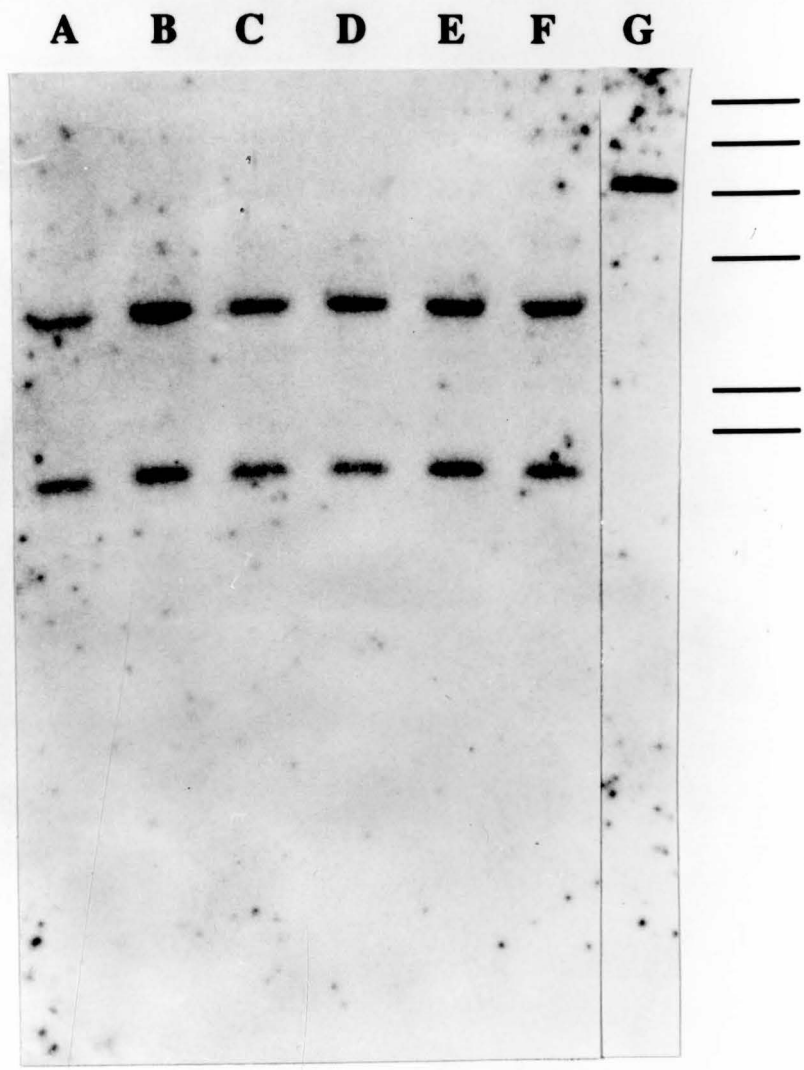
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Figure 15. Retention of E6, E7 ORFs in FEPL cell lines. High molecular DNA prepared from FEPL derived cell lines was double digested with Pst I and Eco RI and subjected to Southern blot analysis. DNA was probed with the Pst I-B fragment of HPV-16 which spans, ORFs E6, E7 and the LCR. Lanes (A) FEPL-N, (B) PTF-2, (C) PPC-1 (D) PPC-2, (E) PBE-1, (F) Pras. Size markers, lambda digested with Hind III, are denoted by bars.

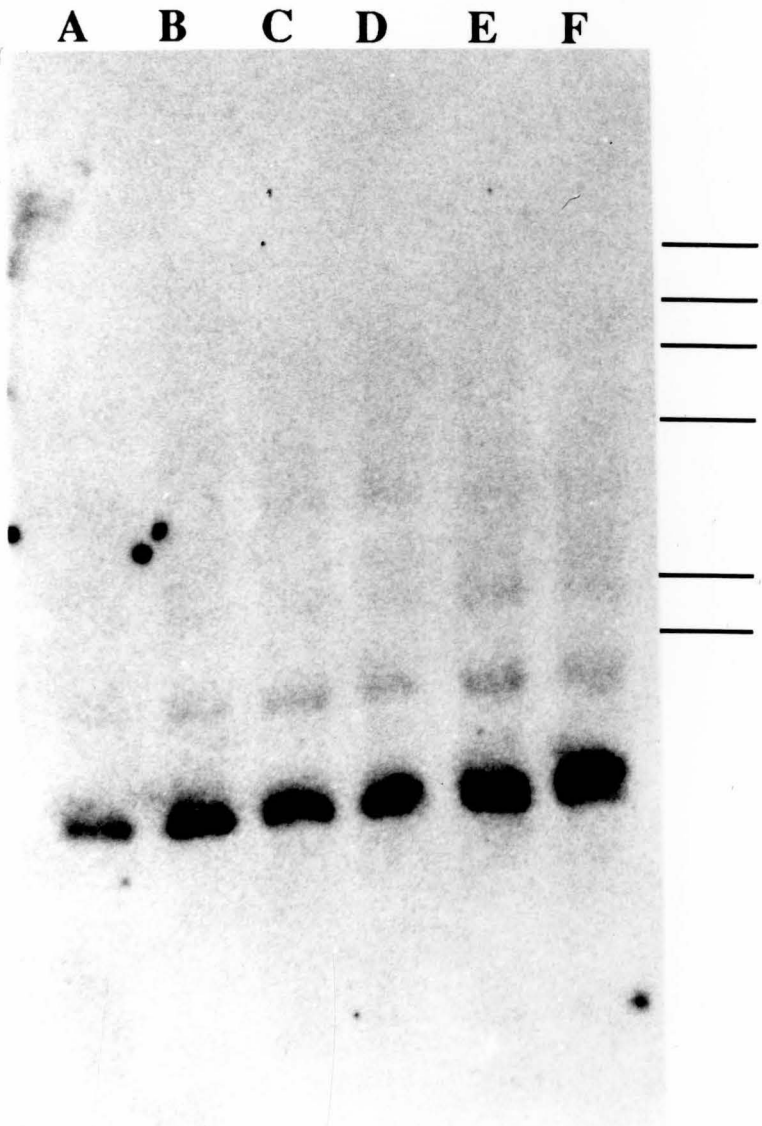
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Figure 16. Retention of ORF E1 in FEPL cell lines. Genomic DNA was prepared from FEPL derived cell lines, double digested with Pst I and Eco RI and Southern blot analysis was performed. The 2.8 kb Kpn I-A fragment, spanning the entire E1 ORF was used the probe. Lane (A) FEPL-N, (B) PTF-2, (C) PPC-1, (D) PPC-2, (E) PBE-1, (F) Pras. Size markers, lambda DNA digested with Hind III, are denoted by lines.

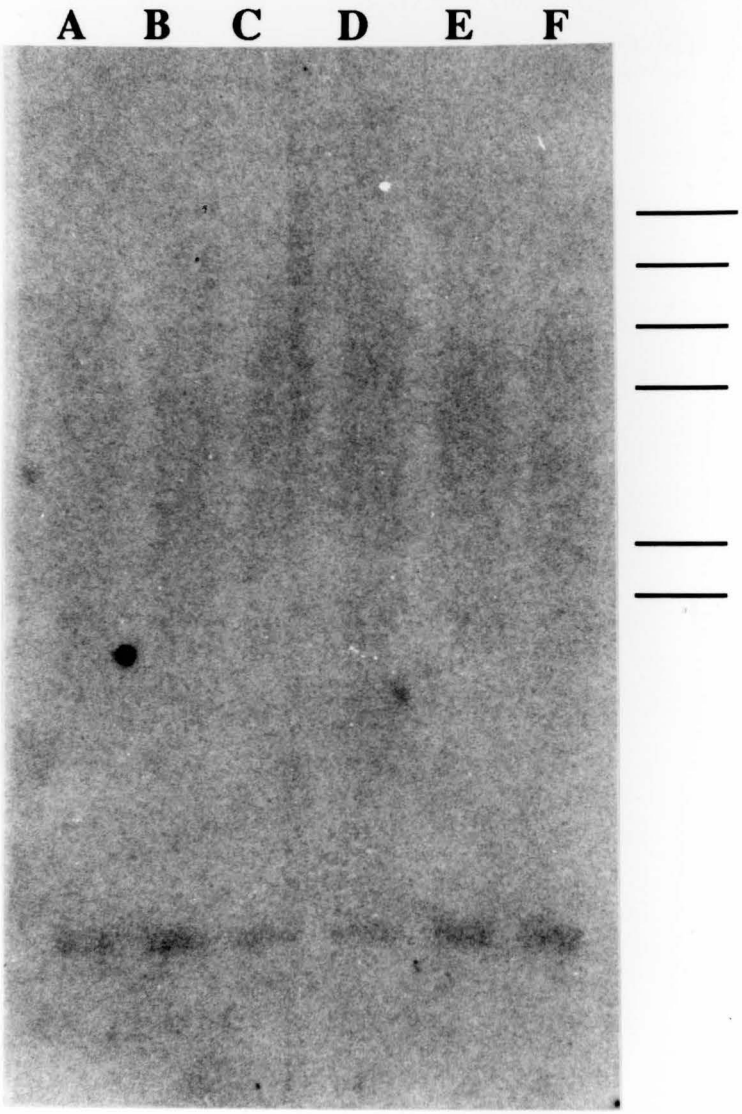
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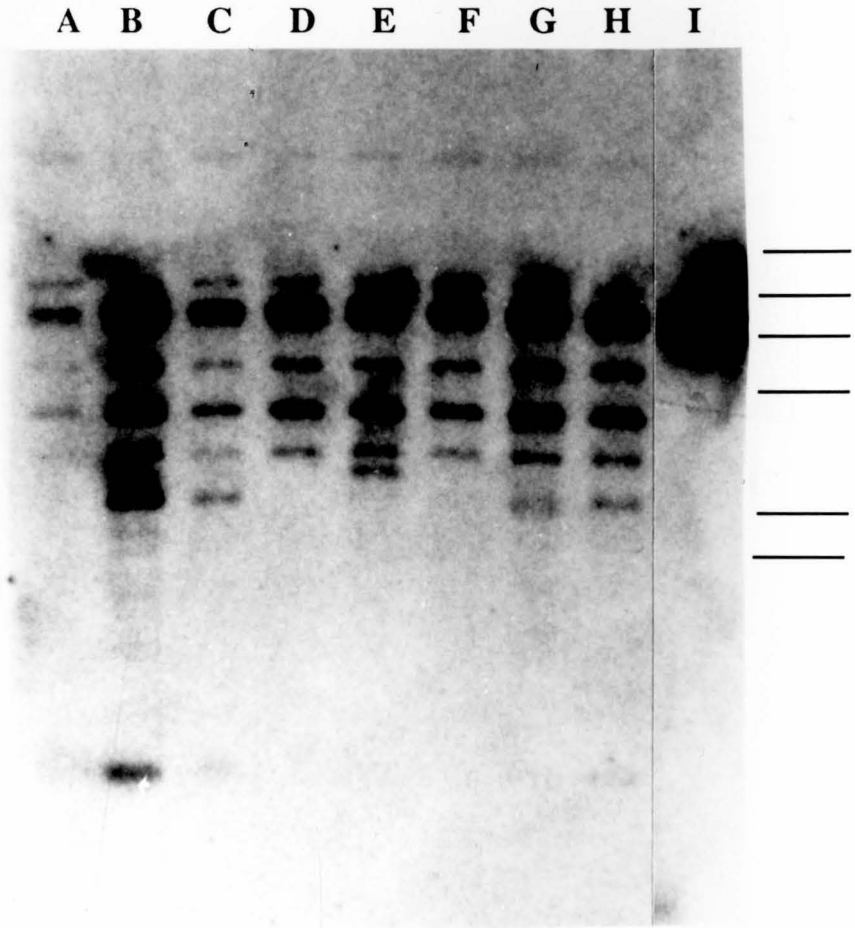
Figure 17. Rearrangment of HPV-18 DNA in transformed keratinocyte cell lines. Genomic DNA prepared from FEA-derived cell lines, digested with Pst I, was subjected to Southern blotting. The 7.9 kb HPV-18 genome was utilized as the probe. Lane (A) FEA-N, (B) ATF-1, (C) ATF-2, (D) APC-1, (E) ABE-1, (F) ABE-2, (G) Aras, (H) Aneo, (I) 7.9 kb fragment of HPV-18. Size markers, lambda DNA digested Hind III, are designated by lines.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shows the results of the Southern blot analysis. Rearrangements of viral DNA were detected in transformed cells (lanes B-F) when compared to immortalized (lane A) and HPV/H-ras (lane G) cell lines. Deletions were detected in transformed cells with the loss of a 2.3 kb band in APC-1 (lane D) and ABE-1 (lane F). In contrast, cell line APC-2 contained an additional 2.6 kb hybridizing fragment (lane E). Similar results were seen when genomic DNA was digested with Hinc II (Figure 18). However, when genomic DNA of FEA cell lines was hybridized to a HPV-18 probe spanning E6, E7 and LCR, no changes were detected (Figure 19) as expected since this region remains intact in a majority of HPV-18 cell lines. In summary, no detectable changes were present in the HPV-16 DNA of transformed cell lines. However, rearrangments were detected in the HPV-18 DNA after FEA cells were transformed by HSV-2 mtr III sequences.

Retention of HSV-2 DNA sequences in transformed cell lines. HSV-2 DNA sequences have not consistently been detected in transformed cells, however, significant evidence has demonstrated a correlation between HSV-2 infection and malignant transformation (DiLuca et al., 1989, Hildesheim et al., 1991). The inconsistent nature of HSV-2 DNA retention has led to a "hit and run" hypothesis for HSV-2 mediated transformation (reviewed by MacNab, 1987). Southern blot analysis using HSV-2 DNA sequences as probes failed to detect viral DNA (Figure 20). Since PCR analysis is a more sensitive

Figure 18. Hinc II digest of DNA from FEA-derived cell lines. Genomic DNA was prepared from FEA-derived cell lines, digested with Hinc II and subjected to Southern blotting. Lane (A) FEA-N, (B) ATF-1, (C) ATF-2, (D) APC-1, (E) APC-2, (F) ABE-1, (G) ABE-2, (H) Aras. The probe used was the 7.9 kb HPV-18 genome. Molecular size markers, lambda DNA digested with Hind III, are denoted by lines.

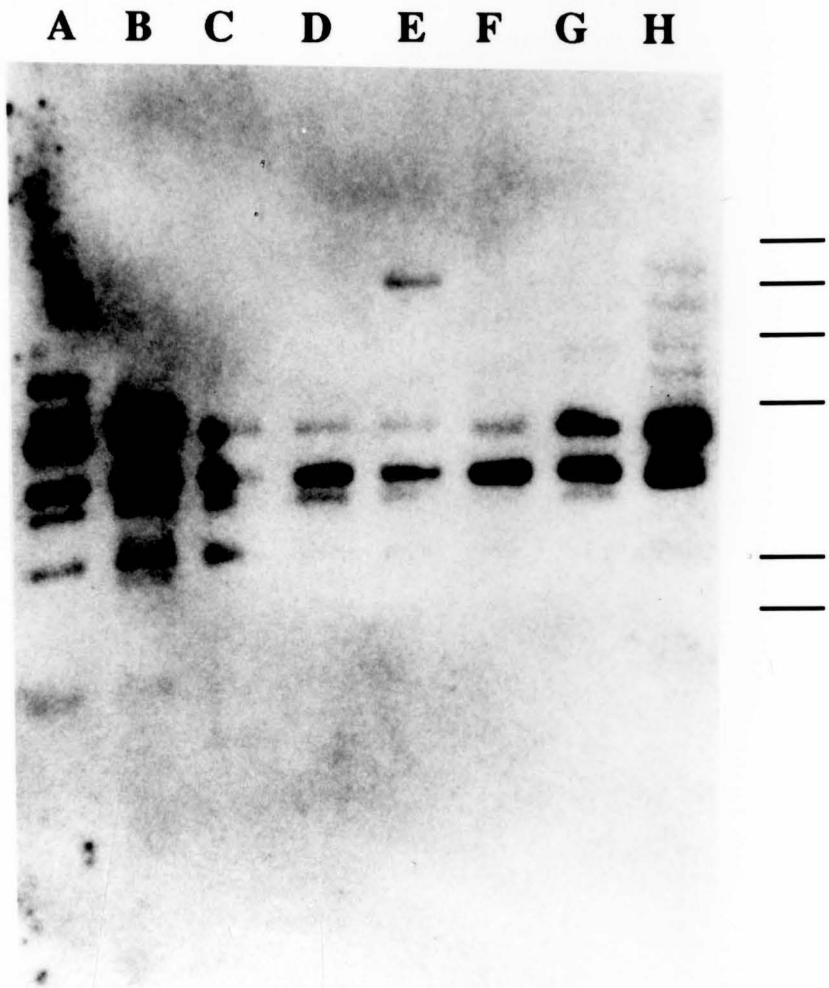
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Figure 19. Retention of HPV-18 sequences in keratinocyte cell lines. High molecular weight DNA prepared from HPV-18 keratinocyte cell lines, digested with Pst I, was subjected to Southern blotting analysis. A 2.4 Eco RI/Bgl I fragment spanning the HPV-18 LCR, E6, E7 ORFs. Lane (A) FEA-N, (B) ATF-1, (C) ATF-2, (D) APC-1, (E) APC-2, (F) ABE-1, (G) Aras. Size markers, lambda DNA digested with Hind III, are denoted by lines.

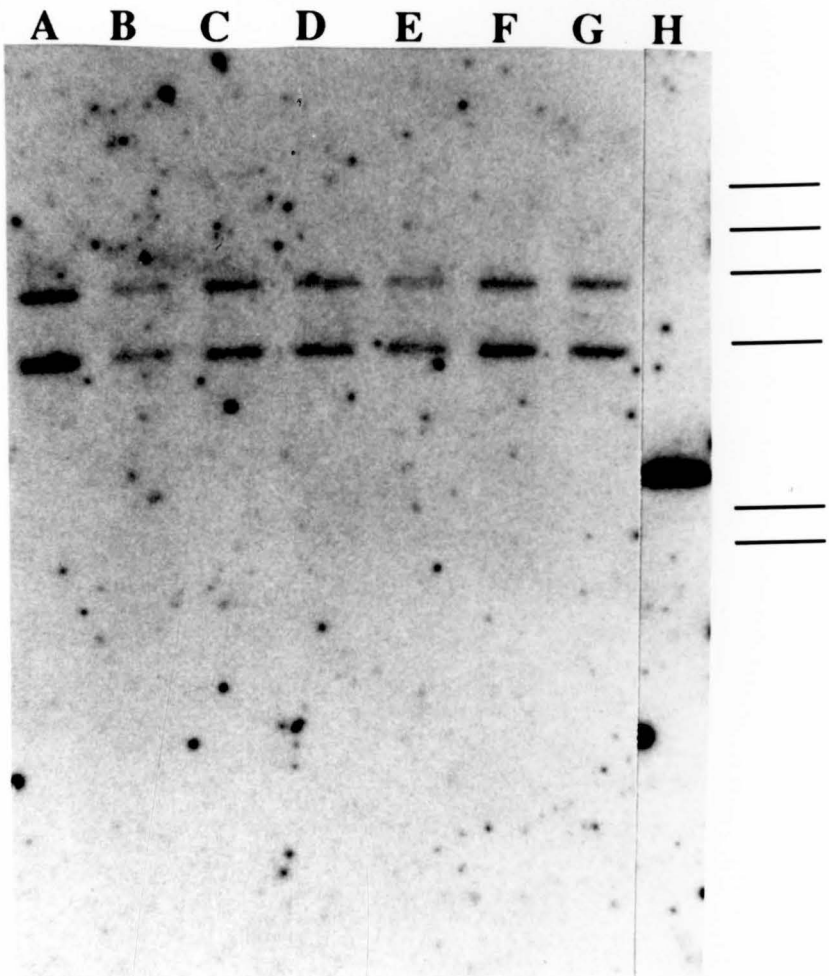
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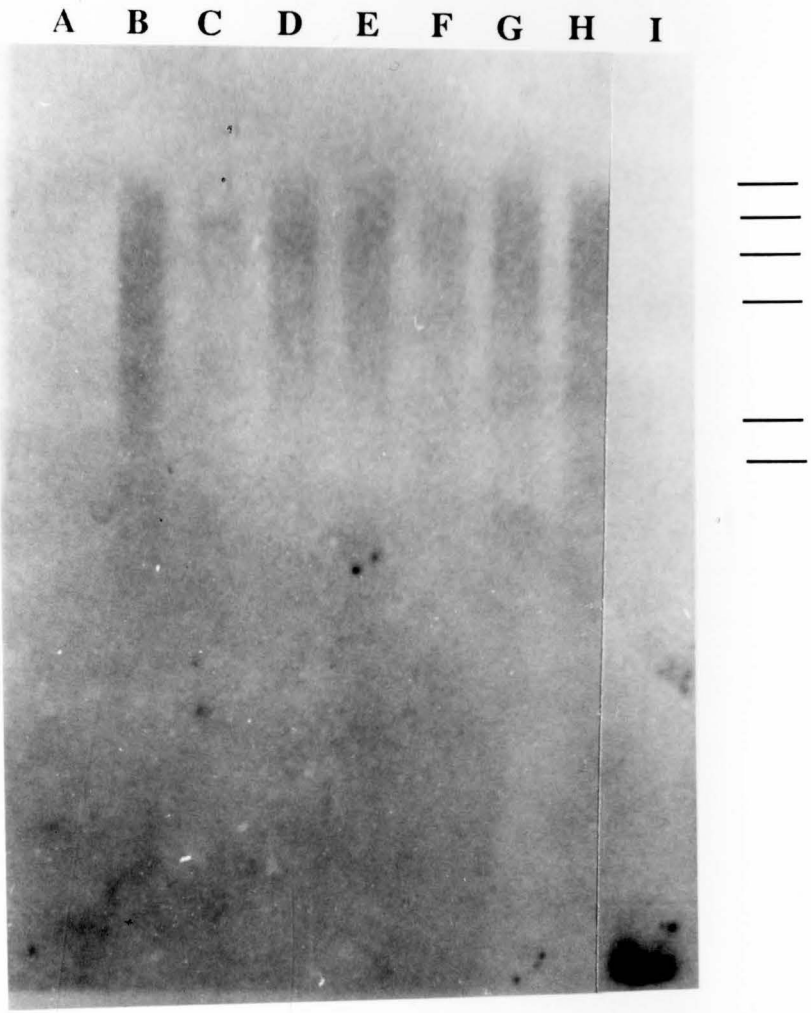
Figure 20. Retention of HSV-2 DNA sequences in FEA-derived cell lines. High molecular weight DNA prepared from FEA derived keratinocyte cell lines, digested with Eco RI, was analyzed by Southern blot analysis to detect retention of HSV-2 DNA sequences. The HSV-2 mtr III 486 bp Pst I-Sal I fragment (486 TF) was used as the probe. Lane (A) FEA-N, (B) ATF-1, (C) ATF-2, (D) APC-1, (E) APC-2, (F) ABE-1, (G) Aras, (H) Aneo, (I) 486 TF. Molecular size markers, lambda DNA digested with Hind III, are denoted by bars.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assay to detect DNA sequences present in very low quantities, this technique was employed. High molecular weight DNA digested with Eco RI was subjected to PCR analysis using primers derived from HSV-2 486 TF. Analysis of FEPL and FEA derived cell lines indicated HSV-2 retention in only three FEA cell lines (Figure 21). None of the 5 HPV-16/HSV-2 cell lines retained HSV-2 DNA, whereas, 3/5 HPV-18/HSV-2 cell lines retained HSV-2 DNA sequences, ATF-2, APC-2, ABE-2. The amplified band migrated as a 366 bp fragment. Neither the immortalized keratinocyte cell lines, normal keratinocyte DNA nor the HPV/H-ras had 486 TF hybridizing fragments. These results demonstrated HSV-2 DNA sequences were present in low concentrations in some transformed cell lines.

Analysis of cellular protooncogene c-myc in transformed keratinocyte cell lines. Earlier studies have demonstrated that integration of high risk HPVs occurs near the c-myc protooncogene or led to disruption of c-myc gene (Riou et al., 1987, Durst et al., 1987, Crook et al., 1990). To determine if the c-myc locus was disrupted after FEA or FEPL cells were transformed by HSV-2 mtr III, organization of the c-myc gene was examined. Two c-myc probes, a 1.6 kb Sac I fragment of the 5′ untranslated region and a 420 bp Pst I fragment of exon 2 were utilized to detect alterations in gene organization of c-myc DNA. Figures 22 and 23 show the Southern blot results when high molecular weight DNA was digested with restriction enzymes (HPV-16, Pst I; HPV-18, Hinc II) and hybridized to the

Figure 21. Polymerase chain reaction (PCR) analysis of transformed keratinocyte cell lines. High molecular weight DNA was prepared from the respective cell lines, digested with Pst I and 1 ug of digested DNA was used for the PCR reaction. Primers were derived from HSV-2 mtr III 486 TF. Lane (A) contains 0.1 ug pCPS plasmid (486 TF cloned into Pst I and Sal I sites of pUC19). Lanes B-Q contains DNA prepared from FEPL and FEA derived cell lines. Lane (B) PTF-1, (C) PPC-1, (D) PPC-2, (E) PBE-1, (F) PBE-2, (G) Pras, (H) Pneo, (I) ATF-2, (J) APC-1, (K) APC-2, (L) ABE-1, (M) ABE-2, (N) Aras, (O) Aneo, (P) normal primary keratinocyte DNA, (Q) "no template" control. The probe used was the 486 TF fragment. Bars indicate size markers, PhiX174 DNA digested with Hae III (bp) (1353, 1078, 872, 603, 310, 281, 234, 174, and 118).

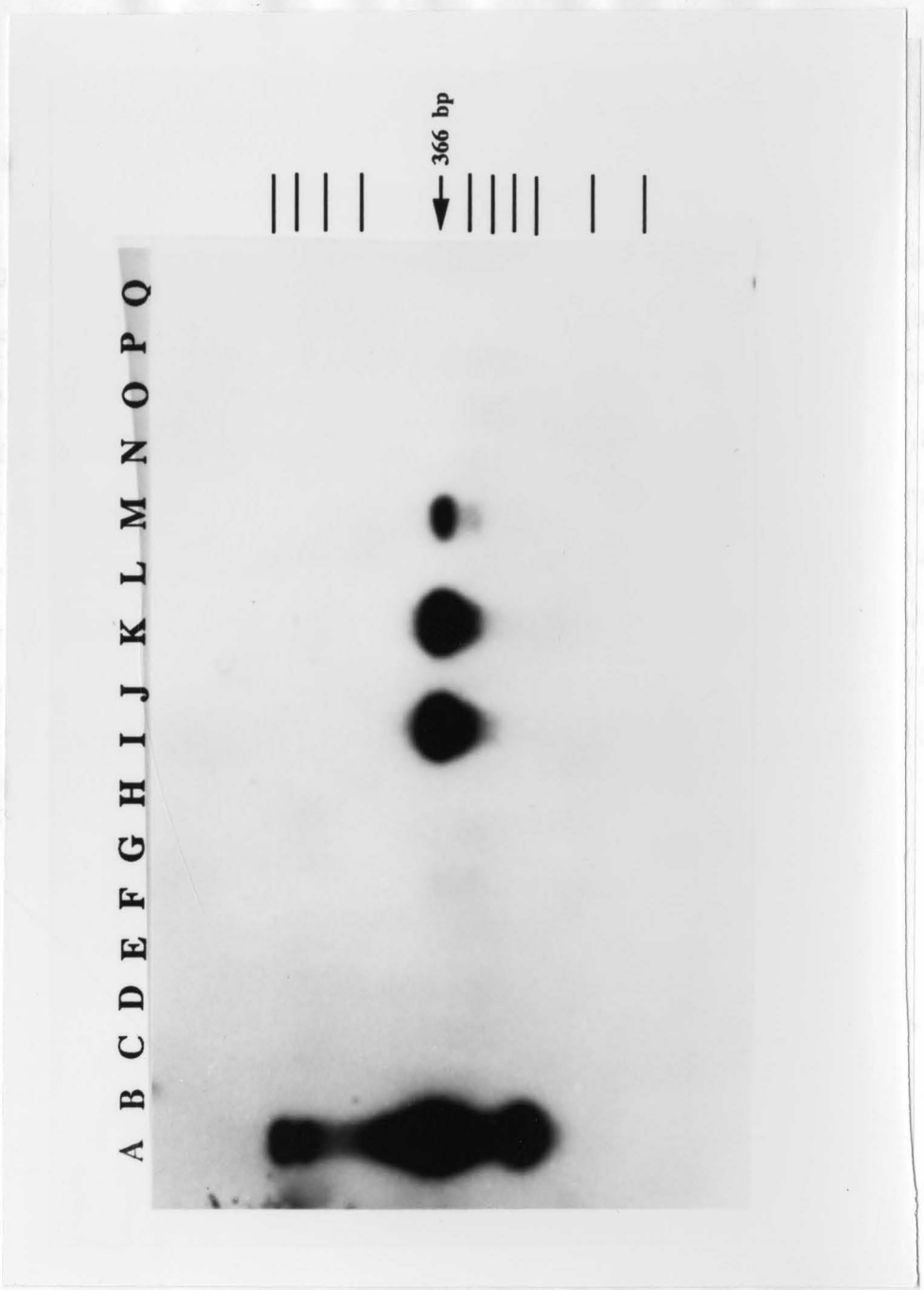
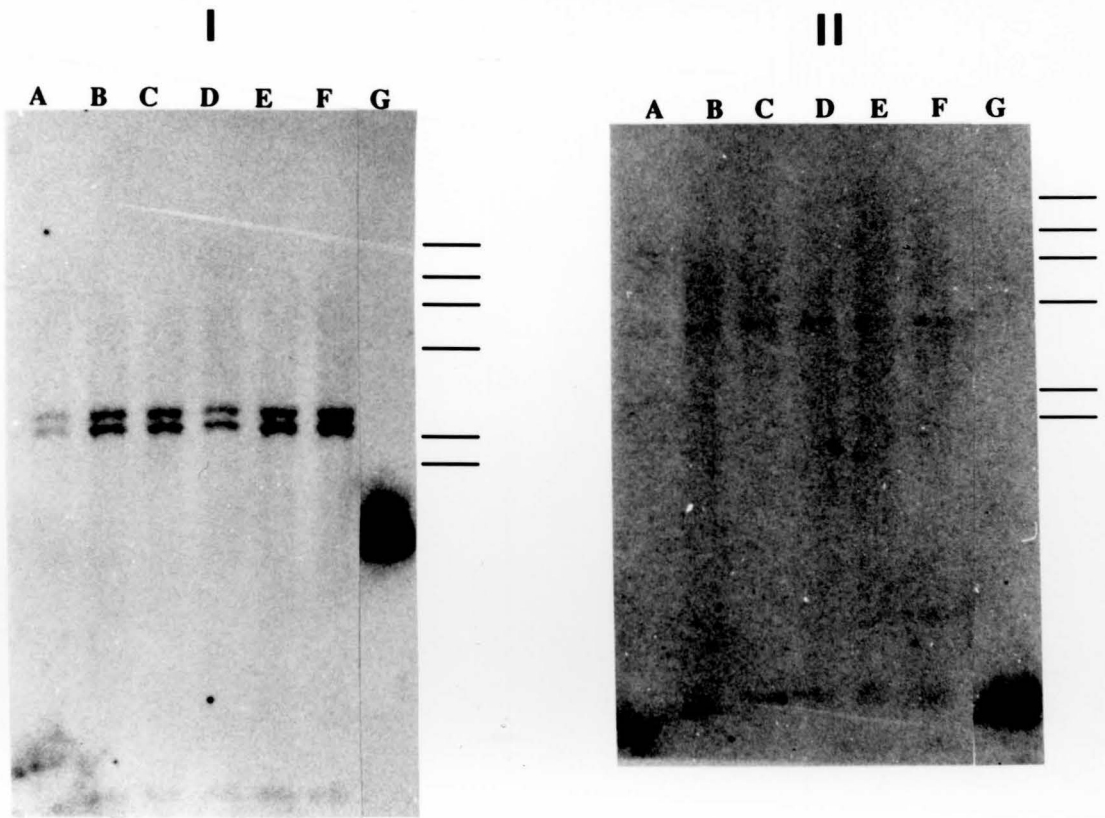

366 bp
A B C D E F G H I J K L M N O P Q

Figure 22. C-myc gene organization in FEPL-derived cell lines. High molecular weight DNA from FEPL cell lines was prepared and digested with Eco RI and Pst I, followed by Southern blotting analysis. Lanes (A) FEPL-N, (B) PTF-2, (C) PPC-1, (D) PPC-2, (E) PBE-2, (F) Pneo, (G) either 5'-untranslated region (I) or exon 2 in (II). Probes used in hybridization were (I) a 1.6 kb Sac I fragment of the 5'-untranslated region and (II) a 420 bp Pst I fragment of exon 2. Size markers, lambda DNA digested with Hind III, are denoted by lines.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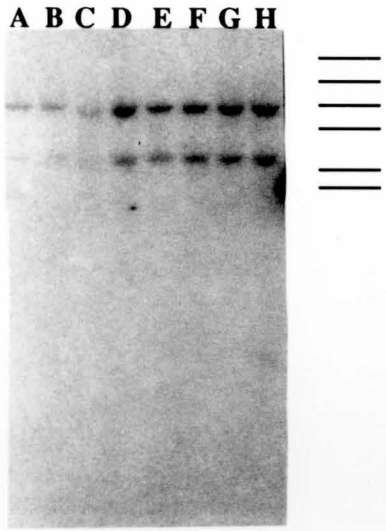
A B C D E F G
II
A B C D E F G

Figure 23. C-myc gene organization in FEA-derived cell lines. High molecular weight DNA was prepared from primary keratinocytes and FEA derived cell lines, digested with restriction enzymes, and Southern blot analysis was performed. Size markers, lambda DNA digested with Hind III, are denoted by lines.

(I) Genomic DNA was digested with Pst I and probed with the 1.6 kb Sac I fragment of the 5'-untranslated region of c-myc. Lane (A) normal primary keratinocyte DNA (NKD), (B) FEA-N, (C) ATF-1, (D) ATF-2, (E) APC-2, (F) ABE-1, (G) ABE-2, (H) Aras.

(II) Genomic DNA was digested with Hinc II and probed with the 420 bp Pst I fragment of exon 2 of c-myc. Lane (A) NKD, (B) FEA-N, (C) ATF-1, (D) ATF-2, (E) APC-2, (F) ABE-1, (G) ABE-2, (H) Aras.

I



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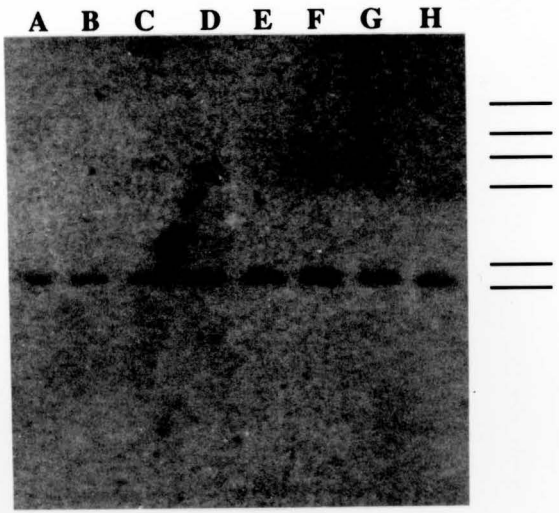

c-myc probes. No detectable rearrangments were observed in any cell lines, either HPV-16 or HPV-18 derived. Furthermore, c-myc organization of immortalized and transformed cell lines was compared to primary keratinocyte DNA and no changes were apparent. Multiple restriction digests (Eco RI, Eco RI/Pst I, Eco RI/Kpn I) were anaylzed.

Analysis of hp53 in transformed keratincyte cell lines. Mutations in the tumor suppressor gene, p53, have been associated with hyperproliferation and chromosomal instability (Hudson et al., 1990, Woodworth et al., 1990). The E6 early protein of HPV-16/18 binds the p53 gene product and promotes degradation of the cellular protein (Scheffner et al., 1990). Thus, it was of interest to understand what role this cellular gene may have in the pathogenesis of HPV/HSV-2 transformed cell lines. Hence, gene organization of human p53 (hp53) was analyzed. A 1.5 kb Xba I-Sma I fragment from the hp53 cDNA clone was used as probe since it yielded clear results. Southern blot analysis indicated no apparent aberration in hp53 gene organization of either FEPL derived (Figure 24) or FEA derived (Figure 25) cell lines. Multiple digests of genomic DNAs were performed and did not detect rearrangments (data not shown). In summary, rearrangements of the hp53 tumor suppressor gene were not apparent in HPV/HSV-2 transformed cell lines when compared to HPV immortalized cell lines.

RNA analysis of transformed cell lines. Northern blot

Figure 24. Hp53 gene organization in FEPL-derived cell lines. Genomic DNA was prepared from FEPL derived cell lines, double digested with Eco RI and Pst I and Southen blot analysis was performed. The probe used for hybridization was the 1.5 kb Xba I-Sma I fragment of hp53. Lane (A) FEPL-N, (B) PTF-2, (C) PPC-1, (D) PPC-2, (E) PBE-2, (F) Pneo, (G) hp53 probe. Size markers, lambda DNA digested with Hind III, are denoted by lines.

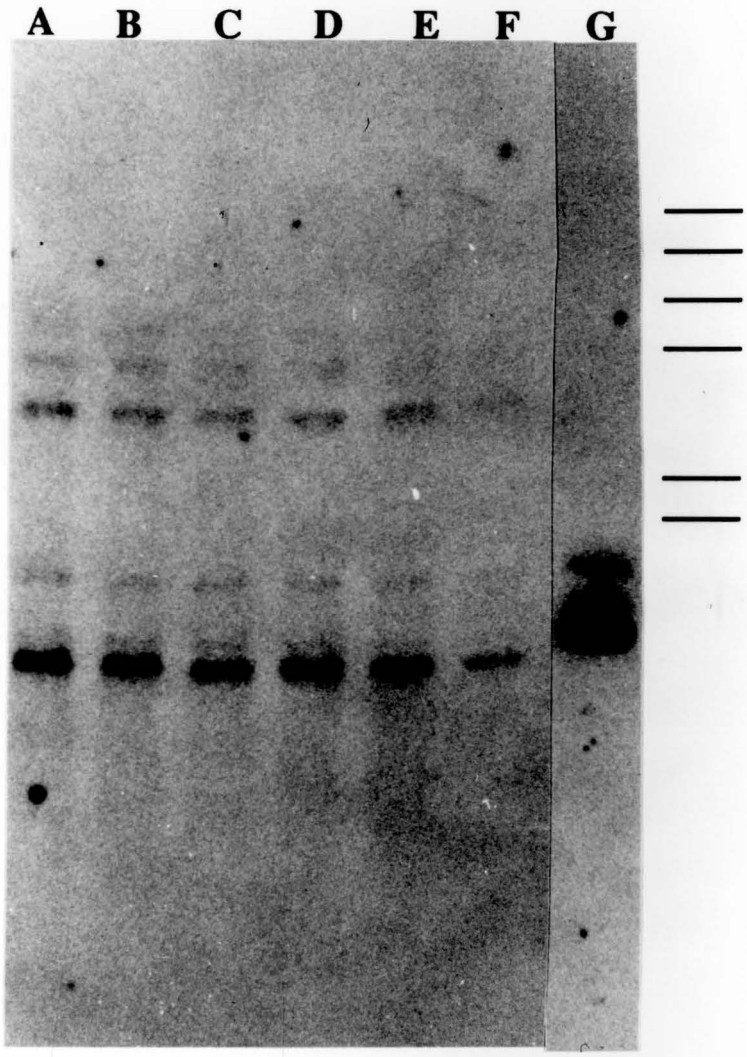
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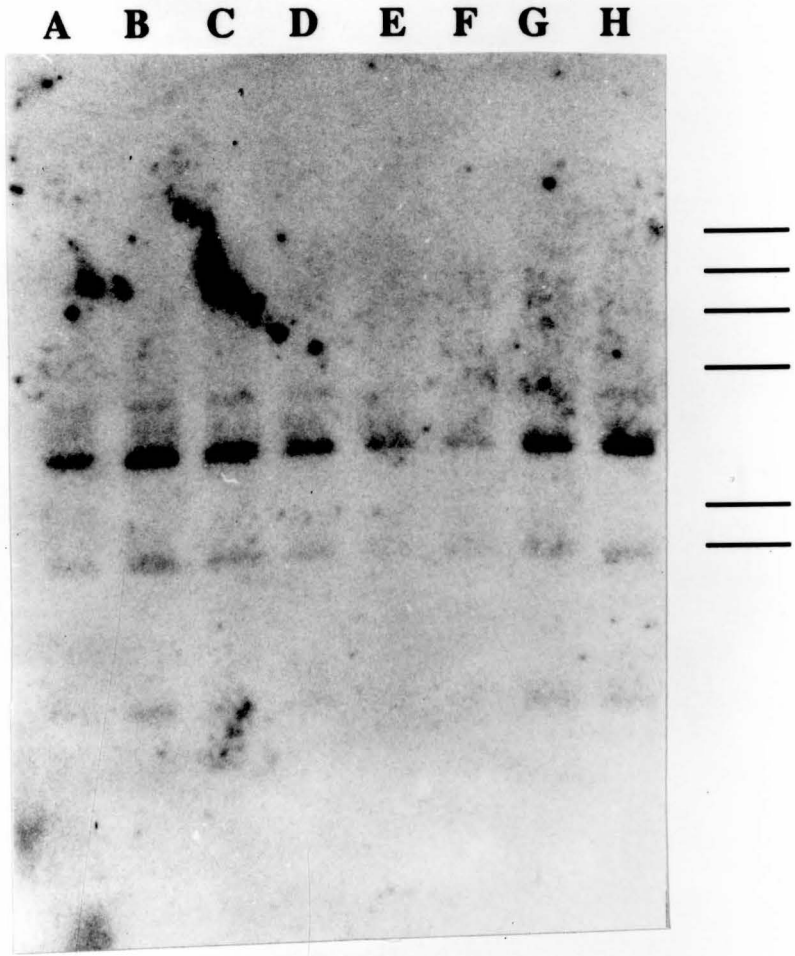
Figure 25. Hp53 gene organization in FEA-derived cell lines. Southern blotting analysis was performed using genomic DNA prepared from FEA cell lines and digested with Pst I. DNA was probed with the 1.5 kb Xba I-Sma I fragment of hp53. Lane (A) FEA-N, (B) ATF-1, (C) ATF-2, (D) APC-1, (E) APC-2, (F) ABE-1, (G) Aras, (H) Aneo. Molecular size markers, lambda DNA digested with Hind III, are denoted by lines.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analysis was performed to determine if RNA transcripts differed in HPV immortalized and HPV/HSV-2 transformed cell lines. Total cellular RNA was hybridized to probes spanning the HPV-16 or -18 E6 and E7 ORFs. Three transcripts were detected ranging in size from 4.5 kb to 1.8 kb (Figure 26). However, no specific differences were observed between immortalized and transformed cell lines.

Tumorigenicity analysis. To determine if the respective cell lines could induce tumors in animals, cell lines were injected subcutaneously into nu/nu nude mice (5 x 10^6 cells/animal). Mice were monitored weekly for the presence of lesions. Eight weeks post injection, mice were anesthesized and lesions excised. FEPL (HPV-16) cells transfected with HSV-2 mtr III sequences were all able to form abnormal tumor-like epithelial lesions (Table 8), in contrast to HPV-16 immortalized cells. FEPL cells transfected with H-ras also formed tumor-like lesions however, they were not invasive, as Matlashewski et al., (1987) had demonstrated with rodent cells. Histological analysis of tumor-like lesions with HPV-16/HSV-2 mtr III sequences indicated benign epithelial cysts. Typically these tumor-like lesions were present 4 weeks post injection.

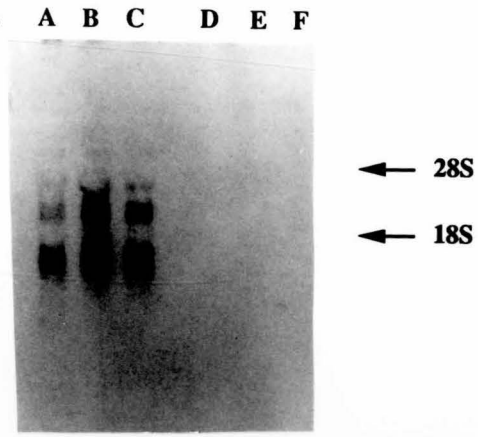
FEA (HPV-18) cells were unable to produce any lesions. Tumor formation in mice injected with HPV-18/HSV-2 mtr III cell lines was not readily observed 8 weeks post injection. Lesions were not observed even in FEA cells transfected with

Figure 26. HPV expression in transformed keratinocytes. Total cellular RNA was extracted from keratinocyte cell lines using the guanidine thiocyanate/cesium chloride method and subjected to Northern blot analysis. Ten ug of total RNA was loaded per lane in a 1% formaldehyde gel. In (I), (II), and (III), lane (A) FEPL-N, (B) PPC-1, (C) PTF-1, (D) FEA-N, (E) APC-2, (F) ATF-2. 28S and 18S ribosomal RNA markers are shown.

(I) Northern blot analysis of FEPL- cells (HPV-16) and FEA- cells (HPV-18). The HPV probe used was derived from HPV-16, the Bam HI-Pst I fragment covering E6, E7 and LCR.

(II) Northern blot analysis FEPL- and FEA- cell lines. The HPV probe used was derived from HPV-18, the Bam HI-Eco RI fragment covering E6, E7, LCR and 2/3 of E1.

(III) Northern blot analysis of FEPL- and FEA- cells using actin as a probe to ensure equal levels of RNA were loaded in each lane.



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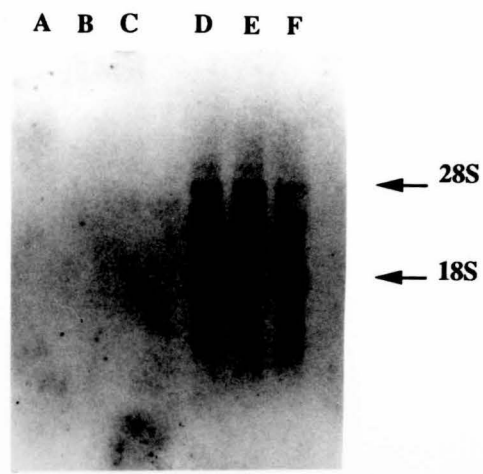

III.

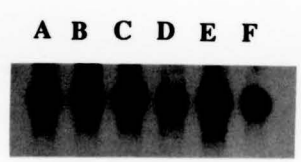

Table 8. Tumorigenicity analysis of keratinocyte cell lines.

Cell Line	Tumor Formation	Type of Lesion
FEPL-N (HPV-16)	0/5	
PTF-1 (HPV-16/486 TF)	2/5	epithelial cyst
PPC-1 (HPV-16/Pst I-C)	5/7	epithelial cyst
PBE-1 (HPV-18/Bam HI-E)	4/5	epithelial cyst
Pras (HPV-16/H-ras)	4/5	epithelial cyst
FEA-N (HPV-18)	0/5	
ATF-2 (HPV-18/486 TF)	0/5	
APC-2 (HPV-18/Pst I-C)	0/5	
ABE-2 (HPV-18/Bam HI-E	0/5	
Aras (HPV-18/H-ras)	0/5	
Hela (HPV-18 cervical carcinoma)	2/2	anaplastic carcinoma

Tumorigenic potential of keratinocyte cell lines was analyzed for production of palpable tumors. 5×10^6 cells were injected subcutaneously into nu/nu mice. Eight weeks post injection, mice were anesthetized and observed for the presence of lesions. Lesions were classified based on histological analysis.

H-ras. However, when Hela cells, HPV-18 containing cervical carcinoma cell line, were injected into nude mice, tumors appeared after two weeks and grew progressively. Histological analysis of Hela lesions indicated anaplastic carcinoma. Figure 27 shows the tumor-like lesions seen upon injection of HPV-16/HSV-2 Pst I-C cells into mice. These tumor-like lesions were similar with other HSV-2 mtr III sequences. Figure 28 demonstrates the histological analysis of normal adjacent tissue (muscle) (I), tumor-like lesions (II), and Hela induced anaplastic carcinoma (III, IV). The tumor-like epithelial lesions formed circular cysts. These contained actively growing, keratin producing cells able to enlarge the lesion with time. Histological analysis of the Hela induced tumor showed characteristics indicative of an invasive carcinoma. Numerous multinucleated cells, abnormal mitotic phases were visible along with necrotic cells near the invasive cells. Figure 29 shows the gross anatomy of the Hela infected mouse, enlarged area of tumor formation and size of tumor. In summary, HPV-16/HSV-2 cell lines were able to form benign epithelial cysts when injected into nude mice in contrast to HPV-18/HSV-2 cell lines where lesions were not readily apparent. This may imply a propensity for HPV-16, more commonly found in cervical carcinoma biopsies than HPV-18, to interact with transforming agents such as H-ras and HSV-2 mtr III sequences in the induction of tumor-like epithelial lesions. In addition, the inability of HPV-18/HSV-

2 or H-*ras* to produce lesions may indicate rejection of these human cells by the mouse.

Figure 27. Photograph of HPV-16/HSV-2 induced epithelial cyst. Arrows indicate tumor-like lesion observed after 8 weeks post injection of 5 x 10^6 HPV-16/HSV-2 Pst I-C cells into nu/nu mouse. The tumor-like lesion was small and white.


300
20

Figure 28. Histological analysis of (I) normal muscle tissue, (II) tissue from the tumor-like lesion induced by HPV-16/HSV-2 Pst I-C cells, (III) tissue from Hela induced anaplastic carcinoma. Arrows indicate invasive cells. (IV) Magnification of Hela tumor showing multinucleate cells and abnormal mitotic phases.

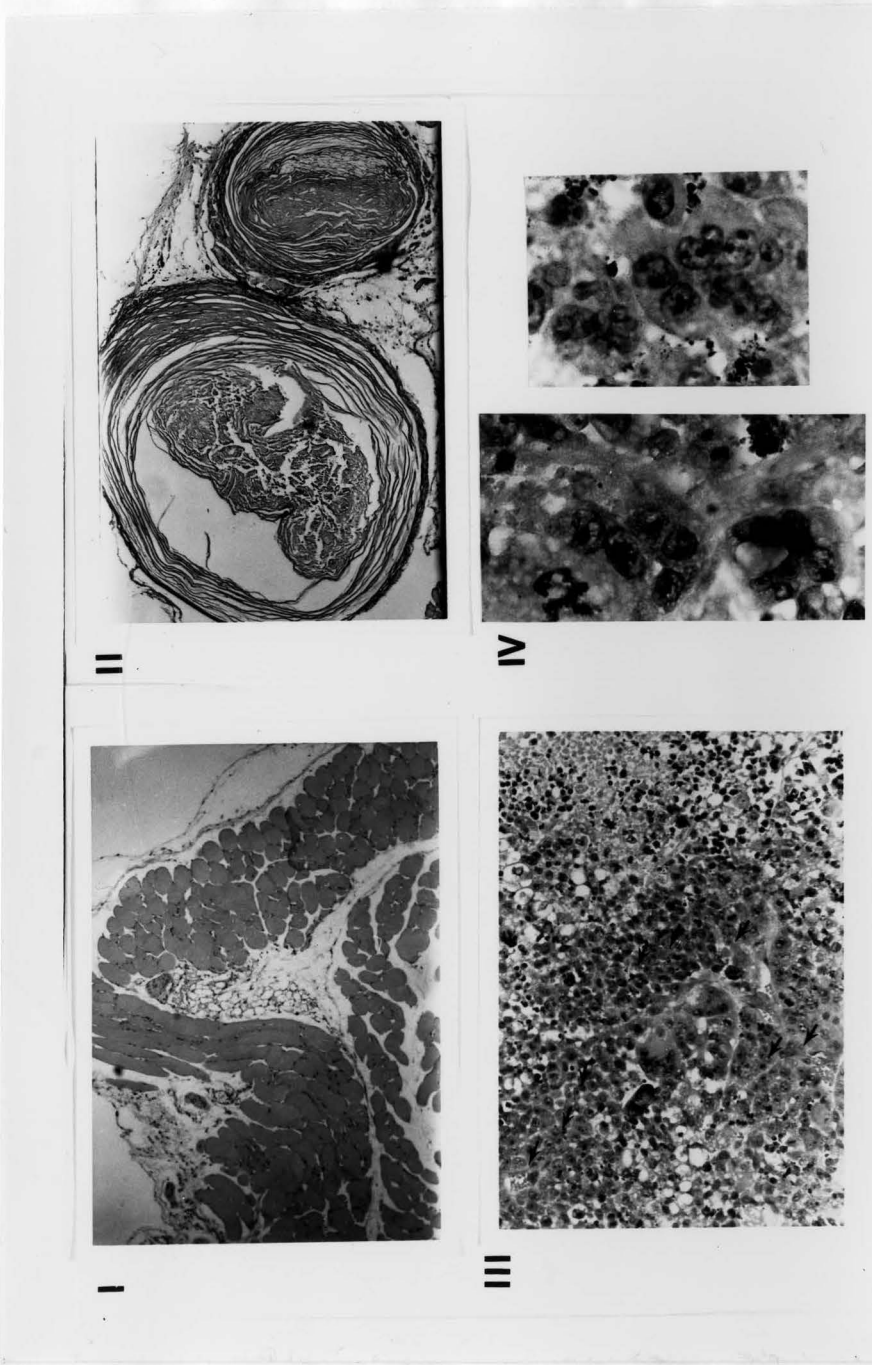
I
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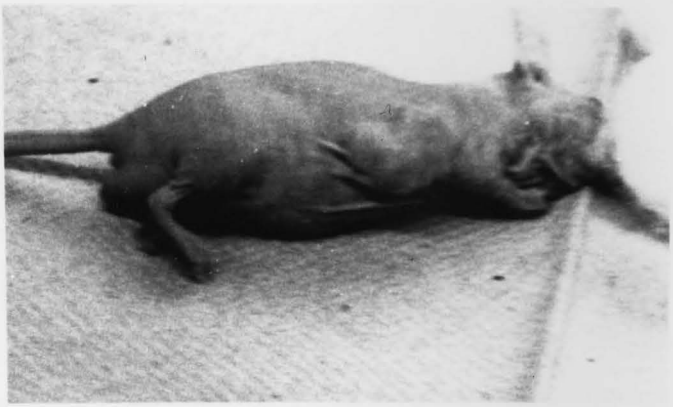
Figure 29. Gross anatomy of Hela induced tumor. (I) 5 x 10^6 cells were injected subcutaneously into nu/nu mice. Arrow indicates large tumor observed 8 weeks post injection. (II) Mice were anesthesized 8 weeks post injection and the tumor was excised. (III) Photograph of anaplastic tumor excised from nu/nu mouse injected with Hela cells, a cervical carcinoma.

A



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DISCUSSION

In this study, cell lines were established from primary human cells transfected with viral DNA constructs from two human DNA tumor viruses, herpes simplex virus 2 and human papillomavirus types 16/18. Characterization of resulting cell lines was performed to determine if viral DNAs act in synergy to morphologically transform primary human cells. Human fibroblasts and keratinocytes were utilized in the analysis. Studies demonstrated that transformed cell lines with both HSV-2 and HPV-16 or 18 had a distinct phenotype when compared to HPV immortalized cells. Transformed cell lines were morphologically distinct, able to grow to higher saturation densities, exhibited anchorage independent growth capabilities, and were able to produce lesions in athymic mice in contrast to their HPV immortalized counterpart.

CHARACTERIZATION OF HUMAN FIBROBLAST CELL LINES.

Gingival fibroblasts were obtained from a (1) normal individual and (2) from an epileptic patient treated with phenytoin, an anti-seizure drug (Delgado-Escueta et al., 1983). Phenytoin treatment of epileptic patients clearly

leads to hyperplastic gingival growth (Hassel et al., 1983) and may be related to an increased incidence of pseudolymphoma syndrome and Hodgkin's disease (reviewed in Wolf et al., 1985). Apart from these observations, little is known concerning the deleterious side effects of prolonged use of phenytoin. Fibroblast cell lines were derived from gingival tissue.

Early passage human cells were cotransfected with plasmids containing HPV-16 and HSV-2 DNA sequences derived from mtr III or the H-ras oncogene. These studies demonstrated that a plasmid containing the early genes of HPV-16 could efficiently immortalize gingival cells derived from hyperplastic tissue (R-30) but not gingival cells derived from a normal individual (N-16) (Table 1). Neither the H-ras oncogene nor various fragments derived from HSV-2 mtr III alone were capable of immortalizing R-30 or N-16 cells. Although the HSV-2 Bgl II-C fragment is capable of fully transforming primary Syrian hamster cells (Jariwalla et al, 1983), it did not immortalize R-30 cells by itself. This suggests that requirements for immortalizing human cells are unique with respect to primary rodent cells. Cell lines containing HPV-16 and H-ras or HSV-2 DNA sequences, grew more efficiently in soft agar, had a higher saturation density, and were more aneuploid when compared to R-30 cells or R-30 cells immortalized by HPV-16.

The finding that HPV-16 was able to immortalize the

hyperplastic R-30 cells more efficiently than N-16 cells implied R-30 cells are predisposed toward malignant transformation. It is tempting to speculate that the chromosomal translocation in R-30 cells and overexpression of c-myc plays a role in efficient immortalization by HPV-16. Southern blot analysis using two c-myc probes (5'-untranslated region and a portion of exon 2) showed no major differences in c-myc DNA organization (Figure 8). However, c-myc gene expression in R-30 cells and R-30 derived cell lines was 2-3 fold higher when compared to other low passage human gingival cell lines (N-16 or C-1) (Figure 9). Furthermore, N-16 or C-1 cell strains do not contain obvious chromosomal aberrations. Integration of papillomavirus sequences near the c-myc locus has been hypothesized to play a role in viral transformation (Durst et al., 1987) and as such c-myc appears to play a role in HPV induced transformation. Although c-myc is slightly overexpressed in R-30 cells, the increased levels of c-myc was apparently not sufficient to allow H-ras to transform R-30 cells as described using cotransfection experiments with an activated c-myc and H-ras (Land et al., 1983, Parada et al., 1984). It is not known if the chromosomal translocation in R-30 cells is a common result of phenytoin treatment. Thus, it appears cellular factors can play a crucial role in the efficiency of HZIP-16 induced immortalization of early passage human cells.

All fibroblast cell lines cotransfected with HZIP-16

retained HPV-16 DNA sequences as judged by Southern blotting. Viral DNA was integrated into the cellular genome since HPV-16 sequences hybridized to the high molecular weight DNA of genomic DNA not digested with restriction enzymes (Figures 2, 3). HPV-16 or 18 DNA is usually integrated in malignant lesions in vivo (Durst et al., 1985) and in this respect, the fibroblast cell lines are similar to in vivo findings. Although all cell lines retained HPV-16 DNA, the number of copies of viral DNA/cell varied dramatically among the various cell lines. Cell line 140 and 145 contained similar levels of HPV-16 DNA while cell line 141 and 139 had approximately equivalent amounts (Figure 2) suggesting a random pattern for viral integration into the cellular genome. Random integration of HPV DNA in cervical carcinoma is generally the rule (Durst et al., 1983, Baker et al., 1987). When high molecular weight DNA of fibroblast cell lines was analyzed to map which regions of the HPV-16 genome were retained, the entire genome was retained. The presence of multiple bands which do not migrate with control Pst I probes (Figure 4) indicated integration of viral DNA with cellular DNA and duplications of the HPV-16 genome during transformation. As observed in tissue from cervical cancer, HPV-16 DNA is integrated into the cellular genome in random positions and frequently undergoes duplications or amplification (Wagatsuma et al., 1990).

HSV-2 DNA sequences derived from mtr III were present in

3/3 cell lines cotransfected with HZIP-16 and mtr III containing plasmids (cell lines 140, 141, and 268; Figure 6). These results suggested retention of HSV-2 DNA sequences may lead to growth in soft agar of transformed cells compared to R-30 cells immortalized with HZIP-16 alone (Table 4). HSV-2 DNA retention was not observed when Southern blotting analysis was performed. However, when the more sensitive PCR assay was employed, HSV-2 mtr III specific DNA was detected, suggesting the presence of HSV-2 DNA correlated with very low number of viral copies. The low copy level of HSV-2 DNA may be a result of the excision of viral sequences during replication. HSV-2 has been hypothesized to transform cells by a "hit and run" mechanism where viral sequences are required for transformation but not for the maintenence of the transformed phenotype (Skinner, 1976, Galloway and McDougall, 1983). It is not clear if HSV-2 DNA sequences are integrated or persist as an episome in these cells, however, these cells have undergone extensive passage (at least 80 PD post geneticin selection) prior to PCR analysis.

Several models have been proposed alluding to the role played by tumor suppressor genes in human cancer (Knudson, 1985, Stanbridge, 1990). The E6 and E7 proteins of high risk HPVs (types 16 and 18) interact with cellular tumor suppressor proteins: retinoblastoma with E7 and p53 with E6. An analysis of HPV positive and negative cervical carcinomas revealed HPV positive cancers had relatively low levels of hp53 protein and

an intact hp53 gene (Scheffner et al., 1991) suggesting an intact protein was required to interact with the HPV E6 protein. DNA from fibroblast cell lines indicated no rearrangments of the hp53 gene organization when compared to DNA from R-30 cells (Figure 10). This result is similar to HPV-positive carcinomas where the hp53 gene is not mutated and thus may be able to produce an intact protein which interacts with the HPV E6 protein.

Hyperplastic R-30 cells are a unique model to study multistep carcinogenesis since they do not behave as a typical immortalized cell line. This conclusion was based on the fact that H-ras alone or HSV-2 transforming fragments were unable to stably transform R-30 cells (Table 1). Several studies have demonstrated that H-ras can transform established or immortalized cells but not primary human or rodent cells (Sager et al., 1983, Barbacid, 1987 references therein). When H-ras or HSV-2 DNA fragments were cotransfected with HZIP-16, the resulting cell lines were distinct compared to cells immortalized by HZIP-16. For instance, cotransfection of HZIP-16 with H-ras or HSV-2 DNA sequences consistently led to a higher degree of aneuploidy and these cells also grew better in soft agar when compared to HZIP-16 immortalized R-30 cells (Tables 3, 4).

Although R-30 cells are not immortalized, they may not truly be a primary cell. This cell strain may represent a stage in which a cell has undergone an alteration, a "single

hit", which can allow for subsequent assaults on its phenotype. Thus, phenytoin treatment of R-30 cells and resulting hyperplasia may have resulted in the phenotypic change of its cellular makeup which may allow for further alterations to occur. Extensive genetic analysis of human colorectal cancer has demonstrated that multiple tumor progression events occur during carcinoma. There are a number of alterations the cells undergo from activation of oncogenes to inactivation of tumor suppressor genes (Vogelstein et al., 1988, Stanbridge, 1990, Fearon and Vogelstein, 1990). By employing a normal primary cell strain and a primary-like cell strain in this study, one may be able to better understand the progression of the tumor phenotype seen in human cervical carcinoma. HPV-16 or 18 infection alone is not sufficient to transform primary cells, other factors are thought to play a role (Broker and Botchan, 1986, McCance, 1986). Our data suggest that immortalization of primary cells is quite difficult and may be facilitated by other factors or genetic lesions. In R-30 cells a chromosomal translocation was detected between chromosomes 8 and 18. These cells also had elevated levels of c-myc compared to other low passage gingival cells (N-16 and C-1). It is tempting to suggest these alterations in R-30 cells allowed for more efficient immortalization by HZIP-16.

When HSV-2 mtr III sequences were present, a clear difference in phenotype was demonstrated between HPV-16

immortalized and HPV-16/HSV-2 transformed cell lines. The presence of both viral DNAs clearly enhanced the transformation phenotype of primary human cells in saturation density values, anchorage-independent growth and aneuploidy when compared to cell lines with HPV-16 DNA alone. Based on these observations, HPV-16/HSV-2 fibroblast cell lines were more transformed than HZIP-16 immortalized cells. The inability to form tumors in nude mice may suggest the cells were not completely transformed or the human cells were merely rejected by the mouse. It is also possible that additional alterations may be necessary for complete transformation of fibroblast cell lines. For example, tobacco use induces progression to invasive carcinoma in women with low grade cervical intraepithelial neoplasia (Vessey, 1986, Trevathan et al., 1983).

Although, a synergistic relationship is seen with HPV-16 and HSV-2 in fibroblast cell lines, another model system was employed to verify this synergy. HPVs are epitheliotrophic viruses (reviewed in Broker and Botchan, 1986). Furthermore, HSV-2 replicates in epithelial cells in vivo (Roizman, 1985). Thus, it would be advantageous to examine HPV/HSV-2 interactions in cells of epithelial origin. To achieve this goal, genital keratinocytes were employed.

CHARACTERIZATION OF HUMAN KERATINOCYTE CELL LINES.

To study interactions between HSV-2 and HPV-16/18 in their natural host cell, human epithelial cells were employed. Keratinocytes are differentiating epidermal cells and thus these cells were utilized. Primary genital keratinocytes were immortalized with plasmids containing HPV-16 (FEPL) or HPV-18 (FEA) DNA sequences. For analysis of keratinocyte cell lines, HSV-2 mtr III DNA sequences were transfected into previously HPV immortalized keratinocytes rather than cotransfected with HPV DNA sequences. Thus, cells were already expressing HPV genes and expression was regulated by the LCR of the HPV genome. Two cell lines were used as parental cells, HPV-16 immortalized (FEPL) and HPV-18 immortalized (FEA) cells. Transfection analysis demonstrated that no single region of HSV-2 mtr III was more efficient for transforming either FEPL or FEA cells (Table 6) suggesting the minimal transforming fragment, 486 TF, was sufficient for morphological transformation of human HPV immortalized cells.

Growth characteristics of keratinocyte cell lines were assessed. Saturation density analysis indicated FEPL or FEA cells transformed by mtr III DNA sequences contained two times the number of cells after one week in culture than their immortalized counterpart (Table 7). However, cell lines differed in their ability to grow in soft agar. Neither FEA cells nor transformed derivatives were able to grow in 0.3% agar. In contrast, FEPL transformed cells grew with high efficiency in soft agar (Table 7). Immortalized FEPL cells

also grew in 0.3% agar, however not with the efficiency of their transformed counterpart. The inability of FEA cells to exhibit anchorage independent growth suggested these cells may not be fully transformed compared to FEPL transformed derivatives.

Many studies have established a role for the activation of oncogenes such as c-myc and the inactivation of tumor suppressor genes, p53, in carcinomas (Riou et al., 1987, Crook et al., 1990, Scheffner et al., 1991). We wanted to examine if these genes were altered in the keratinocyte cell lines. Southern blot analysis of DNA from transformed cell lines hybridized to two c-myc probes showed no alterations in this gene when compared to immortalized keratinocytes (Figures 22, 23). Furthermore, no detectable alterations of the hp53 gene were observed in transformed cells (Figures 24, 25). This data suggested that alterations of the hp53 or c-myc genes were not required for transformation of keratinocyte cell lines.

Analysis of HPV DNA organization of transformed cell lines was performed to determine if rearrangments of the HPV DNA occurred. FEPL cells contained 3.4 kb of the HPV-16 genome which encompasses the LCR, ORFs E6, E7 and subregions of E1 and L1 (Kaur et al., 1989). Integration of HPV-16 DNA sequences into the cellular genome seldom disrupts the LCR, E6 and E7 ORFs (Matsukura et al., 1986, Pater and Pater, 1985, Seedorf et al., 1987) since these are necessary for

immortalization. In cells obtained from cervical carcinomas the integration site of HPV DNA is frequently within E2 (Durst et al., 1986, Matsukura et al., 1986, Pater and Pater, 1985). The integration event usually disrupts E2. Since FEPL cells contain just the HPV-16 sequences which are required for immortalization, it was not surprising that rearrangments of HPV DNA were not detected in the HSV-2 transformed cells.

FEA transformed derivatives were also analyzed to examine HPV-18 DNA organization. FEA cells contain the entire genome of HPV-18, 7.9 kb DNA (Kaur and McDougall, 1988). When high molecular weight DNA was hybridized with a probe spanning the entire 7.9 kb genome of HPV-18, rearrangments of HPV DNA in cells transformed by HSV-2 was detected when compared to immortalized cells (Figures 17, 18). However, no changes were detected when DNA was probed with a HPV-18 probe spanning the LCR, E6 and E7 regions (Figure 19). This was predictable since expression of E6 and E7 is required for maintaining an immortalized state. Specific recombination between HSV-2 and HPV-18 may not occur since rearrangments of HPV-18 DNA also occurred in cell line A-ras (Figure 18, lane H), FEA cells transfected with H-<u>ras</u>. Rearrangements in HPV-18 DNA sequences in FEA transformed cell lines may suggest that the presence of the entire genome of HPV-18 provides a greater region available for recombination between the two DNAs in contrast to FEPL cells containing only 3.4 kb HPV-16 DNA.

The retention of HSV-2 DNA sequences in transformed cells

is inconsistent (reviewed by MacNab, 1987). However evidence has demonstrated a correlation between HSV-2 infection and cervical carcinoma (DiLuca et al., 1989, Hildesheim et al., 1991). Thus, keratinocyte cell lines were examined to detect the retention of HSV-2 sequences. Southern blot analysis failed to detect HSV-2 mtr III sequences. When PCR analysis was utilized to detect HSV-2 sequences in transformed cells, not all of the cell lines contained HSV-2 DNA sequences (Figure 21). In fact, analysis of FEPL and FEA transformed derivatives indicated HSV-2 retention in only 3/5 HPV-18/HSV-2 cell lines and 0/5 HPV-16/HSV-2 cell lines. Since all cell lines do not retain HSV-2 DNA, retention is not required for maintenence of the transformed phenotype. Conversely, only small portions of HSV-2 DNA are retained in transformed cell since HSV-2 is known to excise itself and portions of the cellular genome (Jariwalla et al., 1986). This random nature of HSV-2 retention in keratinocyte cell lines seems to confirm the hypothesis that HSV-2 DNA sequences are required for the initiation of transformation but not the maintenance of the transformed state (Skinner, 1976, Galloway and McDougall, 1983). After HSV-2 transfection, transformed cell lines behaved distinctly from immortalized counterparts, even though, HSV-2 DNA was not detected in all transformed derivatives.

The tumorigenic potential of FEA and FEPL cell lines was examined by injecting respective cell lines subcutaneously

into nude mice. FEA cells, immortalized as well as transformed, were unable to produce any tumors. Tumor-like lesions were not detected even with FEA cells transfected with H-ras. In contrast, all FEPL transformed derivatives were able to form abnormal epithelial lesions (Table 8). FEPL cells transfected with H-ras were not invasive as Matlashewski et al., (1987) had demonstrated previously with rodent cells. This may suggest that human cells are more refractile to tumorigenic conversion when compared to rodent cells. Tumor-like lesions were present when HSV-2 transformed FEPL cells were injected into nu/nu mice but not with FEPL cells. It can be implied that alterations have occurred in transformed FEPL cells and the initial stages of the transformed phenotype are being exhibited.

When the tumor-like lesions were examined by histological methods, they were identified as benign cysts. If cells from an invasive cervical carcinoma (Hela) were injected into nu/nu mice, large metastatic tumors developed. The size of the FEPL epithelial cyst was quite small, 3-5 mm in size (Figure 27), whereas, the Hela tumor grew to more than 20 times this size (Figure 29). Both epithelial lesions and Hela tumors were solid masses and thus difficult to dissect. Small nodules were also detected in immortalized FEPL cells 1-2 weeks post injection. However, these small nodules regressed. In contrast, when FEPL transformed cells were injected into nu/nu mice, tumor-like lesions appeared 2-3 weeks post injection and

remained up to 8 weeks with slow continous growth. When Hela cells were injected into mice, 1 week post injection, several tumors developed near the site of injection which grew rapidly. Histological analysis demonstrated differences among lesions. With the Hela tumor, numerous multinucleated cells, abnormal mitotic phases were visible along with necrotic cells near the tumor (Figure 28) indicative of anaplastic carcinoma. In contrast, the tumor-like lesion induced by HSV-2 transformed cells were a keratinized cyst. The cyst contained actively growing cells which enlarged the lesion with time. These results demonstrated that FEPL cells transformed by HSV-2 fragments derived from mtr III were able to form small benign tumors or cysts in nu/nu mice.

The mechanism by which a cell progresses to the malignant phenotype after HPV-16 infection in vivo is not known. The presence of HPV-16 or HPV-18 alone is not sufficient for tumorigenesis (reviewed in Broker and Botchan, 1986). Additional cofactors, environmental or viral, may play a crucial role in tumor progression events. Since HSV-2 is transmitted sexually similar to HPVs, infects the same cells as HPVs, and can induce morphological transformation, progression may be mediated by this virus (zur Hausen, 1983). Studies assessing a posssible synergy between HSV-2 and high-risk HPV infections in the pathology of cervical carcinoma have looked for the presence of both viral DNAs in tumor tissue. Molecular analysis of HPV-16 and HSV-2 demonstrated

that 30% of cervical cancer tissues had homology to HSV-2 mtr II and mtr III sequences (DiLuca et al., 1989). Seroepidemiological studies have demonstrated an etiologic role for HSV-2 in the development of cervical cancer where women with cervical cancer have higher levels of HSV-2 antibodies compared to controls (Kaufman and Adam, 1986). Women that are HSV-2 seropositive and infected with HPV-16/18 have a 60% increased risk of cervical cancer compared to seronegative women infected with HPV-16/18 (Hildesheim et al., 1991). These studies provide further evidence to the role that HSV-2 plays in conjunction with high-risk HPVs, in cervical carcinoma.

In our analysis, we have demonstrated the synergistic role played by HSV-2 and HPV-16/18 in the morphological transformation of primary human cells. Characterization of human cell lines immortalized by HPV-16/18 have a distinct phenotype compared to cells transformed by HPV-16/18 and HSV-2 DNA sequences. The development of tumor-like lesions by transformed HPV-16/HSV-2 keratinocytes and not by immortalized HPV-16 cells suggests transfection of HSV-2 sequences may have altered these cells and led to enhanced invasive properties. HSV-2 is known to induce stable heritable changes in the cell without the need for viral replication or gene expression (Clarke and Clements, 1991). Thus, in FEPL cells which have been already altered by immortalization, the mutagenic abilities of HSV-2 could induce alterations in cellular DNA

and subsequently lead to the transformed phenotype.

However, FEA cells containing the entire genome of HPV-18 did not produce any lesions in mice even though 3/5 cell lines retained HSV-2 DNA sequences and transformed cell lines had HPV-18 DNA rearranged. It may be speculated that several factors have a role in the inability of these lesions to produce tumors. FEA cells with the complete HPV-18 genome can express the E2 protein which acts as a transactivator or repressor of HPV gene expression (Phelps and Howley, 1987, Cripe et al., 1987). Full-length E2 binds to Sp1 sites to transactivate transcription whereas a truncated E2 will repress transcription (Li et al., 1991, Cripe et al., 1987). Thus, if rearrangments within the HPV-18 genome altered the E2 gene, truncated E2 proteins may act not only on the HPV-18 LCR but also on the promoters of HSV-2 mtr III sequences (Jones, 1989) to down regulate transcription.

HPV-16/18 DNAs are associated with the majority of cervical carcinomas, the viral DNA can be detected in at least 85% of cervical carcinoma biopsies (Broker and Botchan, 1986). Of these, HPV-16 accounts for approximately 60% of HPV positive cervical carcinomas while HPV-18 accounts for approximately 10-15% (Villa and Schlegel, 1991). The reason for the prevelance of HPV-16 in cervical carcinoma and not HPV-18 is not known. No significant differences have been found between E6 and E7 proteins of types 16 and 18, nor was there any major disparity in the LCRs of both viruses (Villa

and Schlegel, 1991). It may be that HSV-2 interacts with HPV-16 to produce a malignant phenotype but cannot carry out the same interactions with HPV-18.

In our analysis, the characterization of keratinocyte cell lines imply that HSV-2 can interact with HPV-16 to produce cells with a transformed phenotype but it is not able to do so with HPV-18. Data also suggests that HSV-2 can act as a cofactor with HPV-16 to transform primary human cells. Premalignant papillomavirus lesions in a number of animal models progress to malignancy after interacting with other known mutagens (Jarrett et al., 1980, Rous and Kidd, 1938, Orth et al., 1978). HSV-2, with its ability to act as a biological mutagen, is a biologically relevant cofactor in the development of genital cancer.

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