Characterization of primary human fibroblasts transformed by human papilloma virus type 16 and herpes simplex virus type 2 DNA sequences

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Human papilloma virus type 16 (HPV-16) and herpes simplex virus type 2 (HSV-2) are human viruses implicated in the development of cancer, in particular cervical cancer. The ability of HSV-2 and HPV-16 to transform early passage human cells was examined in this report. For these studies, gingival fibroblasts were utilized. One gingival cell strain was derived from a normal individual (N-16). The second cell strain was derived from hyperplastic gingival tissue of an epileptic individual (R-30) treated with phenytoin, an anti-seizure drug. A common side effect of phenytoin is the induction of gingival overgrowth. R-30 cells contained a stable chromosomal translocation between chromosomes 8 and 18 and expressed higher steady state levels of c-myc. HPV-16 DNA efficiently immortalized R-30 cells but not N-16 cells. R-30 cells cotransfected with HPV-16, and HSV-2 viral DNAs were more aneuploid than R-30 cells transfected with HPV-16 DNA alone. Additionally, R-30 cells cotransfected with both viral DNAs grew better in soft agar than R-30 cells transfected with HPV-16 DNA alone. HSV-2 DNA was detected in transformed cells by polymerase chain reaction. These results suggested R-30 cells were immortalized more efficiently by HPV-16 and further imply that HPV-16 and HSV-2 DNA fragments can cooperate during multistep transformation.

Introduction

Morphological transformation of primary cells is a multistep phenomenon (reviewed in Bishop, 1985; Stanbridge, 1990). In this report, the multistep nature of transformation was examined using two primary human cell types derived from gingival tissue. Gingival fibroblasts were obtained from a normal individual (N-16) and an epileptic patient treated with phenytoin (R-30), an anti-seizure drug (Delgado-Escueta et al., 1983). 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). This investigation was initiated to determine whether R-30 cells and N-16 cells differ with respect to morphological transformation.

Papillomaviruses are small DNA tumour viruses which induce proliferative lesions of the skin and mucosa (reviewed in zur Hausen, 1977; Pfister, 1984). Although many of the lesions from human papillomavirus (HPV) infections are benign, infection with specific types of HPV are associated with invasive carcinoma. For example, HPV sequences are present in 80 to 85% of cervical carcinoma biopsies with HPV types 16 and 18 being the most prevalent (Broker & Botchan, 1986; Durst et al., 1983). Malignant lesions frequently contain HPV DNA integrated into the cellular genome with only a portion of the viral DNA present (Durst et al., 1985). Invariably, two viral genes, E6 and E7, are intact and expressed (Matsukura et al., 1986; Pater & Pater, 1985; Seedorff et al., 1987). It is well established that HPV-16 and HPV-18 can immortalize primary human cells, such as keratinocytes (Durst et al., 1987b; Kaur & McDougall, 1988; Woodworth et al., 1989) and fibroblasts (Pirisi et al., 1987; Watanabe et al., 1989). The E6 and E7 genes are required for this process (Matlashewski et al., 1987; Munger et al., 1989; Watanabe et al., 1989). In addition, a recent study demonstrated the presence of HPV-16 and -18 DNA sequences in oral epithelial tissues (Yeudall & Campo, 1991).

Progression to the malignant phenotype after HPV infection in vivo apparently requires additional cofactors. It has been hypothesized that herpes simplex virus type 2 (HSV-2) cooperates with the oncogenic HPVs to produce a malignant phenotype (zur Hausen, 1983). HSV-2 is an oncogenic DNA virus spread primarily by sexual contact.
(Ginsberg, 1980). Inactivated HSV-2 can also immortalize human cells (epithelial as well as primary fibroblasts) but virus is not recovered (Takahashi & Yamanishi, 1974; Kucera & Gusdon, 1976). Within the HSV-2 genome there are two unique morphological transform- ing regions (mtr) designated as mtr II (map unit 0-585 to 0-63) and mtr III (map unit 0-42 to 0-58) (Galloway & McDougall, 1981; Jariwalla et al., 1983). The HSV-2 mtr II contains a minimal transforming fragment (TF) of 486 bp (486 TF) which apparently does not encode an oncogenic protein (Jones et al., 1986). However, it can function as a complex transcriptional promoter element and therefore transformation is hypothesized to be mediated by altered cellular gene expression (Jones, 1989).

In this study, we examined whether HPV-16 and HSV-2 can transform primary human cells more efficiently together than individually. Although HPVs do not routinely infect fibroblasts, efficient expression of HPV genes in fibroblasts is achieved because gene expression is regulated by the Moloney murine leukemia virus (M-MuLV) long terminal repeat (LTR) sequence (Matlashewski et al., 1987). These studies implied R-30 cells were not immortalized but could be immortalized more efficiently than normal N-16 gingival fibroblasts. Cells transformed by multiple transforming agents contained higher numbers of chromosomes when compared to R-30 cells or HPV-16 immortalized cells. Additionally, retention of HSV-2 DNA sequences was detected in R-30 cells transfected with HPV-16 and HSV-2 DNA but not in cells with HPV-16 alone or the parental R-30 cells. Thus, HSV-2 transforming fragments and the activated H-ras oncogene can have an effect on human cells immortalized by HPV-16.

**Methods**

**Cells and plasmids.** The 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 Earle’s modified Eagle’s medium (Sigma) supplemented with 10% foetal bovine serum, penicillin (10 units/ml), and streptomycin (100 μg/ml). Plasmid HZIP-16 was obtained from Dr G. Matlashewski and contains the early region of HPV-16 genome flanked by the M-MuLV LTR (Matlashewski et al., 1987). To construct HZIP-16, the region between BamHI and EcoRI was deleted. HZIP-16 contains M-MuLV LTRs, neomycin resistance gene (Neo*), origins of replication (ori) of simian virus 40 (SV40) and pBR322 vector sequences in addition to the HSV-16 early region. Within the vector sequences, two more EcoRI sites are present (in addition to those within HPV-16 sequences) such that digestion of HZIP-16 with EcoRI yields three bands (9-4 kb, 6-5 kb and 2-5 kb). The 9-4 kb band contains the HPV-16 sequences plus vector sequences (data not shown). Plasmid pEX2 containing a 420 bp fragment of exon 2 was obtained from Dr Len Erickson.

**Eukaryotic cell transfections.** Low passage R-30 cells or N-16 cells were transfected with 25 μg DNA using calcium phosphate precipitation (Graham & 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 μg/ml) to select for cells containing plasmids. All cultures were cotransfected with pSV2neo (5 μg/60 mm dish) since the neomycin resistance gene of HZIP-16 was not active. Geneticin selection was carried out for 2 to 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 in a 60 mm dish were trypsinized and subcultured as described above in the absence of geneticin. For experiments using a single transforming agent (HZIP-16, H-ras, 486 TF, BgII-C, BamHI-E), 20 μg of the respective plasmid was added to 5 μg pSV2neo and the mixture was used for transfection. If multiple transforming sequences were used (HZIP-16/ H-ras, HZIP-16/486 TF, HZIP-16/BgII-C, HZIP-16/BamHI-E), 10 μg of each respective plasmid was combined with 5 μg pSV2neo and these mixtures were used for transfections. Anchorage-independent growth was performed as previously described (Jones et al., 1986).

**DNA preparation and Southern blot hybridization.** High M, DNA was prepared from mammalian cells (Maniatis et al., 1982). The respective DNA samples were digested to completion with EcoRI (10 units/μg) for 16 to 18 h at 37 °C. The digested DNA samples, approximately 10 μg, were subsequently subjected to electrophoresis in 1-0% agarose gels buffered with Tris-borate (0-089 M-Tris-borate, 0-089 M-boric acid and 0-002 M-EDTA) and the DNA transferred to nitrocellulose (Southern, 1975). 32P-Labelled probes were prepared by random priming in the presence of [α-32P]dCTP (1 x 106 c.p.m./μg DNA) and hybridized to the filter in a solution which contained 50% formamide, 5 × SSC, 2 × Denhardt’s solution, 50 μg/ml sheared salmon sperm DNA, 0-1% SDS and 0-05 M-sodium diphosphate pH 6-8. Hybridization was carried out at 42 °C for 16 h. The filters were washed with 2 × SSC, 0-2% SDS (three times, 20 min each) and subsequently in 0-2 × SSC, 0-5% SDS (three times, 20 min each). All washes were performed at 55 °C. Filters were air-dried and autoradiographed.

**RNA extraction and ribonuclease protection analysis.** Total cellular RNA was extracted from cells by the method of Peppel & Baglioni (1990). Cells were washed with PBS and lysed with a solution containing 2% SDS, 200 mM-Tris–HCl pH 7-5, and 1 mM-EDTA. DNA and proteins were precipitated from the lysate by addition of 4:37 M-potassium acetate. The RNA containing supernatant was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1). The RNA was precipitated with isopropanol, and was stored at -70 °C in formamide.

Ribonuclease protection analysis (RPA) was performed using the RPA kit by Ambion. An in vitro generated RNA probe (7 x 105 c.p.m. [32P]UTP) was mixed with 10 μg total cellular RNA. Samples were ethanol-precipitated and resuspended in a hybridization buffer (80% formamide, 40 mM-PIPES pH 6-4, 400 mM-sodium acetate pH 6-4, 1 mM-EDTA) and hybridized overnight at 45 °C. Hybrids were digested with a solution containing RNase A/RNase T1 to digest unprotected ssRNA. SDS and proteinase K were added and subsequently extracted with phenol:chloroform:isoamyl alcohol (25:24:1). The RNAs were precipitated and run on an 8 M-urea/5% acrylamide denaturing gel to separate RNA species. The gel was dried and subjected to autoradiography.

**Polymerase chain reaction (PCR) analysis.** High M, DNA was prepared from various cell lines as previously described and digested with PstI overnight at 37 °C. All transformed cells had undergone at least 80 population doublings (PDs) post-transfection prior to DNA
isolation. Oligonucleotide primers (Genosys Biotechnologies), 21 bases in length, were chosen to amplify a target sequence of 366 bp located within the HSV-2 mtr III 486 TF sequence. The primers were synthesized according to the published sequence of HSV-2 mtr III PstI-C fragment (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' GCCGTGTCCGGGCTGTGGA 3'). The probe used to detect the amplified sequence was 486 TF (486 bp PstI-SalI fragment). PCR was performed in 100 μl volumes in standard commercial buffer (Promega) containing 1·5 mM-MgCl₂. Cycling parameters for PCR were 1 min at 94 °C for denaturation, 1 min of primer annealing at 60 °C and 2 min at 72 °C for extension. A total of 40 cycles was run. For PCR, 1 μg of template DNA was used. Amplification products were analysed on 2% Nu-Sieve 3:1 agarose (FMC Bioproducts) gels containing 1 x TBE buffer and 0·5 μg/ml ethidium bromide. Subsequently, Southern blot analysis of the gels was done using random primed 32P-labelled 486 TF probes. Prehybridization was for 1 h at 61 °C in 5 x SSPE (1 x SSPE contains 0·18 M-NaCl, 0·01 M-sodium phosphate, 1 mM-disodium EDTA pH 7·7), 5 x Denhardt's and 1% SDS. Blots were hybridized in the prehybridization solution which contained the denatured probe. Filters were washed in 1 x SSPE and 1% SDS three times at room temperature and once at 65 °C. 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. Positive control for all PCRs was a plasmid, pCPS-I (486 TF cloned into PstI and SalI sites of pUC19) (Jones et al., 1986). Negative controls for the PCRs were DNA from normal cells and the 'no template' reactions which contained all components of the PCR reaction except for the template. 'No template' lanes provided a control for PCR performance.

Results

Transfection analysis

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) (Fig. 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 types. 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. Phentoin-treated cells (R-30) were more susceptible to immortalization by HZIP-16 alone in comparison 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 h 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 genenicin-resistant colonies were not observed. The genenicin-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 genenicin-resistant colonies. Since an activated H-ras oncogene will transform immortalized human and rodent cells but ordinarily not primary cells (Newbould & Overell, 1983; Sager et al., 1983), it appears that R-30 and N-16 cells do not behave like immortalized...
Table 1. Transfection analysis of R-30 and N-16 cells*

<table>
<thead>
<tr>
<th></th>
<th>R-30</th>
<th>N-16</th>
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<tbody>
<tr>
<td></td>
<td>Expt. 1 Expt. 2 Expt. 3</td>
<td>Expt. 1 Expt. 2 Expt. 3</td>
</tr>
<tr>
<td>HZIP-16/H-ras</td>
<td>4  7  5</td>
<td>3  6  7</td>
</tr>
<tr>
<td>HZIP-16/486 TF</td>
<td>5  4  6</td>
<td>4  3  5</td>
</tr>
<tr>
<td>HZIP-16/BglII-C</td>
<td>6  2  7</td>
<td>0  3  0</td>
</tr>
<tr>
<td>HZIP-16/BamHI-E</td>
<td>7  5  3</td>
<td>2  2  1</td>
</tr>
<tr>
<td>HZIP-16</td>
<td>5  7  4</td>
<td>0  1  0</td>
</tr>
<tr>
<td>H-ras</td>
<td>1  0  0</td>
<td>0  0  0</td>
</tr>
<tr>
<td>HSV-2 486 TF</td>
<td>0  0  0</td>
<td>0  0  0</td>
</tr>
<tr>
<td>HSV-2 BglII-C</td>
<td>0  0  0</td>
<td>0  0  0</td>
</tr>
<tr>
<td>HSV-2 BamHI-E</td>
<td>0  0  0</td>
<td>0  0  0</td>
</tr>
<tr>
<td>pSV2neo</td>
<td>0  0  0</td>
<td>0  0  0</td>
</tr>
</tbody>
</table>

* R-30 or N-16 cells (5 × 10⁶ cells/60 mm dish) were transfected with 25 µg of plasmid DNA using the calcium phosphate precipitation procedure (Graham & Van der Eb, 1973) followed by geneticin selection (400 µg/ml).

cells. When genetin-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/BgllII-C, HZIP-16/ BamHI-E), stable cell lines were not obtained regardless of the transforming sequences introduced into these cells. Typically these cells senesced after only four or five passages. In contrast, genetin-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 to 80 PDs after the original colony was trypsinized in the absence of genetin. 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 cells

The various cell lines were analysed 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 that all transformed cell lines retained HPV-16 DNA (Fig. 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 & Campo, 1991), it was important to determine whether HPV-16 sequences were present in R-30 cells. As expected, normal R-30 cells did not hybridize to the HPV probe. 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 BgllII-C) and cell line 139 (R-30 cotransfected with HZIP-16 and H-ras) also had equivalent amounts of HPV-16 specific DNA; however, these were at lower levels compared to cell lines 140 and 145. When uncut genomic DNA of the respective cell lines was probed with an HPV probe, the hybridizing fragment migrated with the high M₅ genomic DNA suggesting integration had occurred (data not shown). 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.

Fig. 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. Lane 1, R-30; 2, 139; 3, 140; 4, 141; 5, 145. The probe was derived from the PstI-A and PstI-B fragments of HPV-16. Asterisks denote the site of migration of EcoRI digestion products of HZIP-16. Molecular size markers, λ DNA/HindIII, are shown in kb.
Table 2. Chromosome number per cell of the transformed cell lines

<table>
<thead>
<tr>
<th>Cell strain</th>
<th>Chromosome number in a cell*</th>
</tr>
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<tbody>
<tr>
<td>39-50</td>
<td>51-70</td>
</tr>
<tr>
<td>71-100</td>
<td>101-200</td>
</tr>
<tr>
<td>X &gt; 200</td>
<td></td>
</tr>
<tr>
<td>R-30 (HZIP-16/H-ras)</td>
<td>17 0 3 0 0</td>
</tr>
<tr>
<td>139 (HZIP-16/HSV-2 486 TF)</td>
<td>9 0 5 6 0</td>
</tr>
<tr>
<td>140 (HZIP-16/HSV-2 BglII-C)</td>
<td>2 0 13 5 0</td>
</tr>
<tr>
<td>141 (HZIP-16/HSV-2 BamHI-E)</td>
<td>2 0 11 4 3</td>
</tr>
<tr>
<td>145 (HZIP-16)</td>
<td>10 4 6 0 0</td>
</tr>
</tbody>
</table>

* 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.

Fig. 3. PCR analysis of transformed R-30 cells. High M, DNA was prepared from the respective cell lines, digested with PstI and 1 µg of digested DNA used for the PCR reaction. Primers were derived from 486 TF described previously. Lane 1 contains 0.1 µg pCPS-1 plasmid (486 TF cloned into PstI and SalI sites of pUC19). Lanes 2 to 7 contain DNA prepared from cell lines 139, 140, 141, 145, 227, and 268, respectively. Lanes 8 and 9 contain R-30 cells and C-1 cells, respectively. Lane 10 contain the 'no template' control. Lane 11 contains φX174 DNA digested with HaeIII. The positions of these fragments are denoted by lines (1353, 1078, 872, 603, 310, 234, 174 and 118 bp).

The retention of HSV-2 DNA sequences in transformed cells was examined by PCR. The stable retention of HSV-2 DNA in transformed cells has not been consistently observed and thus HSV-2 transformation has been postulated to be mediated by a hit and run mechanism (reviewed by Macnab, 1986). Southern blot analysis using HSV-2 DNA sequences as probes failed to detect HSV-2 DNA (data not shown). However, when high M, DNA was subjected to PCR analysis using primers derived from 486 TF, retention of HSV-2 DNA was detected in three cell lines. All three had been transfected with HSV-2 DNA sequences: (i) cell line 140 (HZIP-16 + HSV-2 486 TF), (ii) cell line 141 (HZIP-16 + HSV-2 BglII-C) and (iii) cell line 268 (HZIP-16 + HSV-2 BamHI-E) (Fig. 3). 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 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 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 2) 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 analysed 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 3). 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 and regions of HSV-2 mtr III sequences (140, 141 or 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 in soft agar. Furthermore, saturation density of R-30 cells transfected with
Fig. 4. Karyotype analysis of N-16 and R-30 cells. (a) Arrows indicate aberrations in chromosome 8 and 18 of R-30 cells. All R-30 cells contained the aberrant chromosome 8 and 18. (b) 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.

Table 3. Growth characteristics of transformed R-30 cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Colony formation (%)* in 0-3% agarose</th>
<th>Saturation density†</th>
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<tbody>
<tr>
<td>R-30</td>
<td>1 ± 1</td>
<td>1 × 10⁶ ± 1 × 10⁵</td>
</tr>
<tr>
<td>139 (HZIP-16/H-ras)</td>
<td>30 ± 5</td>
<td>4.9 × 10⁶ ± 1.3 × 10⁵</td>
</tr>
<tr>
<td>140 (NZIP-16/486 TF)</td>
<td>15 ± 3</td>
<td>5.2 × 10⁶ ± 1.8 × 10⁵</td>
</tr>
<tr>
<td>141 (HZIP-16/BgII-C)</td>
<td>15 ± 4</td>
<td>4.3 × 10⁶ ± 2.0 × 10⁵</td>
</tr>
<tr>
<td>268 (NZIP-16/BamHI-E)</td>
<td>15 ± 3</td>
<td>5.1 × 10⁶ ± 1.5 × 10⁵</td>
</tr>
<tr>
<td>145 (HZIP-16)</td>
<td>3 ± 1</td>
<td>2.4 × 10⁶ ± 0.6 × 10⁵</td>
</tr>
<tr>
<td>181 (HZIP-16)</td>
<td>4 ± 1</td>
<td>2.7 × 10⁶ ± 1.1 × 10⁵</td>
</tr>
</tbody>
</table>

* The ability of R-30 cells and the various transformed derivatives of R-30 cells were analysed for growth in soft agar. 1 × 10⁵ cells of each line were plated in medium containing 0-3% agarose and scored after 10 days. Numbers indicate the approximate percentage of cells which formed colonies. To be considered a colony, the diameter of the colony was at least 1 mm. Values are the average of three independent experiments.

† Saturation density was the number of cells/100 mm² dish at confluence. Plates were seeded with 3 × 10⁵ cells and the various cultures were fed two times a week. After two weeks in culture, the cells were trypsinized and counted using a haemocytometer. Values are the average of three independent experiments.

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 × 10⁶ to 8 × 10⁶ cells/animal), tumour formation was not readily observed after 6 weeks (data not shown). 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 that 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 N-16 and R-30 cells

Since R-30 cells were immortalized more efficiently by HZIP-16 than by N-16 cells, a comparison of the two cell types was subsequently made. No obvious differences were detected in morphological characteristics [fluorescence-activated cell sorting (FACS), 90° versus forward scattering ratios], growth rates, or saturation densities (data not shown). 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 (Fig. 4). Approximately 15% of R-30 cells contained more than 50 chromosomes. Only 5% of N-16 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; Durst et al., 1987a; 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 was performed. Two c-myc probes, a 1.6 kb SacI fragment of the 5'-untranslated
region and a 420 bp PstI 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 (data not shown). 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 PstI 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 (Fig. 5). 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 threefold 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.

Discussion

In this report, early passage human cells were transfected with plasmids containing HPV-16, H-ras oncogene, or HSV-2 DNA sequences derived from mtr III. 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). Neither the H-ras oncogene nor HSV-2 mtr III DNA sequences alone were capable of immortalizing R-30 or N-16 cells. Although the HSV-2 BglII-C fragment is capable of fully transforming primary Syrian hamster cells (Jariwalla et al., 1983), it could not immortalize R-30 cells by itself. This suggests that requirements for immortalizing human cells are unique with respect to primary rodent cells. When HPV-16 containing plasmids were cotransfected with H-ras or HSV-2 DNA sequences, the resulting cell lines 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 may be 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. However, c-myc gene expression in R-30 cells and R-30 derived cell lines was two- to threefold higher when compared to N-16 or C-1 (cyclosporin-treated) cells. Both of these primary 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., 1987a) 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 whether the chromosomal translocation in R-30 cells is a common result of phenytoin treatment. 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. Thus, it appears cellular factors can play a crucial role in the efficiency of HZIP-16-induced immortalization of early passage human cells.

Hyperplastic R-30 cells could be a unique model to study multistep carcinogenesis since they do not behave as a typical immortalized cell line. This conclusion is 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 and references therein). When H-ras or HSV-2 DNA fragments were cotransfected with HZIP-16, the resulting cell lines were distinct when 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. In summary, DNA sequences derived from HSV-2 mtr III or H-ras played a role in efficient growth of R-30 cells when cotransfected with HZIP-16.

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 usually not sufficient for tumorigenesis (reviewed in Broker & Botchan, 1986). Additional cofactors, environmental or viral, may play a crucial role in tumour progression events. In fact, progression may be mediated by infection with another virus, HSV-2 (zur Hausen, 1983). A recent study demonstrated by Southern blot analysis that 16-7% of invasive cervical carcinoma biopsies which hybridized to HPV-16 also hybridized to HSV-2 mtr III DNA sequences, BamHI-E (Di Luca et al., 1989).

In our analysis, HSV-2 DNA sequences derived from mtr III were present in each of the three cell lines cotransfected with HZIP-16 and mtr III containing plasmids (cell lines 140, 141 and 268; Fig. 3). These results suggested retention of HSV-2 DNA sequences may lead to aberrant growth characteristics of R-30 cells in soft agar compared to R-30 cells immortalized with HZIP-16 alone. It is not clear whether 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. Experiments to address the physical state and stability of HSV-2 sequences are currently in progress. It appears HSV-2 DNA sequences derived from mtr III were consistently retained in R-30 cells and can have a profound effect on R-30 cells when cotransfected with HZIP-16.

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References


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