Induction of chromosome abnormalities in mouse and human epidermal keratinocytes by the human papillomavirus type 16 E7 oncogene

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Cytogenetic abnormalities associated with human papillomavirus (HPV) type 16 were studied using primary human and mouse epidermal keratinocytes. The E7 transforming gene of HPV-16 was found to induce chromosome duplication in epidermal keratinocytes; little or no detectable chromosome disorganization was associated with the function of the E6 gene. These results suggest that the E7 gene-linked cytogenetic effect reflects HPV-16-associated pathogenicity in the early phase of transformation.

Introduction

Human papillomavirus type 16 (HPV-16) is a DNA tumour virus that has been identified as an aetiological agent of human squamous cell carcinoma (Durst et al., 1983; zur Hausen & Schneider, 1987). HPV-16-associated malignant tumours usually appear more than 10 years after primary virus infection (zur Hausen & Schneider, 1987). Viral DNA transforms an array of cell types, including human cells, in vitro (Yasumoto et al., 1986, 1987; Matlashewski et al., 1987; Durst et al., 1987; Pirisi et al., 1987; Schlegel et al., 1988), but does not readily produce malignancy (Yasumoto et al., 1987; Noda et al., 1988); the transforming activity has been mapped to the E6 and E7 genes of HPV-16 (Bedell et al., 1987; Hawley-Nelson et al., 1989; Munger et al., 1989; Watanabe et al., 1989). Growth promotion, immortalization and altered differentiation are detectable E6 and E7 protein-associated biological functions in human keratinocytes (McCance et al., 1988; Hawley-Nelson et al., 1989; Munger et al., 1989), and E7 and E6 genes can cooperate in the transformation of epidermal keratinocytes in vitro (Hawley-Nelson et al., 1989; Munger et al., 1989). Using in vitro transcription/translation systems, the E7 gene product of HPV-16 has been shown to be able to bind to and coprecipitate with the cellular p105-Rb protein (retinoblastoma susceptibility gene product), which is responsible for growth regulation (Dyson et al., 1989). This E7 product-associated biological function is thought to play a critical role in the transforming activity of HPV-16 (Phelps et al., 1988; Kanda et al., 1988; Sato et al., 1989a), although its functions in vivo are unknown. Other unknown E6 gene-associated functions or cellular oncogenes may cooperate to produce a more advanced transformed state (Crook et al., 1988, 1989; Munger et al., 1989). More recently, the E6 protein has been shown to bind the cellular p53 protein (Werness et al., 1990), which can form a complex with the large T (LT) antigen of simian virus 40 (SV40) or the E1B protein of adenovirus 5 (Lane & Crawford, 1979; Linzer & Levine, 1989; Sarnow et al., 1982). These protein–protein interactions are implicated in the uncontrolled cell growth which follows virus infection. However, the growth promoting activity of the HPV-16 E6 and E7 oncogenes is not linked directly to the immortalization of primary cells, including human keratinocytes, because not all cells carrying E6 or E7, or both, are immortalized (Munger et al., 1989; Hawley-Nelson et al., 1989). Thus, E6 and E7 gene-associated progressive transformation in humans is not fully understood.

Histocytogenetic examination of HPV-associated genital lesions has demonstrated that a shift in ploidy from diploid to polyploid occurs in early neoplastic hyperproliferation in the lesion. Since almost all malignant tumours carry aneuploid cells, changes in ploidy patterns (diploid to polyploid to aneuploid) associated with HPV-16 may be implicated in a high incidence of tumour development in genital regions (Fu et al., 1981; Reid et al., 1984). Changes in ploidy have also been observed in HPV-16-immortalized human epithelial cells in vitro (Durst et al., 1987; Hawley-Nelson et al., 1989; Smith et al., 1989). These accumulated pathobiological observations raise the question of whether a causal relationship between HPV functions and changes in ploidy is present. In this study, we focused on correlating cytogenetic abnormalities with functions of the E6 and E7 genes, and found that the effect of HPV-16 on changes in ploidy was correlated with E7 gene functions, but not with those of the E6 gene.
Methods

Cells and cell culture. Primary epidermal keratinocytes were prepared from human foreskin epidermis or surgically dissected adult human skin regions, and epidermis from the skin of the back of newborn BALB/c mice by the collagenase-floating technique (Pirisi et al., 1987). The cells were maintained in complete MCDB152 medium containing a hormone mixture of epidermal growth factor (10 ng/ml), insulin (5 µg/ml), transferrin (10 µg/ml), hydrocortisone (0.2 µM), o-phosphoethanolamine (5 µM), ethanolamine (5 µM), and supplemented with 0.5% chexlex-treated foetal calf serum and bovine pituitary extract (50 µg/ml). The culture medium was replaced every other day and the cells were subcultured at a ratio of 1:5 when 70 to 80% confluent. Only growing keratinocytes can be maintained under these culture conditions and fibroblast contamination was negligible.

Construction of recombinant plasmids. The DNA segment of 630 bp containing the E6 open reading frame (ORF) was excised by DdeI digestion. The sticky ends of the DdeI fragment [nucleotides (nt) 24 to 654] were filled using the Klenow fragment of DNA polymerase I and the DNA segment was cloned into the BamHI site of the pcD2 vector (Chen & Okayama, 1987) using BamHI adaptors. The PvuII-PstI fragment (nt 551 to 875) containing the 324 bp E70RF was blunt-ended using S1 nuclease and cloned at the blunt-ended BamHI site of the pcD2 vector, partially cleaved using BamHI, so that E7 expression occurs from the SV40 promoter (Hashida & Yasumoto, 1990). These recombinants were designated pDE6 and pDE7, respectively (Fig. 1). Plasmids pMHPV16d and pMHPV16s contain dimeric and monomeric HPV-16 DNA, respectively, as described previously (Yasumoto et al., 1987).

DNA transfection and establishment of keratinocyte cell lines. DNA (10 to 15 µg) was used to transfect 1 x 10^6 human foreskin keratinocytes by the modified calcium phosphate coprecipitation method described previously (Pirisi et al., 1987), or by electroporation using the Baekon 2000 Advanced Gene Transfer system (Baekon) (Hashida & Yasumoto, 1990; Yasumoto et al., 1991). Three clonal lines, PHK16-0b, PHK16-1, and PHK16-11, were isolated from the G418-selected HPV-16 transformants. Two other clonal lines, PHK16-1 and PHK16-2, were established as continuous lines without G418 selection after transfecting cells with pMHPV16s DNA. These cell lines grew for more than 300 population doublings. Mouse keratinocytes were transfected with DNA using the modified calcium phosphate precipitation method and then selected for G418 resistance (100 µg/ml).

RNA analysis. Total cellular RNA was extracted with 4 M-guanidine isothiocyanate as described previously (Yasumoto et al., 1987), electrophoresed in a 1.2% agarose gel containing 2.2 M-formaldehyde and subjected to Northern blot analysis (Thomas, 1980). Nitrocellulose filters were hybridized with nick-translated 32P-labelled DNA (10⁸ c.p.m./µg DNA) under stringent conditions (50% formamide in the presence of 10% dextran sulphate at 42 °C for 16 h). The filter was washed three times in 2 x SSC (1 x SSC is 150 mM-NaCl, 15 mM-sodium citrate, pH 7.0) containing 0.1% SDS at room temperature for 15 min and twice in 0.1 x SSC containing 0.1% SDS at 50 °C for 30 min. The filters were exposed to Fuji X-ray film at -80 °C under an intensifying screen.

Immunoprecipitation of E7 protein. Subconfluent cell cultures were labelled with 0.5 mCi[^35S]cysteine for 4 h in complete medium lacking only unlabelled cysteine. Cells were then lysed in HSN buffer pH 7.4 containing 250 mM-NaCl, 0.1% NP40, 50 mM-HEPES, 1 mM-PMSF, 1 µg/ml antipain. After 30 min incubation on ice, the cells were scraped off the plates and the debris was removed by centrifugation at 10000 r.p.m. for 10 min at 4 °C. Supernatants were subjected to immunoprecip-
Chromosome duplication induced by HPV-16 E7

pitation using polyclonal anti-E7 protein rabbit serum (Sato et al., 1989b). The immunoprecipitated proteins were subjected to SDS-PAGE and fluorography.

Cytogenetic methods. Metaphase cells were enriched by treatment with colcemid (0.05 μg/ml) for 1 h at the given passage number. Chromosome analysis was performed by the conventional cytogenetic method. Chromosome preparations on glass slides were stained with Giemsa (10% in 0.05 M-phosphate buffer pH 6.8, 15 min) or Quinacrine for microscopic examination. Chromosome spreads of developing microcolonies were prepared using the method of in situ chromosome preparation (Oshimura et al., 1988). The plasmid DNA-transfected microcolonies consisting of 50 to 200 cells (average 100) were selected in the presence of 100 μg/ml G418 for 1 week after introducing plasmid DNA carrying the neo gene.

Results

Chromosomal abnormalities in HPV-16-immortalized human keratinocyte cell lines

To correlate HPV-16 infection and cytogenetic disorganization during tumour progression, we examined karyotypes of human keratinocyte cell lines PHK16-I, PHK16-II, PHK16-0b, PHK16-L1 and PHK16-L2, which carried transcriptionally active dimeric or monomeric full-length HPV-16. A predominantly expressed mRNA from the HPV-16 genomes in these cell lines contained the E6 and E7 ORFs. At least 100 metaphase cells from five individual clonal lines were examined. Abnormal karyotypes carrying numerical and structural chromosome disorders were found in all human keratinocyte cell lines. Some of these chromosomal abnormalities contained endoreduplications, chromatid gaps and breakages (Fig. 2a). Dicentric chromosomes and centric rings were also found in an independent chromosome preparation (Fig. 2b and c). A particular clone, PHK16-0b, persisted in the diploid state, but numerical and structural chromosome abnormalities were also found; a typical PHK16-0b karyotype is shown in Fig. 3. The other four cell lines contained tetraploid cells at markedly high frequencies; such cells appeared to be predominant (Table 1). These results raise the question.

Fig. 2. Chromosome abnormalities in HPV-16-immortalized human keratinocyte cell lines. Chromosomes were stained with Quinacrine. (a) Endoreduplicated metaphase figure which also contained a number of chromatid gaps and breakage (arrow heads). (b) Dicentric chromosome. (c) Centric ring.
Fig. 3. A typical karyotype of an HPV-16-immortalized human keratinocyte cell line, PHK16-0b, with a diploid range. Loss of part or all of chromosomes 2 (m), 4 (m), 8, 9, 12, 15, 22 and Y is shown; chromosome 5 is trisomic. Five marker chromosomes (mar) exist. The chromosome preparation was performed after 300 propagation doublings and stained with Quinacrine.

Table I. Numerical and structural chromosome abnormalities* induced by HPV-16 genes in human keratinocytes

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Passage number†</th>
<th>Polyploid (%)</th>
<th>Endoreduplication (%)</th>
<th>Abberations‡ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHEK</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PHK16-0b</td>
<td>9</td>
<td>11</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PHK16-I</td>
<td>18</td>
<td>24</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>NHK16-I</td>
<td>25</td>
<td>97</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>PHK16L-1</td>
<td>5</td>
<td>93</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>PHK16L-2</td>
<td>18</td>
<td>86</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>PDE7-1</td>
<td>9</td>
<td>22</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>PDE7-2</td>
<td>9</td>
<td>27</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PDE7-5</td>
<td>9</td>
<td>17</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>PDE7-6</td>
<td>6</td>
<td>11</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>PSV-1</td>
<td>3</td>
<td>31</td>
<td>17</td>
<td>2</td>
</tr>
</tbody>
</table>

* More than 100 metaphase cells from each cell line were analysed for numerical and structural chromosome abnormalities.
† Passage numbers of DNA-transfected cells are the number after isolating a single colony which showed growth advantage in culture.
‡ Aberrations include chromatid gaps, fusion and breakage.

To determine whether the cytogenetic abnormalities, particularly chromosome duplication in human cells, could be related to functions of HPV-16 oncogenes, the two putative transforming genes, E6 and E7, were cloned separately into the expression vector pcD2, resulting in plasmids pDE6 and pDE7, respectively. These plasmids ensured that transcriptional activity was driven by the SV40 promoter, terminating at the SV40 polyadenylation signal (Chen & Okayama, 1987). Plasmid DNA from pDE6, pDE7 and pSV40ori− (Giuzman et al., 1980) was introduced separately into primary human keratinocytes. The E7 gene induced several clonal cell lines which had an apparently extended life span, whereas no
Chromosome duplication induced by HPV-16 E7

Fig. 4. Detection of E7 protein in extracts of E7 transformants PDE7-1, PDE7-2, PDE7-5 and PDE7-6. Lanes 4 (a) and 6 (b) SiHa cell extracts of an established human cell line derived from a cervical carcinoma. Lanes 1 (a) and 2 (b) NHK extracts. SDS-PAGE was performed using a 15% polyacrylamide gel. (a) E7 protein detected in PDE7-1 (lane 2) and PDE7-2 (lane 3) extracts. (b) E7 protein in PDE7-1 (lane 3), PDE7-5 (lane 4) and PDE7-6 (lane 5) extracts. (c) Detection of E7 mRNA in PDE7-1 (2) and PDE7-2 (3) extracts by dot hybridization. Extracts were probed with the HPV-16 EcoRI-PstI fragment (nt 7454 to 875, 1.3 kb), specific for the E6 and E7 ORFs. 1, NHK mRNA as a control.

Fig. 5. Northern blotting of E7 and E6 mRNA expressed in pDE7- and pDE6-transfected mouse keratinocytes. Total cellular RNA (25 μg/lane) extracted from five pDE7 lines (PMKH7-1, 2, 3, 4 and 5; lanes 6 to 10) and poly(A+) mRNA (2.5 μg/lane) purified from pDE6 lines (PMKH6-1, 3 and 5; lanes 2 to 4) were probed with the 1.3 kb DNA segment as described in Fig. 4. Multiple mRNA species were detected in all PMKH6 lines, possibly because of differential splicing within the E6 ORF (Taniguchi & Yasumoto, 1990). Lane 1, NMK RNA; lane 5, PMKH6-3 RNA.

Transformants induced by the E6 gene were expanded. Transfection with pSV40ori− DNA, used as a positive control, produced stable transformants at an efficiency 10-fold or more greater than that of HPV-16.

Karyotype analysis was performed similarly using those cell lines (pDE7 clones, 1, 2, 5 and 6) which expressed the E7 protein (Fig. 4). An individual clonal line contained mosaic patterns of chromosome sets with a range of diploid and polyploid; the percentage of tetraploid cells in at least 100 metaphases cells was 11% to 27% in all E7 gene-transformed clonal lines (Table 1). This value was significantly higher than that of normal human keratinocyte cultures (NHEK). These results suggest that polyploidy is induced by HPV-16 functions and that the E7 oncogene alone is involved in chromosome duplication in HPV-16-immortalized human keratinocyte cell lines. Moreover, we found that metaphase figures contained diplochromosome sets in all E7 transformants, as well as in PHK16 lines containing monomeric or dimeric full-length HPV-16 DNA, although at low frequency (Table 1). No diplochromosome set has been found in untransformed primary human keratinocytes. Another DNA tumour virus, SV40, also transforms primary human keratinocytes and a number of tetraploid cells as well as significantly high numbers of diplochromosome sets were found (Table 1). These results suggest that the virus-encoded oncogenes, SV40 LT or HPV-16 E7, play a role in the induction of cytogenetic abnormalities in infected cells. Since the E6 gene alone does not produce stable human cell lines (Hawley-Nelson et al., 1989; Munger et al., 1989; and our unpublished results), we could not examine the effect of the E6 gene in human cells.

E6 oncogene does not induce chromosome duplication in mouse keratinocytes

Primary mouse keratinocytes can be cultivated in the same culture conditions as human cells and we have successfully established a number of mouse keratinocyte lines harbouring the E6 gene alone. We found that all clonal mouse keratinocytes harbouring transcriptionally active E6 retained the diploid state (Fig. 5, Table 2; PMKH6-1, 3 and 5). Although E6 mRNA species could not be identified, plasmid pDE6 was biologically active because cotransfection with pDE7 produced immortalized human keratinocytes whereas pDE7 alone did not (S. Yasumoto et al., unpublished results). Karyotype stability was also observed in spontaneously immortalized mouse keratinocytes (NMK) and in cells transfected with vector DNA alone (PMKPM-1) (Table 2). In contrast, changes in ploidy were found in clonal lines PMK16d, PMKH7 and PMKSV, which carry full-length HPV-16, the E7 gene and pSV40ori− DNA, respectively. Most notably, all clonal mouse keratinocytes except the E6 clones contained polyploid chromosome sets, including hyper- and hypo-tetraploids. Unlike
human cells, mouse keratinocytes appeared to be highly susceptible to the HPV-16-associated cytogenetic effect.

**E7 oncogene may be a causative agent of chromosome duplication in mouse keratinocytes**

To determine more directly whether changes in ploidy were due to the E7 oncogene alone, changes in chromosome number were examined in primary mouse keratinocytes after transfection with pDE7, pDE6 and pCD2 vector DNA alone. The transfected mouse cells were enriched by a brief G418 selection for the neo gene. Metaphase cell preparation was performed using the method of in situ chromosome spreading (Fig. 6). G418-resistant cells were allowed to develop into microcolonies each consisting of approximately 100 cells on average. On day 9 (approximately 10 population doublings), 67% of metaphase figures in G418-resistant cells carried polyploids; on day 21 (25 population doublings), the number of polyploid cells increased to 84%. This indicates that E7 gene-transfected mouse keratinocytes underwent at least seven population doublings shortly after transfection and that polyploid cells subsequently became predominant in culture. Since microcolonies transfected with E6 gene or vector DNA alone contained only 10% polyploid cells, which is comparable with that of spontaneously established NMK lines, it seems likely that chromosome duplication is induced by an E7 gene function, the exact mechanisms of which are unknown.

**Progressive chromosome loss during serial passage of tetraploid mouse keratinocytes**

The loss of specific chromosomes has been implicated in the development of malignant tumours (Koufos et al., 1984; Stanbridge et al., 1981; Oshimura et al., 1985; Yokota et al., 1987). Although malignantly transformed keratinocytes could not be developed from HPV-16-immortalized cell lines in our system, to determine whether progressive loss of chromosomes could be associated with keratinocytes carrying duplicated chromosomes, the possibility that chromosome numbers could change from polyploid to aneuploid with loss or gain of chromosomes, was examined. The result was clearest in mouse keratinocyte cell lines containing 100% tetraploid cells (Fig. 7). Karyotypes of these clones, PMK16d-3 harbouring full-length HPV-16 and PMKH7-2 carrying the E7 gene alone, were examined at different passage numbers. Modal chromosome numbers decreased from the tetraploid range to the hypotetraploid range in both PMK16d-3 and PMKH7-2.

**Discussion**

We have shown that cytogenetic instability, especially changes in ploidy, is a detectable somatic disorganization in HPV-16-immortalized cell lines. Since changes in ploidy to aneuploid have been found in almost all HPV-16-associated malignant tumours in vivo (Fu et al., 1981;}

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Table 2. Changes in ploidy of mouse keratinocytes induced by the E7 and E6 genes of HPV-16*

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Passage number†</th>
<th>%</th>
<th>Modal chromosome number (range)‡</th>
<th>%</th>
<th>Modal chromosome number (range)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMK</td>
<td>25</td>
<td>90</td>
<td>40 (39-41)</td>
<td></td>
<td>ND§</td>
</tr>
<tr>
<td>PMKPM-1</td>
<td>8</td>
<td>91</td>
<td>40 (38-41)</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>PMK16d-2</td>
<td>9</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>80 (78-82)</td>
</tr>
<tr>
<td>PMK16d-3</td>
<td>9</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>80 (74-82)</td>
</tr>
<tr>
<td>PMK16d-4</td>
<td>12</td>
<td>0</td>
<td>100</td>
<td>78</td>
<td>78-82</td>
</tr>
<tr>
<td>PMK16d-7</td>
<td>14</td>
<td>0</td>
<td>100</td>
<td>80</td>
<td>80 (71-80)</td>
</tr>
<tr>
<td>PMK16d-3</td>
<td>9</td>
<td>0</td>
<td>100</td>
<td>74</td>
<td>72-77</td>
</tr>
<tr>
<td>PMK16d-3</td>
<td>9</td>
<td>0</td>
<td>100</td>
<td>78</td>
<td>71-82</td>
</tr>
<tr>
<td>PMKH7-2</td>
<td>8</td>
<td>0</td>
<td>100</td>
<td>80</td>
<td>78-82</td>
</tr>
<tr>
<td>PMKH7-2</td>
<td>8</td>
<td>0</td>
<td>100</td>
<td>80</td>
<td>80 (78-81)</td>
</tr>
<tr>
<td>PMKH7-5</td>
<td>18</td>
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<td>100</td>
<td>80</td>
<td>76-82</td>
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<tr>
<td>PMKH6-1</td>
<td>8</td>
<td>100</td>
<td>41 (39-42)</td>
<td></td>
<td>ND§</td>
</tr>
<tr>
<td>PMKH6-3</td>
<td>14</td>
<td>97</td>
<td>40 (39-42)</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>PMKH6-5</td>
<td>16</td>
<td>91</td>
<td>40 (39-42)</td>
<td></td>
<td>ND§</td>
</tr>
<tr>
<td>PMKSV-1</td>
<td>3</td>
<td>0</td>
<td>100</td>
<td>80</td>
<td>76-80</td>
</tr>
<tr>
<td>PMKSV-3</td>
<td>3</td>
<td>0</td>
<td>100</td>
<td>80</td>
<td>78-80</td>
</tr>
</tbody>
</table>

* Karyotypes were examined in at least 100 metaphase cells.
† The passage number of each line is the number after isolating a single clone resistant to G418.
‡ Chromosome numbers represent the mode of at least 20 metaphase cells.
§ ND, Not determined.
Reid et al., 1984) and HPV-16-immortalized human keratinocytes in vitro (Durst et al., 1987; Hawley-Nelson et al., 1989; Smith et al., 1989), the occurrence of numerical (aneuploidy) and structural chromosomal disorganization may somehow reflect changes in ploidy in HPV-16-associated neoplasia in vivo. By using primary epidermal keratinocytes, we have shown that the E7 gene alone is responsible for the cytogenetic abnormalities induced. This was shown most clearly using mouse epidermal keratinocytes; rodent cells appear to be more susceptible than human cells to these virus oncogenes. This may reflect the different sensitivity to various carcinogenic agents of rodent and human cells (DiPaolo, 1983). Human keratinocytes harbouring a transcriptionally active E6 gene alone failed to become stable cell lines, in marked contrast to E6 gene-transfected mouse keratinocytes. All mouse keratinocyte lines harbouring a transcriptionally active E6 gene alone grew stably and subsequently became immortal. These E6 transformants of mouse keratinocytes enabled us to analyse the progressive cytogenetic changes in a clonal lineage compared with E7 gene-transfected cells. In all clonal mouse keratinocyte lines harbouring the E7 gene alone (PMKH7 series), 100% of metaphase cells were tetraploid. In contrast, all clonal lines containing the E6 gene alone consisted of fairly stable diploid chromosome sets comparable with those of NMK cells. All conditions for chromosome preparation were consistent and there was no significant difference in the growth rate between cells and cell lines. The effect of the antibiotic G418 on karyotype instability was negligible. It is therefore reasonable to assume that the change in ploidy is closely related to E7 gene-associated biological functions.

Although the mechanisms of the conversion of somatic cells to polyploidy are unknown, one possible explanation is that there is a defect in cytokinesis followed by chromosome duplication in the cell cycle. This has often been observed in cells treated with inhibitors of cytokinesis, such as colcemid or vinblastine (Oksala & Therman, 1974). It is true that colcemid treatment for a few hours often produced polyploid cells in the subsequent population. However, since no endoreduplication appeared from such populations, this event could be an alternative and likely process of polyploidization in the oncogenic virus-stimulated kera-
tinocytes. This cytogenetic event gives rise to a diplochromosome set juxtaposed to the duplicated alleles that have been implicated in DNA duplication prior to the following mitosis (Okasala & Therman, 1974); in some cases tetrachromosome sets were also detected. The frequency of endoreduplication was found to be between 1 and 5% of 100 human metaphase cells; this value is relatively lower than that in SV40-transformed human keratinocytes, but significantly higher than that in controls. No endoreduplication was found in primary human and mouse keratinocytes, possibly because it is an extremely rare event. It thus seems likely that E7 gene-associated functions induce diplochromosome formation in both human and mouse keratinocytes. Although the exact mechanism of endoreduplication remains unclear, these results in part correlate E7 gene function with the event of endoreduplication, which may result from either duplicated DNA synthesis in the S phase, or dysfunction of the kinetochore in somatic chromosome segregation.

The E7 gene encodes the major oncoprotein of HPV-16 and HPV-18, and its cooperation with the E6 gene is necessary to transform rodent cell lines fully (Bedell et al., 1987; Crook et al., 1989; Kanda et al., 1988; Munger et al., 1989; Hawley-Nelson et al., 1989). Growth stimulation is an apparent biological function associated with the E6 and E7 genes in a variety of cells (Hawley-Nelson et al., 1989; Munger et al., 1989; Sato et al., 1989a). It is therefore attractive to speculate that the E7 oncprotein may be directly involved in the process of genome duplication, inducing uncontrolled DNA synthesis, because one of the known E7 protein functions is related to the stimulation of cellular DNA synthesis in rodent cell lines (Sato et al., 1989a). Recently, it has been suggested that a near-tetraploid population of human keratinocyte lines transformed by HPV-16 or HPV-18 appears owing to reduplication in the original diploid clone (Smith et al., 1989). Taken together with these observations in vitro, our experimental results appear to coincide in part with changes in ploidy of the HPV-associated histopathological spectra in vivo (Reid et al., 1984). It is still unclear how these polyploid populations become predominant. Changes in sensitivity to transforming growth factor β could be one possible explanation for the growth advantage (unpublished results).

Another potent DNA tumour virus, SV40, is also known to produce stable human keratinocyte lines (Steinberg & Defendi, 1979; Banks-Schlegel & Howley, 1983). The SV40-transformed human keratinocytes established here showed frequent endoreduplication and chromosomal aberrations in an early phase of transformation, as has been documented in a previous report (Okasala & Therman, 1974). Since SV40 LT and HPV-16 E7 proteins both bind p105-Rb (Ludlow et al., 1988; Whyte et al., 1988; Dyson et al., 1989), these transforming gene products may directly or indirectly elicit cytogenetic disorganization by common mechanisms.

In conclusion, we have shown that changes in ploidy are a critical E7 gene-associated cytogenetic event which may be related to the oncogenic activity of HPV-16. Although malignant transformation has not been correlated with specific chromosome loss in our system, apparent chromosome loss occurred on serial passage of a clonal cell lineage, possibly by random chromosome segregation or mutation. Some of our immortalized premalignant keratinocytes could be used to correlate malignant transformation with specific changes in chromosome number.

We thank K. Yoshihke for the gift of anti-E7 anti-serum, and M. Oshimura and T. Utakoji for advice on chromosome analysis. We also thank H. Andoh for technical assistance. This work was supported in part by a grant-in-aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

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(Received 4 September 1990; Accepted 11 March 1991)