The EMBO Journal vol.12 no.5 pp.1847–1852, 1993

# Enhanced degradation of p53 protein in HPV-6 and BPV-1 E6-immortalized human mammary epithelial cells

**Vimla Band[1], Sorab Dalal[2], Laurie Delmolino and Elliot J.Androphy[2,3]**

Division of Radiation and Cancer Biology, Departments of Radiation Oncology, [2]Microbiology and Molecular Biology and [3]Dermatology, New England Medical Center Hospitals and Tufts University School of Medicine, 750 Washington Street, Boston, MA 02111, USA

[1]Corresponding author

Communicated by L.Crawford

**Normal mammary epithelial cells are efficiently immortalized by the E6 gene of human papillomavirus (HPV)-16, a virus commonly associated with cervical cancers. Surprisingly, introduction of the E6 gene from HPV-6, which is rarely found in cervical cancer, or bovine papillomavirus (BPV)-1, into normal mammary cells resulted in the generation of immortal cell lines. The establishment of HPV-6 and BPV-1 E6-immortalized cells was less efficient and required a longer period in comparison to HPV-16 E6. These HPV-6- and BPV-1 E6-immortalized cells demonstrated dramatically reduced levels of p53 protein by immunoprecipitation. While the half-life of p53 protein in normal mammary epithelial cells was ~3 h, it was reduced to ~15 min in all the E6-immortalized cells. These results demonstrate that the E6 genes of both high-risk and low-risk papilloma viruses immortalize human mammary epithelial cells and induce a marked degradation of p53 protein *in vivo*.**

*Key words*: E6/human papillomavirus/half-life/mammary epithelial cells/p53 protein

## Introduction

Breast cancer is one of the most common lethal malignancies of women in North America and Europe. The molecular events involved in the development of early breast cancer are largely unknown, primarily due to the lack of suitable *in vitro* models. Immortalization is a crucial early event in tumorigenesis that allows cells to proliferate continuously. To examine this stage of mammary cell oncogenesis we first defined culture conditions for the growth of normal and transformed mammary epithelial cells (Band and Sager, 1989; Band *et al.*, 1990a). Using this *in vitro* system it was demonstrated that the normal human mammary epithelial cells were immortalized by the introduction of human papillomavirus (HPV) type 16 or 18 DNA (Band *et al.*, 1990b). These HPVs are often referred to as 'high risk' since they are frequently isolated in human cervical cancers. HPVs such as types 6 and 11 are common in genital and cervical papillomas and are referred as 'low risk', since these lesions rarely progress to frank malignancy.

Several studies in rodent cell culture systems and in human keratinocytes have shown that the E6 and E7 genes of HPV-16 and HPV-18 were critical for the establishment of their immortal state (Beddel *et al.*, 1987; Matlashewski *et al.*, 1987; Kaur and McDougall, 1988; Münger *et al.*, 1989a; Barbosa and Schlegel,1989; Hawley-Nelson *et al.*, 1989; Hudson *et al.*, 1990; Barbosa *et al.*, 1991; Halbert *et al.*, 1991). The proteins encoded by the E6 and E7 genes have been shown to complex *in vitro* with the two known tumor suppressor gene products, the p53 and retinoblastoma protein (Rb), respectively (Münger *et al.*, 1989b; Werness *et al.*, 1990). Binding of E6 to p53 and of E7 to Rb is thought to result in the loss of their normal growth inhibitory functions. In contrast to keratinocytes which require both the E6 and E7 genes for efficient immortalization (Münger *et al.*, 1989a), transfection studies with mutant HPV-16 constructs suggested that the HPV-16 E6 gene was essential and sufficient for the immortalization of normal mammary epithelial cells (Band *et al.*, 1991).

Using the *in vitro* reticulocyte lysate system, it has been reported that HPV-16 E6 induced p53 degradation through the ubiquitin-dependent pathway (Scheffner *et al.*, 1990; Crook *et al.*, 1991b). Consistent with these *in vitro* results, HPV-16 E6-immortalized mammary epithelial cells showed nearly undetectable levels of p53 protein, as opposed to the normal parent cells which have relatively high levels of the p53 protein (Band *et al.*, 1991). This system, therefore, provides a model to understand the biochemical basis of immortalization by E6 in the absence of potential interactions with other viral gene products such as E7.

The degradation of p53 protein *in vitro* by the E6 proteins of the high risk viruses appears to be biologically relevant since the E6 proteins of low-risk viruses, such as HPV-6, are not competent to mediate its degradation (Scheffner *et al.*, 1990; Crook *et al.*, 1991b). However, although BPV-1 E6 does not bind human p53 in these *in vitro* assays (Werness *et al.*, 1990), it efficiently induces the oncogenic transformation of murine C127 cells (Schiller *et al.*, 1984). These BPV E6 transformed cells have levels of p53 equal to non-transformed or v-ras transformed C127 cells (Schiller *et al.*, 1986; E.J.Androphy, unpublished data). Similarly, the E6 gene from HPV 8, an oncogenic virus causing squamous cell cancer in epidermodysplasia verruciformis, transforms C127 cells but does not bind p53 (Steger and Pfister, 1992). The HPV-6 and BPV-1 E6 genes, therefore, provide naturally occurring variants to address the role of p53 in E6-induced mammary cell immortalization. Here, we demonstrate that these E6 genes were able to induce the immortalization of mammary epithelial cells. In the resultant cell lines the half-life of p53 protein was markedly reduced, suggesting that augmented p53 degradation was required for immortalization.

## Results

### *Immortalization of normal mammary epithelial cells by HPV-16, HPV-6 and BPV-1 E6*

Our previous studies have demonstrated that a plasmid containing a β-actin promoter-regulated HPV-16 E6 open

reading frame (ORF) with a disrupted E7 ORF efficiently immortalized normal human mammary epithelial cells (Band *et al.*, 1991). Because the disrupted E7 gene contained the amino-terminal Rb binding domain, we were unable to exclude the possibility that it was contributing to the immortal phenotype. Therefore, the coding sequences of the E6 genes of HPV-16, HPV-6 and BPV-1 were cloned into p1318 vector (Münger *et al.*, 1989a) downstream from the β-actin promoter. The normal mammary epithelial cell strain 76N was transfected with these plasmids by calcium phosphate coprecipitation. Following G418 selection in DFCI-1 medium for 10 days, individual colonies and a pooled population were transferred to the defined medium D2. Normal mammary epithelial cells do not proliferate in this medium (Band *et al.*, 1990b, 1991). The efficiency of immortalization was determined as the number of G418-resistant colonies that gave rise to immortal clones.

The results of a representative experiment are presented in Table I. All (10/10) of the G418-resistant colonies tested from the HPV-16 E6 transfection gave rise to immortal clones as did the pooled population. When G418-resistant colonies from the HPV-6 E6 transfection were transferred to D2 medium, a period of selection was observed during which a large proportion of the cells died. After this 'crisis' period, two of the 10 colonies yielded a population of cells that could be grown in D2 medium without further cell death. Similar results were obtained with the pooled cell population. These proliferating cells have been serially propagated in D2 medium for >20 passages (>100 population doublings) for the individual clones and 40 passages (>200 population doublings) for the pooled HPV-6 E6-transfected cell population without any evidence of senescence, clearly indicating that the cells became immortal. Similar results were obtained in one other normal mammary epithelial cell strain 70N (data not shown).

To determine whether BPV-1 E6 transforming function would be manifest in human mammary epithelial cells, this gene was transfected into 76N cells. When individual colonies and a pooled population were plated in D2, they exhibited a 'crisis' period comparable to that observed in the HPV-6 E6 transfectants. While none of the individual colonies could be established as an immortal line (Table I), a population of cells was established from pooled colonies after the 'crisis' period. These have been maintained continuously in D2 medium (>20 passages or ~100 population doublings), indicating that they were immortal.

Considering the biological differences we observed during HPV-16, HPV-6 and BPV-1 E6 immortalization, the possibility that the HPV-6 or BPV-1 E6-immortalized cell lines resulted from contamination with HPV-16 E6 (which was used as a positive control in each transfection experiment) was tested. A differential PCR was performed on DNA extracted from the immortalized lines as described in Materials and methods. All PCR reactions contained a mixture of oligonucleotides specific for each of the three E6 genes. As shown in Figure 1, the E6 primers did not amplify a specific product using DNA isolated from non-immortalized cells (lane 3). The BPV specific 206 bp fragment could be amplified from the BPV-1 E6-immortalized cells (lane 4), and the HPV-6 E6 specific 482 bp fragment was amplified from DNA isolated from HPV-6 E6 immortalized cells (lane 5). Only DNA from HPV-16 E6-immortalized cells produced the 16-E6 specific 188 bp fragment (lane 6). This demonstrates that the phenotype exhibited by the HPV-6- and the BPV-1 E6-immortalized cells was not due to the presence of an HPV-16 E6 gene. In addition the HPV-6 E6 gene product was cloned from H6E6-P, cells sequenced and confirmed to be wild type. In Northern blot analyses, E6-specific RNA transcripts were observed in all E6-immortalized cells, demonstrating that E6 genes were expressed. As expected, the normal parent cells did not contain any HPV specific RNAs (data not shown).

**Table I.** Immortalization of normal mammary epithelial cells by transfection of papilloma virus E6 genes

| Plasmid | No. of immortal colonies | Immortal pooled cells |
|---|---|---|
| HPV-16 E6 | 10 (out of 10) | + |
| HPV-6 E6 | 2 (out of 10) | + |
| BPV-1 E6 | 0 (out of 10) | + |

$10^6$ cells were co-transfected with the indicated plasmids and pSV2neo. After 48 h G418-resistant cells were selected for 10 days. Individual G418-resistant colonies (column 2) or pooled population of these cells (column 3) were grown in D2 selection medium to determine the immortal phenotype. Pooled cells in all the three cases gave rise to immortal cell lines. However, on the basis of the number of immortal clones, the E6 gene of HPV-16 was the most efficient and BPV-1 E6 was the least efficient.

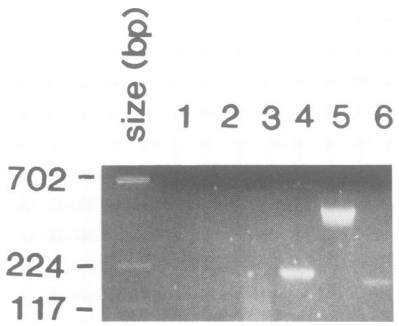


**Fig. 1.** Differential PCR. PCR reactions were performed on DNA isolated from either 76N (lane 3), BE6-P (lane 4), H6E6-P (lane 5) and H16E6-P (lane 6) as described in Materials and methods. The E6 primers did not amplify a specific product from 76N cells. The E6-immortalized cells showed a single specific product that corresponded to the gene transfected (BPV-1 E6 206 bp, HPV-6 E6 482 bp and HPV-16 E6 188 bp). Lane 2 is a control where the PCR mix was primed with distilled water instead of DNA and lane 1 is the reaction mix with the PCR reagents alone.

A possible explanation for the 'crisis' period during HPV-6 or BPV-1 E6-induced immortalization was that these cells acquired a p53 mutation. To determine whether the p53 gene expressed by these immortal cells was wild type, RNA was isolated from HPV-6- and HPV-16-immortalized cells and used as a template for synthesizing cDNA (as described in Materials and methods). The entire coding sequences from 10 independent cDNA clones of p53 from both HPV-16- and HPV-6 E6-immortalized cells were sequenced and confirmed to be identical to parent wild-type gene in 76N (sequence not shown). The p53 alleles in 76N cells were found to be wild type by direct sequence analysis (L.Delmolino *et al.*, submitted).

### *Status of p53 protein in E6-immortalized cells*

The E6 genes of the high risk viruses HPV-16 and HPV-18 cause the degradation of p53 protein *in vitro*. Consistent with these *in vitro* results, we have demonstrated that the levels

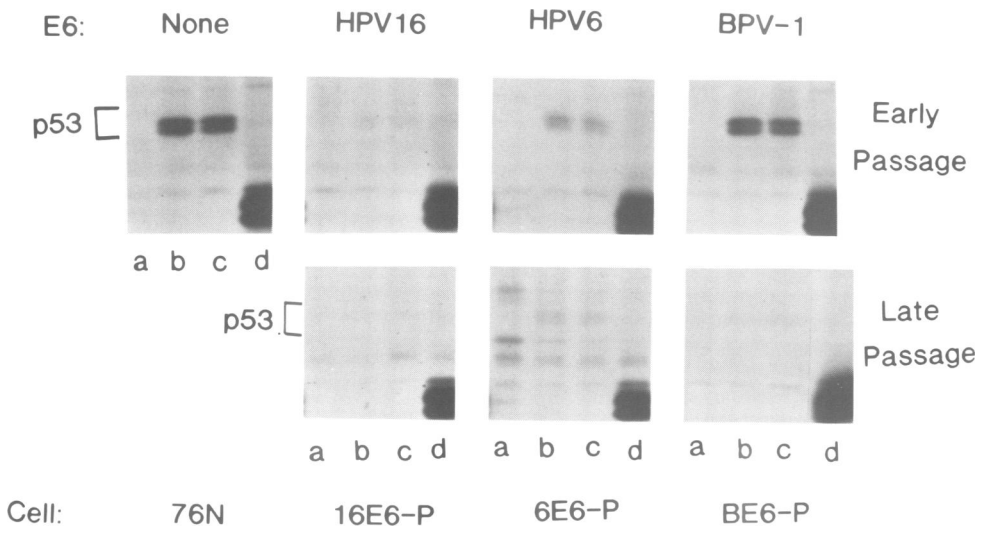


**Fig. 2.** Immunoprecipitation analysis of p53 protein in E6-immortalized cells. NETN lysates of metabolically labelled cells at early (before crisis) or late passages (established immortal) were immunoprecipitated using anti-p53 mAb, PAb 122 (lane b) and PAb 1801 (lane c). In each case lane a, represents immunoprecipitation with P3 (negative control) or lane d, with W6/32 (anti-HLA class I; used as a control). The position of p53 protein is indicated. Note that both early and late passage HPV-16 E6-transfected cells showed nearly undetectable p53. In contrast, early passages of HPV-6 and BPV-1 E6-transfected cells showed a reduced but detectable levels of p53; at later passages (post-crisis) these cells showed marked p53 loss comparable to HPV-16 E6 transfectants.

of p53 protein were drastically reduced in HPV-16 E6-immortalized mammary cells (Band *et al.*, 1991). Conflicting *in vitro* data suggest that the E6 protein from a low-risk virus such as HPV-6 either does not bind p53 (Werness *et al.*, 1990) or binds p53 less efficiently than HPV-16, but is unable to target p53 for degradation (Crook *et al.*, 1991b). BPV-1 E6 does not bind human p53 protein *in vitro* (Werness *et al.*, 1990). These *in vitro* results would imply that HPV-6 or BPV-1 E6 might not affect p53 levels *in vivo*. To examine the state of p53 in these cells, the levels of p53 in the E6-immortalized cells were analyzed by immunoprecipitation with the p53-specific monoclonal antibodies PAb 122 and PAb 1801, both before and after the 'crisis' period.

As reported previously (Band *et al.*, 1991), immunoprecipitation with these antibodies showed abundant p53 protein in 76N cells (Figure 2). In contrast, a pooled population of HPV-16 E6-immortalized cells, H16E6-P, showed nearly undetectable levels of metabolically labeled p53 at both early and late passages (Figure 2). When a pooled population of HPV-6 E6 transfected cells, H6E6-P, was examined at early passage (before crisis), p53 protein was decreased relative to 76N cells, but was still detectable. At later passages (post-crisis) the same pooled population showed barely detectable levels of p53, which were comparable to HPV-16 E6-immortalized cells. The pooled population from BPV-1 E6 transfected cells, BE6-P, showed high levels of p53 when examined at an early passage. When the pooled population was examined at a later passage (post-crisis), the p53 protein was barely detectable by immunoprecipitation (Figure 2).

### *Normal p53 RNA expression in E6-immortalized cells*

To demonstrate that loss of immunoprecipitable p53 protein in E6-transfectants was not due to the reduction in the expression of its mRNA, p53 mRNA levels in normal parent (76N) and HPV-16, HPV-6 and BPV-1 E6-immortalized cells were analyzed by Northern blot analysis. p53 mRNA was equally abundant in 76N and E6-immortalized cell lines

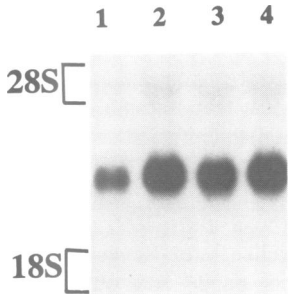


**Fig. 3.** p53 mRNA expression in normal and E6-immortalized cells. Total cellular RNA isolated from normal parent cells (lane 1), 16E6-P (lane 2), 6E6-P (lane 3) and BE6-P (lane 4) was resolved on an agarose gel and transferred to a nylon membrane (Hi-bond N, Amersham) and hybridized with a 1.8 kb human p53 cDNA. Locations of the ribosomal RNAs (28S, 4850 bp and 18S, 1740 bp) are indicated.

(Figure 3). Thus, reduction of p53 protein levels in E6-immortalized cells was not due to change in p53 gene expression.

### *The half-life of p53 protein is reduced in E6-immortalized cells*

While HPV-16 E6 induced the degradation of p53 *in vitro*, neither HPV-6 E6 nor BPV-1 E6 demonstrated this activity. To assess whether the decrease in p53 *in vivo* was due to an enhanced rate of degradation in the E6-immortalized cells, we determined the stability of p53 protein in these cells.

A pulse-chase analysis demonstrated that the half-life of p53 in 76N cells was ~3 h (Figure 4), considerably longer than the 15 min half-life reported in rodent fibroblasts such as NIH 3T3 cells (Levine and Momand, 1990; L.Delmolino *et al.*, submitted). Under identical conditions, p53 protein in MDA-MB-231, a human mammary tumor cell line carrying an Arg to Lys mutation at codon 280, showed a half-life of ~30 h (L.Delmolino *et al.*, submitted). Thus,

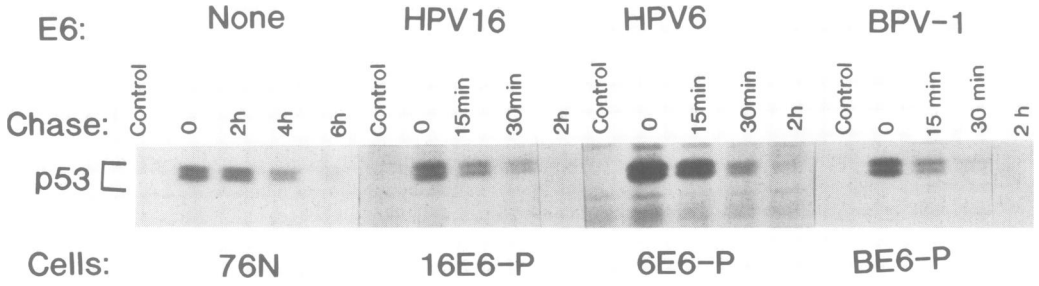


**Fig. 4.** Papilloma virus E6-immortalized cells show markedly enhanced degradation of p53 protein. 76N cells and E6-immortalized cells were metabolically pulse-labelled for 15 min and then chased for various time periods as described in Materials and methods. Equal amounts of cell lysates (c.p.m) were immunoprecipitated using the anti-p53 mAb PAb 122 and separated on SDS−7.5% polyacrylamide gels. Note that in 76N cells (half-life of ~3 h), p53 can be detected even after 4 h of chase. In contrast, E6-transfected cells show marked loss of p53 within 15 min, with little detectable by 2 h and virtually no p53 was detected after 4 h (not shown).

the methodology used accurately measured the kinetics of p53 catabolism. The half-life of p53 was reduced to 15 min in H16E6-P, indicating enhanced degradation. A similar analysis of p53 stability in H6E6-P and BE6-P demonstrated that its half-life was also about 15 min in these cell lines (Figure 4). These results demonstrate that *in vivo* both HPV-6 and BPV-1 E6 induce an increase in p53 degradation.

## Discussion

The generation of immortal human cell lines from normal human cells is a rare phenomenon. The HPV E6 and E7 oncoproteins appear to possess a unique ability to prolong the life span of human epithelial cells (Kaur and McDougall, 1988; Barbosa and Schlegel, 1989; Hawley-Nelson *et al.*, 1989; Münger *et al.*, 1989a; Band *et al.*, 1990b; Hudson *et al.*, 1990; Barbosa *et al.*, 1991; Halbert *et al.*, 1991) and smooth muscle cells (Perez-Reyes *et al.*, 1992). The results presented in this paper and our previous observations demonstrate that the HPV-16 E6 oncoprotein can efficiently immortalize normal human mammary epithelial cells. The HPV-16 E7 gene was neither sufficient nor necessary for their immortalization (Band *et al.*, 1991). In this regard human mammary epithelial cells differed from primary human keratinocytes which required both E6 and E7 for efficient immortalization. Mutant forms of Rb are rarely found in breast cancer (Bartek *et al.*, 1990), while there is a high frequency of p53 mutations or deletions in breast cancer (Crawford *et al.*, 1984; Bartek *et al.*, 1990; Runnebaum *et al.*, 1991). Since HPV-16 E6 and E7 presumably inactivate p53 and Rb function, respectively, these observations would suggest that Rb may not have an important tumor suppressor function in mammary epithelial cells. The mammary cell immortalization provides a unique model that is strictly E6 dependent and thus facilitates analyses of the biochemical basis of immortalization as well as the interactions of E6 with cellular factors such as the p53 tumor suppressor gene product.

In this report we have demonstrated that not only the E6 gene from HPV-16, but also the E6 genes of HPV-6 and BPV-1, can immortalize these cells. HPV-6 and BPV-1 E6 were significantly less efficient than HPV-16 E6 in induction of the immortal phenotype, and the HPV-6 and BPV-1 E6-transfected cultures revealed an ongoing cell death at early passages. This phase, referred to as the 'crisis' period, was not observed during HPV-16 E6 induced immortalization. The efficiency of immortalization *in vitro* by these HPV E6 genes correlates with their natural biology, since HPV-16 infection is associated with cervical cancer while HPV-6 only rarely eventuates in malignancy (zur Hausen, 1989). This mammary cell system, therefore, provides a unique model to study transforming functions shared between HPV and BPV E6 genes, as well as their distinguishing properties. It is of interest that HPV-6 E6 in cooperation with HPV-16 E7 have been recently shown to have a weak immortalizing activity for human foreskin epithelial cells (Halbert *et al.*, 1992). Furthermore, a higher proportion of the tumors of vulva and anus have been reported to harbor HPV-6 compared with cervical cancers (Rando *et al.*, 1986; Kasher and Roman, 1988; Beckman *et al.*, 1989).

Previous analyses by Scheffner *et al.* (1990) have demonstrated that the E6 proteins of HPV-16 or HPV-18 complex with p53 *in vitro* and induce its degradation through the ubiquitin-dependent pathway. HPV-6 E6 did not induce p53 degradation *in vitro* because it was not competent to bind p53 (Scheffner *et al.*, 1990). However, when expressed as an E7 fusion protein, HPV-6 E6 was able to induce degradation of the E7 complexed Rb protein (Scheffner *et al.*, 1992). In contrast Crook *et al.* (1991b) have reported that HPV-6 E6 bound p53 *in vitro* with reduced affinity, but failed to induce p53 degradation. A further complexity is that while BPV-1 E6 is capable of inducing morphologic transformation of the C127 murine cell line (Schiller *et al.*, 1984), it did not bind p53 *in vitro* (Werness *et al.*, 1990). Interestingly, C127 cells were derived from normal mammary tissue of an RIII mouse (Lowy *et al.*, 1978). These genes therefore serve as variants for analyzing the role of p53 in immortalization and correlation of the biological functions of E6 with its *in vitro* activity.

The reduction in the levels of newly synthesized p53 in E6-immortalized mammary epithelial cells was due to a decrease in p53 half-life. A similar decrease in p53 half-life has recently been reported in HPV-16 E6 and E7-immortalized primary keratinocytes (Hubbert *et al.*, 1992; Lechner *et al.*, 1992). In contrast the HPV-6 or BPV-1 E6-transfected cells showed reduced but detectable levels of p53 by immunoprecipitiation before cells went through the 'crisis' phase. After the cells were carried through the 'crisis' stage and became immortal, p53 became barely detectable by immunoprecipitation. On comparing the half-lives of p53 in cells immortalized with different E6 genes, we observed that in all the immortal cells the half-life was drastically

reduced to ~15 min compared with ~3 h in normal epithelial cells. Together with unchanged levels of p53 mRNA, these results provide direct evidence that a decrease in the half-life of p53 protein in mammary epithelial cells immortalized with the E6 genes of HPV-16, HPV-6 and BPV-1 is due to enhanced degradation. Some cervical carcinoma cell lines that contain the E6 gene have very low steady-state levels of p53 (Crook *et al.*, 1991a; Scheffner *et al.*, 1991). Hubbert *et al.* (1992) and Lechner *et al.* (1992) have demonstrated that steady-state levels of p53 are variable in HPV-16 E6 and E7-immortalized keratinocytes despite the fact that the half-life of p53 is also dramatically reduced in these same cells. Our preliminary data indicate that in E6-immortalized mammary cell lines the steady-state levels of p53 were comparable to those found in the parental 76N cells by Western blot analysis (data not shown). These data suggest that only the newly synthesized p53 is being targeted for degradation and this may be critical for immortalization.

The data suggest that the E6 genes of HPV-16, HPV-6 and BPV-1 exhibit similar immortalizing functions in mammary epithelial cells. One possible explanation for these results is that the HPV-6 and BPV-1 E6 genes may interact with and stimulate the degradation of p53 *in vivo*. The efficiency of E6 genes to immortalize mammary epithelial cells might reflect their relative ability to form a complex with p53 *in vitro* (Crook *et al.*, 1991b). An alternative explanation is that E6 may provide another immortalizing function in mammary epithelial cells. One line of evidence that supports this argument is the observation that a mutant p53 gene (valine to alanine at amino acid 143, Baker *et al.*, 1990) did not substitute for HPV-16 E6 in the immortalization assay in mammary epithelial cells (V.Band, unpublished results). Additionally, this mutant p53 gene did not substitute for HPV-16 E6 as a transforming gene in NIH3T3 cells (Sedman *et al.*, 1992). In contrast, in primary keratinocytes the same mutant p53 gene could substitute for E6 in co-transfection with HPV-16 E7 for their immortalization (Sedman *et al.*, 1992). Furthermore, SV40 large T antigen, which binds and presumably inactivates the p53 protein (Lane and Crawford, 1979) does not efficiently immortalize human cells including mammary epithelial cells (O'Brien *et al.*, 1986; V.Band, unpublished data). Taken together these observations suggest that in mammary epithelial cells, E6 may target other protein(s) in addition to its interaction with p53.

The studies reported here show an increasing loss of p53 protein during progression towards immortalization with different E6 genes. Together with the observation that E7-induced loss of Rb function is not required for mammary cell immortalization, these results strongly suggest that p53 performs an important function to regulate cell proliferation and/or senescence in mammary epithelial cells. This conclusion is consistent with the high frequency of p53 mutations or deletions in breast cancers (Bartek *et al.*, 1990; Runnebaum *et al.*, 1991) and the increased susceptibility of Li-Fraumeni syndrome patients with germ line p53 mutations to develop familial breast cancer (Malkin *et al.*, 1990). Understanding the biochemical pathways of p53 function should provide insights into how this protein performs its critical roles in mammary cells. Further analysis in this model may reveal the biochemical basis of the immortalizing function of the E6 oncogene.

## Materials and methods

### *Cells and cell culture*

Derivation of normal epithelial cell strains 76N from reduction mammoplasties has been described previously (Band and Sager, 1989). These cells were grown in DFCI-1 medium (Band and Sager, 1989). HPV and BPV-1 E6-immortalized cells were grown in a modified version of DFCI-1 medium called D2 medium (Band *et al.*, 1990, 1991).

### *Plasmid constructs*

The HPV-16, HPV-6 and the BPV-1 E6 genes were expressed from the β-actin promoter in the p1318 plasmid (obtained from Dr P.Howley). HPV-16 E6 was PCR amplified from HPV-16 E6 genomic DNA (gift of Dr H.zur Hausen) using specific oligonucleotides (5′ ggg gtcgacATGCACCAAAAGAGAAC 3′ which corresponds to nt 83–99 and 5′ cgcgaagcTTACAGCTGGGTTTC 3′ which is complementary to nt 559–545; the lower case letters represent introduced linker sequences). The HPV-16 E6 gene was then cloned as a *Sal*I and *Hin*dIII cassette. The HPV-6 E6 ORF which corresponds to nt 102–554 on the HPV-6 E6 genome and the BPV-1 E6 gene corresponding to nt 91–504 in the BPV-1 genome were cloned into p1318.

### *Antibodies*

Anti-human p53 mAb used in this study and their sources are: PAb 122 ($IgG_2a$ from ATCC). PAb 1801 (IgG1 from Dr Lionel Crawford, ICRF, London). P3 (Kohler and Milstein, 1975, control antibody), rat anti-mouse kappa chain antibody 187.1 (Yelton *et al.*, 1981), and anti-HLA antibody W6/32 (ATCC) were from Dr Hamid Band, Harvard Medical School, Boston, MA.

### *Transfection and selection*

Normal cell strains cultured in DFCI-1 medium were released from culture dishes with trypsin/EDTA and plated at $10^6$ cells/100 mm diameter dish 18 h prior to transfection. 8 μg of linearized plasmid DNA was co-transfected with 2 μg of linearized pSV2neo (to provide a selectable marker) by calcium phosphate co-precipitation. After 6 h, cells were treated with 15% (v/v) glycerol for 4 min, and then fresh medium was added. After 48 h of transfection, we began selection in 50 μg/ml of G418 (GIBCO) and continued for 10 days. The surviving cells were then shifted to D2 medium.

### *Differential PCR*

A differential PCR was performed on genomic DNA isolated from various cells using a mixture of oligonucleotides specific for either HPV-16 E6, HPV-6 E6 or BPV-1 E6. The oligonucleotides were HPV-16 E6: 5′ ggggtcgacATGCACCAAAAGAGAAC 3′and 5′ ACTATGCATAAATCCCG 3′ which is complementary to nt 261–245; HPV-6 E6: 5′ gggggatccgaattcATGGAAAGTGCAAATGC 3′ which corresponds to nt 102–118 and 5′ gtgaagcttggatccTAGGGTAACATGTCTTCC 3′ which are complementary to nt 553–536; BPV-1 E6: 5′ ctctgatcatgGCTGAATTATTGCATGGC 3′ which corresponds to nt 313–330 and 5′ggggtcgaCTATGGGTATTTGGACC 3′ complementary to nt 504–488. The PCR was performed on 2 μg of DNA under standard conditions using *Taq* polymerase (Perkin-Elmer, Cetus) and the products visualized after electrophoresis on a 1.5% agarose gel by staining with ethidium bromide.

### *Sequencing of the HPV-6 E6 gene product*

HPV-6 E6 was PCR amplified from HPV-6 E6-immortalized cells as described above and cloned as a *Bam*HI cassette into pUC19 (NEB). The clones were then sequenced using Sequenase version 2.0 (US Biochemical) and the m13 positive and negative strand primers.

### *Immunoprecipitation of p53 protein*

Exponentially growing cells in 100 mm diameter dishes at 50–70% confluency were washed once with cysteine- and methionine-free αMEM, and then incubated in this medium for 30 min at 37°C. 250 μCi of [$^{35}$S]cysteine and [$^{35}$S]methionine (NEN, Express$^{35}$S$^{35}$S) were added in the same medium and labelling was carried out for 3 h. Cells were washed with cold phosphate-buffered saline (PBS) and lysed in 2 ml/100 mm dish of NETN lysis buffer (20 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% NP40, 1 mM PMSF) (components from Sigma). Lysates were precleared once with *Staphylococcus aureus* Cowan 1 strain (Pansorbin, Calbiochem) and once with protein A–Sepharose 4B (Pharmacia) (200 μl of 10% suspension of each per 2 ml lysate). For specific immunoprecipitations, precleared lysates were incubated with optimal amounts of culture supernatants of different p53 antibodies. For IgG1 antibodies, rat antimouse kappa chain monoclonal antibody 187.1 was added to allow protein A binding. After 1 h at 4°C, 50–75 μl of 10% suspension

of protein A–Sepharose were added, and incubation continued for 45 min at 4°C. Immune complexes were washed six times in lysis buffer, boiled in SDS–PAGE sample buffer with 5% 2-mercaptoethanol and resolved on SDS–7.5% polyacrylamide gels.

***Sequencing of the p53 gene from HPV E6-immortalized cells***

Ten μg of total cellular RNA from the HPV E6-immortalized cell lines (H16E6-P, H6E6-P) were used as a template to synthesize cDNA using reverse transcriptase. Five sets of oligonucleotide primers were used to generate overlapping p53 specific PCR products, which were cloned into the M13mp18 and M13mp19 vectors. At least 10 single-stranded DNA templates were sequenced for each fragment using M13 specific primers and Sequenase as described above. The primer combinations used for PCR and their positions in the p53 coding sequence (Lamb and Crawford, 1986) are as follows: 1. sense: 5′-ggggatccATTGGCAGCCAGACTGCC-3′(# −32 to −15), antisense: 5′-gggaattcAGGGACAGAAGATGACAG-3′ (#294–277); 2. sense: 5′-ggggatccGATGAAGCTCCCAGAAT-3′(# 181 to 197), antisense: 5′-gggaattcCTGCTTGTAGATGGCCAT-3′ (#495–478); 3. sense: 5′-ggggatccGTGCAGCTGTGGGTTGAT-3′ (#427–444), antisense: 5′-gggaattcTCCCAGTAGATTACCACT-3′ (#798–781); 4. sense: 5′-ggggatccATCACACTGGAAGACTCC-3′ (#763–790), antisense: 5′-gggaattcACGGATCTGAAGGGTGAA-3′(#999–982); 5. sense: 5′-ggggatccAAACCACTGGATGGAGAA-3′ (#961–978), antisense: 5′-ggggaatTCAGTCTGAGTCAGGCC-3′ (#1182–1166).

***RNA isolation and analysis***

Total RNA was isolated from 50–60% confluent cell monolayers, and Northern (RNA) blot hybridizations were carried out as described previously (Band *et al.*, 1990b).

***Pulse–chase for half-life determination***

Exponentially growing cells were starved in cysteine- and methionine-free medium for 30 min, and labelled as above with [$^{35}$S]methionine and [$^{35}$S]cysteine for 15 min at 37°C. After 15 min, cells were washed twice with cold PBS, and then incubated with complete medium (DFCI-1, D2 or α-MEM) supplemented with unlabelled L-cystine (120 μg/ml) and L-methionine (75 μg/ml) (GIBCO) for various time periods. At each time interval, cells were washed with cold PBS and lysed in NETN (2 ml/100 mm dish), precleared and equal c.p.m. of lysates were immunoprecipitated and resolved on SDS–polyacrylamide gels as above.

## Acknowledgements

We thank Drs P.Howley, L.Crawford, H.zur Hausen and H.Band for critical reagents; Dr Band for help in sequencing and immunoprecipitations, Dr C.Lamberti for assistance in cloning the HPV E6 genes, and Ms S.Bembenek for editing the manuscript and for art work. This work was supported in part by S07-RR05598-24 awarded by the Biomedical Research Support Program, Division of Research Resources, NIH and NIH R29 CA56803-01 (to V.Band) and R01-CA44174 and American Cancer Society FRA #393 to E.A.

## References

Baker,S.J., Markowitz,S., Fearon,E.R., Willson,J.K.V. and Vogelstein,B. (1990) *Science*, **249**, 912–915.

Band,V., De Caprio,J.A., Delmolino,L., Kulesa,V. and Sager,R. (1991) *J. Virol.*, **65**, 6671–6676.

Band,V. and Sager,R. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 1249–1253.

Band,V., Zajchowski,D., Swisshelm,K., Trask,D., Kulesa,V., Cohen,C., Connolly,J. and Sager,R. (1990a) *Cancer Res.*, **50**, 7351–7357.

Band,V., Zajchowski,D., Kulesa,V. and Sager,R. (1990b) *Proc. Natl. Acad. Sci. USA*, **87**, 463–467.

Barbosa,M.S. and Schlegel,R. (1989) *Oncogene*, **4**, 1529–1532.

Barbosa,M.S., Vass,W.C., Lowy,D.R. and Schiller,J.T. (1991) *J. Virol.*, **65**, 292–298.

Bartek,J., Iggo,R., Gannon,J. and Lane,D.P. (1990) *Int. J. Cancer*, **46**, 839–844.

Beckman,A.M., Daling,J., Sherman,K., Maden,C., Miller,B., Coates,R., Kiviat,N., Myerson,D., Weiss,N., Hislop,T., Beagrie,M. and McDougall,J.K. (1989) *Int. J. Cancer*, **43**, 1042–1049.

Bedell,M.A., Jones,K.H. and Laimins,L.A. (1987) *J. Virol.*, **61**, 3635–3640.

Crawford,L.V., Pim,D. and Lamb,P. (1984) *Mol. Biol. Med.*, **2**, 261–272.

Crook,T., Wrede,D., Tidy,J., Scholefield,J., Crawford,L. and Vousden,K.H. (1991a) *Oncogene*, **6**, 1251–1257.

Crook,T., Tidy,J.A. and Vousden,K.H. (1991b) *Cell*, **67**, 547–556.

Halbert,C.L, Demers,G.W. and Galloway,D.A. (1991) *J.Virol.*, **65**, 473–478.

Halbert,C., Demers,G.W. and Galloway,D.A. (1992) *J. Virol.*, **66**, 2125–2134.

Hawley-Nelson,P., Vousden,K.H., Hubbert,N.L., Lowy,D.R. and Schiller,J.T. (1989) *EMBO J.*, **8**, 3905–3910.

Hubbert,N.L., Sedman,S.A. and Schiller,J.T. (1992) *J. Virol.*, **66**, 6237–6241.

Hudson,J.B., Bedell,M.A., McCance,D.J. and Laimins,L.A. (1990) *J. Virol.*, **64**, 519–526.

Kasher,J.S. and Roman,A. (1988) *Virology*, **165**, 225–233.

Kaur,P. and McDougall,J.K. (1988) *J. Virol.*, **62**, 1917–1924.

Kohler,G. and Milstein,C. (1975) *Nature*, **256**, 495–497.

Lamb,P. and Crawford,L. (1986) *Mol. Cell. Biol.*, **6**, 1379–1385.

Lane,D. and Crawford,L. (1979) *Nature*, **278**, 261–263.

Lechner,M.S., Mack,D.H., Finicle,A.B., Crook,T., Vousden,K.H. and Laimins,L.A. (1992) *EMBO J.*, **11**, 3045–3052.

Levine,A.J. and Momand,J. (1990) *Biochim. Biophys. Acta*, **1032**, 119–136.

Lowy,D.R., Androphy,E.J. and Scolnick,F.M. (1978) *J. Virol.*, **26**, 291–298.

Malkin,D., Li,F.P., Strong,L.C., Fraumeni,J.F., Nelson,C.E., Kim,D.H., Kassel,J., Gryka,M.A., Bischoff,F.Z., Tainsky,M.A. and Friend,S.H. (1990) *Science*, **250**, 1233–1238.

Matlashewski,G., Schneider,J., Banks,L., Jones,N., Murray,A. and Crawford,L. (1987) *EMBO J.*, **6**, 1741–1746.

Münger,K., Phelps,W.C., Bubb,V., Howley,P.M. and Schlegel,R. (1989a) *J. Virol.*, **63**, 4417–4421.

Münger,K., Werness,B.A., Dyson,N., Phelps,W.C., Harlow,E. and Howley,P.M. (1989b) *EMBO J.*, **8**, 4099–4105.

O'Brien,W., Stenman,G. and Sager,R. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 8659–8663.

Perez-Reyes,N., Halbert,C.L., Smith,P.P., Benditt,E.P. and McDougall,J.K. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 1224–1228.

Rando,R.F., Groff,D.E., Chirikjian,J.G. and Lancaster,W.D. (1986) *J. Virol.*, **57**, 353–356.

Runnebaum,I.B., Nagarajan,M., Bowman,M., Soto,D. and Sukumar,S. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 10657–10661.

Scheffner,M., Münger,K., Byrne,J. and Howley,P.M. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 5523–5527.

Scheffner,M., Münger,K., Huibregtse,J.M. and Howley,P.M. (1992) *EMBO J.*, **11**, 2425–2431.

Scheffner,M., Werness,B.A., Huibregtse,J.M., Levine,A.J. and Howley,P.M. (1990) *Cell*, **63**, 1129–1136.

Schiller,J.T., Androphy,E.J., Vass,W.C. and Lowy,D.R. (1986) *Cancer Cells/DNA Tumor Viruses*, **4**, 571–579.

Schiller,J.T., Vass,W.C. and Lowy,D.R. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 7880–7884.

Sedman,S.A., Hubbert,N.L., Vass,W.C., Lowy,D.R. and Schiller,J.T. (1992) *J. Virol.*, **66**, 4201–4208.

Steger,G. and Pfister,H. (1992) *Arch. Virol.*, **125**, 355–360.

Werness,B.A., Levine,A.J. and Howley,P.M. (1990) *Science*, **248**, 76–79.

Yelton,D.E., Desaymard,C. and Scharff,M.D. (1981) *Hybridoma*, **1**, 5–11.

zur Hausen,H.(1989) In Klein,G. (ed.), *Advances in Viral Oncology*, Raven Press, Ltd, New York, Vol. 8, pp. 1–26.

*Received on November 9, 1992; revised on December 17, 1992*