Expression of the papillomavirus E2 protein in HeLa cells leads to apoptosis

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The papillomavirus E2 protein plays a central role in the viral life cycle as it regulates both transcription and replication of the viral genome. In this study, we showed that transient expression of bovine papillomavirus type 1 or human papillomavirus type 18 (HPV18) E2 proteins in HeLa cells activated the transcriptional activity of p53 through at least two pathways. The first one involved the binding of E2 to its recognition elements located in the integrated viral P_{105} promoter. E2 binding consequently repressed transcription of the endogenous HPV18 E6 oncogene, whose product has been shown previously to promote p53 degradation. The second pathway did not require specific DNA binding by E2. Expression of E2 induced drastic physiological changes, as evidenced by a high level of cell death by apoptosis and G_1 arrest. Over-expression of a p53 *trans*-dominant-negative mutant abolished both E2-induced p53 transcriptional activation and E2-mediated G_1 growth arrest, but showed no effect on E2-triggered apoptosis. These results suggest that the effects of E2 on cell cycle progression and cell death follow distinct pathways involving two different functions of p53.
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Introduction

Papillomaviruses are small DNA viruses which induce proliferative lesions of the skin and mucosa. The viral E2 open reading frame (ORF) encodes a protein which regulates viral transcription when bound to a palindromic DNA sequence present in several copies in the regulatory region of all papillomaviruses (reviewed by Ham *et al.*, 1991b). E2 also acts, in concert with E1 and cellular replication factors, in the initiation of viral DNA replication (Ustav and Stenlund, 1991; Chiang *et al.*, 1992; Del Vecchio *et al.*, 1992). The E2 protein contains two domains of relatively high amino acid conservation, separated by a non-conserved region of variable length called the 'hinge' (Giri and Yaniv, 1988). The amino-terminal part of E2 is required for transactivation, replication and association with E1 (Benson and Howley, 1995), while the carboxy-terminal region is responsible for the dimeriz-

ation of the protein and its specific binding to DNA (Androphy *et al.*, 1987; Dostatni *et al.*, 1988; McBride *et al.*, 1989).

Bovine papillomavirus type 1 (BPV-1) has been studied extensively as a model for papillomavirus replication, transcription and cell transformation. Viral gene expression is controlled by seven promoters, four of which are activated by the full-length 48 kDa E2 protein. BPV-1 also encodes two truncated forms of E2 of 28 and 31 kDa (Lambert *et al.*, 1989). An alternative splicing gives rise to the 28 kDa protein. The 31 kDa product (E2TR) is generated by transcription initiated upstream of an internal ATG codon, and lacks most of the N-terminal transactivation domain. It antagonizes E2-activated transcription by competitive binding to its recognition element and/or heterodimerization (Lambert *et al.*, 1987; Ham *et al.*, 1991b).

In humans, >70 types of papillomavirus (HPVs) have been isolated, a third of which infect the genital tract (de Villiers, 1994). Genital HPVs can be classified into two categories: the 'high risk' types, such as HPV16, 18, 31 and 33, are associated with carcinomas, while the 'low risk' HPVs, such as HPV 6, 11 and 13, cause benign condylomas. In HPV18, the viral transforming genes E6 and E7 are expressed from a single promoter, termed P_{105}, which is regulated by an upstream 800 bp long control region (LCR) (Thierry *et al.*, 1987a). This region contains a keratinocyte-specific enhancer, upstream of promoter elements (TATA and Sp1) crucial for transcription (Garcia-Carranca *et al.*, 1988). Four E2 binding sites are located in the LCR, two of which lie within the promoter, tightly flanked 5' by the Sp1 and 3' by the TATA motifs.

The HPV18 promoter has been shown to be strongly repressed by the BPV-1 E2 and E2TR proteins which presumably hinder the formation of the transcription initiation complex (Thierry and Yaniv, 1987; Dostatni *et al.*, 1991). A moderate repression is also observed with the homologous HPV E2 products (Bernard *et al.*, 1989; Thierry and Howley, 1991; Demeret *et al.*, 1994). The E2 function is almost invariably lost during carcinogenic progression as a result of the viral DNA integration into the cellular genome and the concomitant disruption of the E2 ORF (Schwarz *et al.*, 1985; Berumen *et al.*, 1994). Loss of E2 is believed to increase the level of E6 and E7, whose continuous expression is necessary to maintain the transformed state of the cells (Bosch *et al.*, 1990). These two viral proteins induce cell transformation by neutralizing tumour suppressor proteins which control cell proliferation. E7 binds to the hypophosphorylated form of the retinoblastoma (Rb) protein (Dyson *et al.*, 1989), while E6 associates with the cellular p53 protein and induces its degradation through the ubiquitin pathway (Scheffner *et al.*, 1990). p53 is a transcription factor which binds specific DNA sequences identified in the promoters of

several target genes (Farmer *et al.*, 1992; Funk *et al.*, 1992; Kastan *et al.*, 1992; Barak *et al.*, 1993; El-Deiry *et al.*, 1993). Its level rises following a genotoxic stress, leading the cell either to arrest at the G_1/S transition or to commit apoptosis (Diller *et al.*, 1990; Yonish-Rouach *et al.*, 1991; Kuerbitz *et al.*, 1992; Eizenberg *et al.*, 1995).

In the present study, we showed that transient expression of BPV-1 and HPV18 E2 proteins in the HPV18-positive HeLa cell line activated co-transfected p53-inducible reporter plasmids. Analysis of mutant E2 proteins indicated that the E2-induced activation of endogenous p53 transcriptional activity could be mediated by two different pathways, one of which is independent of the transcriptional repression of the endogenous viral E6 and E7 genes. At the cellular level, we observed that E2 could trigger apoptosis independently of p53 transcriptional activity.

Results

HPV18 and BPV-1 E2 proteins repress endogenous E6/E7 transcription in HeLa cells

It has been shown previously in co-transfection experiments that the HPV18 and BPV-1 E2 proteins repress the activity of a reporter gene transcribed from the HPV18 P_{105} promoter (Thierry and Yaniv, 1987; Thierry and Howley, 1991; Demeret *et al.*, 1994). In HeLa cells, several copies of HPV18 DNA are integrated into the genome. The P_{105} promoter constitutively transcribes the E6 and E7 oncogenes, as illustrated schematically in Figure 1. These cells do not express the endogenous E2 protein as a result of an interruption in the E1–E2 ORFs (Schwarz *et al.*, 1985). In the present study, we investigated whether the ectopically expressed E2 protein was able to repress endogenous E6/E7 transcription in HeLa cells. Our main concern was that the HPV18 regulatory region integrated into the genomic DNA could present a chromatin structure different from that adopted on a transiently transfected plasmid, leading to a modified access of E2 to its cognate DNA sequences.

To make such an experiment feasible, successfully transfected HeLa cells were selected by cell sorting for their immunoreactivity towards a surface antigen (the MHC class I H2Kd molecule) which was co-expressed with E2. Primer extension analysis was performed with an E6-specific probe on the total RNA extracted from H2Kd-positive cells (Figure 1). Both HPV18 (lane 5) and BPV-1 (lane 3) E2 reduced the steady-state level of E6-specific RNA initiated at the P_{105} promoter (upper arrows). A comparable level of repression was obtained with the truncated form of BPV-1 E2 (E2TR), which lacks most of the amino-terminal activation domain (lane 2). No decrease in the RNA levels of the β-actin control gene was observed. These results indicate that E2 and its deleted counterpart E2TR can both repress endogenous E6/E7 transcription, supporting the conclusions previously drawn from co-transfection experiments.

E2 increases p53 transcriptional activity in HeLa cells

HeLa cells contain two wild-type p53 alleles but, despite the abundance of p53 transcripts in these cells, the encoded protein is not detectable because it is degraded by a ubiquitin-mediated pathway activated by E6 (Scheffner
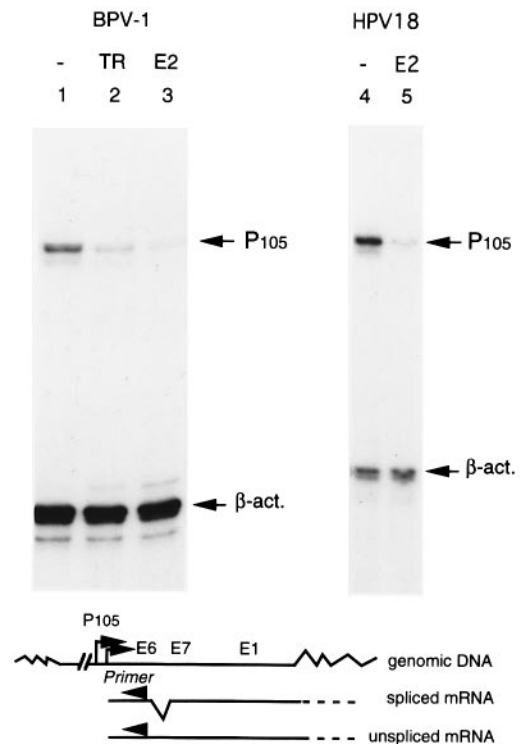

Fig. 1. Both HPV18 and BPV-1 E2 proteins repress the endogenous HPV18 P_{105} promoter in HeLa cells. HeLa cells in 10 cm plates were transfected with 1 μg of the H2Kd expression plasmid and 1 μg of vectors coding for either BPV-1 E2 (lane 3), BPV-1 E2TR (lane 2), HPV18 E2 (lane 5) or the negative control E1TTL (lanes 1 and 4). Total RNA extracted from transfected cells was reverse transcribed with a primer hybridizing upstream of the splice site in the E6 ORF, as schematically shown at the bottom. The upper arrow points to the 129 nucleotide E6/E7-specific product of the primer extension. A primer complementary to the 5′-end of the β-actin mRNA, included as an internal control in each reaction, gave rise to a 93 nucleotide reverse transcription product (lower arrow), demonstrating that equivalent amounts of total RNA were used in each lane.

et al., 1991). However, endogenous transcriptionally active p53 can be activated when these cells are exposed to genotoxic agents (Butz *et al.*, 1995). Since E2 could repress E6 expression, we next investigated whether this resulted in stabilization of p53. The activity of p53 was assayed via co-transfected p53-responsive CAT reporter plasmids. As shown in Figure 2A, expression of full-length BPV-1 E2 resulted in increased CAT activity from plasmids driven by the naturally p53-responsive MDM2 promoter, or a synthetic promoter flanked by 13 binding sites for p53 (PG13-CAT). To confirm that the activation of PG13-CAT was indeed mediated by p53, we co-transfected a plasmid encoding a 17 kDa product corresponding to the carboxy-terminal part of p53 (c-p53). This truncated p53 protein was immunodetected by the p53-specific monoclonal antibody Ab421, which also recognized full-length p53 protein in cells transfected with a p53 expression vector (Figure 2B). Such short truncated products act as *trans*-dominant-negative mutants by forming oligomers with the wild-type protein and consequently inactivate the transactivation and tumour suppressor functions of p53 (Shaulian *et al.*, 1992). Expression of c-p53 (Figure 2C) indeed completely abolished the E2-induced p53 transcriptional activity, confirming that the effect of E2 on PG13-CAT was mediated by p53.
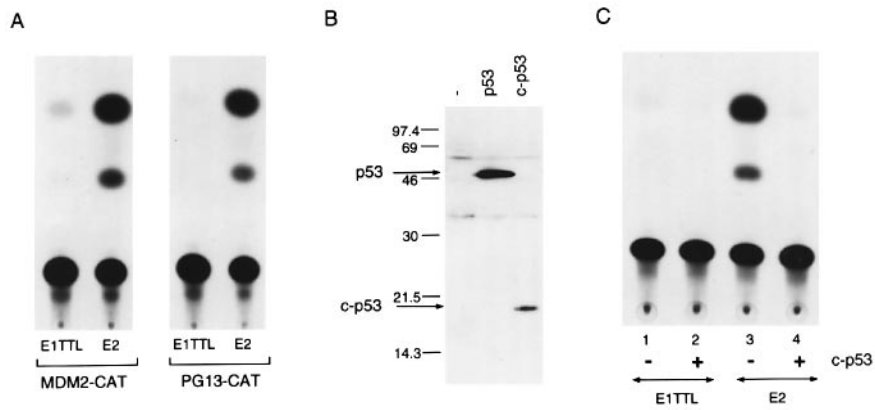
Fig. 2. E2 activates p53-responsive promoters. (**A**) Representative CAT assay performed with extracts from HeLa cells transiently transfected with 2 µg of CAT reporter plasmids containing the MDM2 promoter or the polyomavirus early promoter flanked by 13 p53 binding sites (PG13-CAT), and 0.2 µg of plasmids expressing BPV-1 E2. The assay with MDM2-CAT was carried out with 15 times less extract than that with PG13-CAT. Relative CAT activities were 34, 319, 1 and 40 for MDM2 + E1TTL, MDM2 + E2, PG13 + E1TTL and PG13 + E2, respectively. (**B**) Western blot. HeLa cells were co-transfected with 1 µg of expression vectors for p53, a C-terminal fragment of p53 (c-p53) or vector alone (−). Total cell proteins were probed with the Ab421 p53-specific monoclonal antibody. The arrows point to the full-length p53 protein and c-p53. (**C**) CAT assay. HeLa cells were co-transfected with 2 µg of PG13-CAT with 0.5 µg of expression vectors for BPV-1 E2 (lanes 3 and 4) or E1TTL (lanes 1 and 2) along with 1 µg of a plasmid encoding the *trans*-dominant-negative c-p53 (lanes 2 and 4) or vector alone (lanes 1 and 3). CAT assays were performed with 1/5 of the cellular extracts for 1 h.
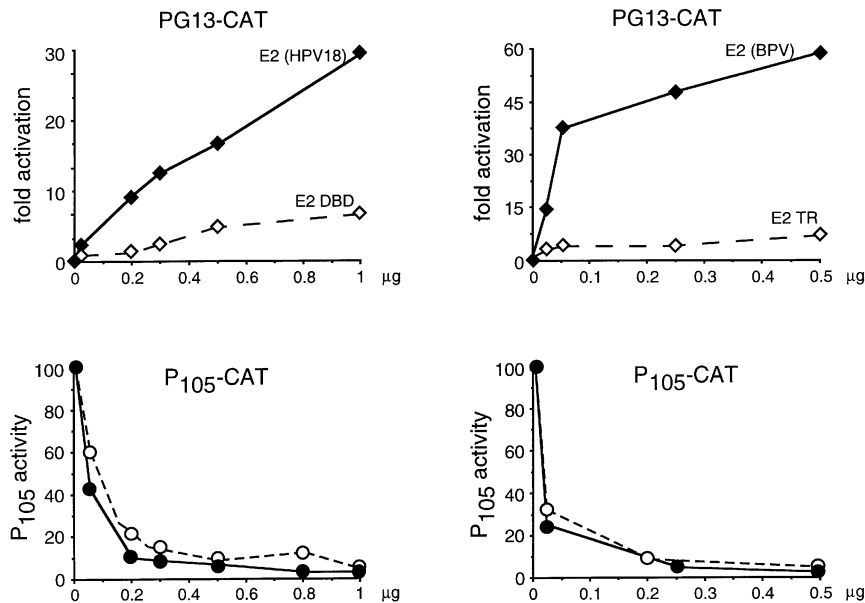

Fig. 3. Differential activation of PG13-CAT by full-length and amino-terminally truncated E2. Increasing amounts of plasmids expressing HPV18 (left panel) or BPV-1 (right panel) full-length E2 (filled symbols) or the amino-terminally truncated forms of E2 (open symbols) were transfected into HeLa cells with 2 µg of PG13-CAT (upper panels) or P_{105}-CAT (lower panels). Values representing the mean of at least 3–8 independent transfections were calculated relative to the CAT activities in the presence of the pCGE1TTL negative plasmid.

E2 and E2TR differ in their abilities to induce p53

Both the full-length and truncated E2 proteins repress E6 expression with comparable efficiencies (Figure 1). If E2 activates p53 solely by repressing endogenous E6 transcription, then both forms of E2 should be able to induce high levels of p53-dependent transcriptional activation. Surprisingly, this was not the case, as illustrated in Figure 3. The full-length HPV18 (left upper panel) and BPV-1 (right upper panel) E2 proteins increased the activity of PG13-CAT up to 30- and 60-fold, respectively, in a dose-dependent manner. The short forms produced only a moderate activation of PG13-CAT (up to 5- and 7-fold for HPV18 and BPV-1, respectively). Under these conditions, however, the truncated forms of E2 (lower

panels, open circles), HPV18 E2DBD or BPV-1 E2TR, repressed in a dose-dependent manner, as efficiently as their full-length counterparts (lower panels, filled circles), the CAT activity of a P_{105} reporter plasmid. In the range between 0.2 and 0.8 µg of E2 expression plasmids, P_{105} activity was repressed still further ~3-fold by both full-length and truncated E2 proteins, although this effect was minimized by the graphic representation. Repression specifically depended on the interaction between E2 and its target DNA sequence, as indicated by two additional experiments. First, mutation of the three most proximal E2 binding sites within the P_{105} promoter completely abolished repression by E2 (data not shown). Second, a point mutation destroying the DNA binding capacity of
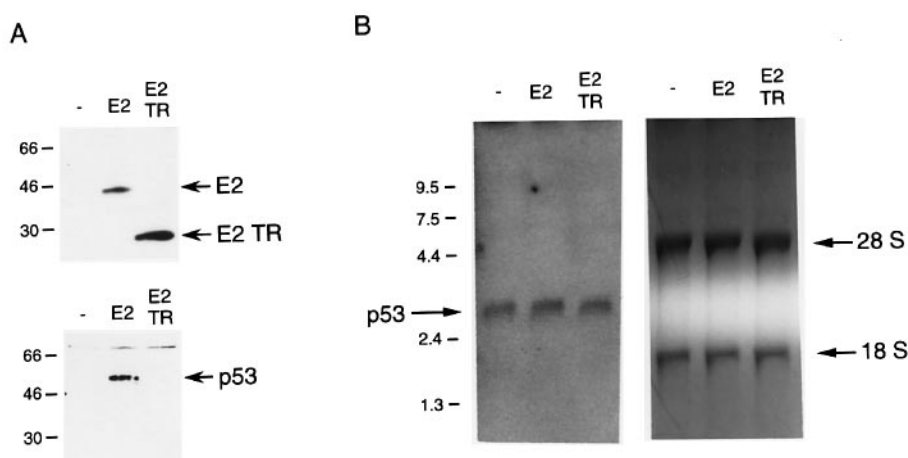
Fig. 4. E2 increases the levels of the p53 protein without affecting transcription of the p53 gene. HeLa cells were transfected with 0.5 µg of an H2Kd expression plasmid and 1.5 µg of vectors coding for E1TTL (–), BPV-1 E2 or BPV-1 E2TR. The population of transfected cells was enriched by sorting the H2Kd-positive cells. Half of the cells were resuspended in Laemmli buffer for protein analysis, while the other half was used to extract RNA. (**A**) Western blots. Membranes were incubated with an E2-specific antiserum (upper panel) or the p53 Ab1801 antibody (lower panel). (**B**) Northern blot. Five µg of RNA were resolved on a 1% MOPS–agarose gel. After transfer, the membrane was incubated with a ^{32}P-labelled p53 probe. In the left panel, the arrow points to p53 transcripts. The positions of the RNA molecular weight markers are indicated on the left. The photograph of the gel in the right panel shows that equivalent amounts of 28S and 18S RNAs were present in the three lanes.

Table I. Moderate enhancement of p53 activity by E2 in HPV-negative cell lines

Cells	Relative CAT activities PG13-CAT/MG13-CAT	Fold activation of PG13-CAT by p53	Fold activation of PG13-CAT by E2
HeLa	5	100	30–60 ($n = 30$)
HepG2	100	2	2 ($n = 5$)
NIH3T3	8	10.6	2.4 ($n = 6$)
C33	0.3	12.4	3[a] ($n = 2$)
SW13	1	3.3	4.5[a] ($n = 7$)
HaCaT	1	50	3[a] ($n = 9$)
SAOS	1	50	2.2[a] ($n = 2$)

Cells were transfected with MG13-CAT or PG13-CAT together with plasmids encoding the negative control E1TTL, E2 or human wild-type p53. The level of endogenous p53 transcriptional activity was determined by comparing the CAT activities in cells transfected with PG13-CAT or MG13-CAT. PG13-CAT activation by E2 or p53 was calculated relative to the negative control E1TTL. Mean values of activities (first column) or fold activation are indicated. n represents the number of independent transfection experiments.
[a]In p53-negative cell lines, a human p53 expression plasmid was co-transfected with PG13-CAT and pCGE2 or pCGE1TTL.

the BPV-1 E2 protein impaired the repression of P_{105} activity (data not shown). The difference between full-length and truncated forms of E2 in p53 activation did not result from a difference in their level of expression, since Western blots showed that both products were present at similar concentrations in transfected cells (Figure 4A, upper panel). We also checked that in extracts of transfected cells, both E2 proteins bound target DNA sequences with similar efficiencies (data not shown). The difference in activation of p53 transcriptional activity was correlated with the steady-state level of the p53 protein, as judged by Western blot analysis (Figure 4A, lower panel). This accumulation of p53 protein did not result from a transcriptional activation of the gene, since the amounts of p53 RNA did not vary in cells expressing both E2 products as compared with cells transfected with the negative control vector (Figure 4B). These data thus show that E2 activates p53 at the post-transcriptional level and that this is not only due to the decrease of the endogenous E6-specific mRNA. Repression of the P_{105} promoter (achieved by both full-length and N-terminal-deleted forms of E2) is not sufficient to confer full p53 activation. This suggests that another function of E2, which probably requires the N-terminal transactivation domain, is also involved in the induction of p53.

Two domains of E2 functionally complement each other to activate p53-mediated transcription

Since the DNA binding domain of E2 alone was not able to fully activate the transcriptional activity of p53, we examined whether E2 could activate p53 independently of its sequence-specific binding. For this purpose, we used a mutant BPV-1 E2 protein (E2K344), in which the arginine residue at position 344 has been changed to a lysine. This mutation in the α-helical recognition segment of the carboxy-terminal domain (Hegde *et al.*, 1992) abolishes DNA binding without altering the dimerization of the protein (Dowhanick *et al.*, 1995). We confirmed by immunofluorescence that this protein accumulated normally in the nucleus (data not shown). At optimal concentrations, E2K344 induced PG13-CAT up to 20-fold (Figure 5A). This activation was 3-fold lower and 7-fold higher than that observed with wild-type E2 and E2TR, respectively. However, co-expression of 0.2 µg of E2TR with increasing doses of E2K344 resulted in a synergistic activation (up to 45-fold) of PG13-CAT activity. Compared
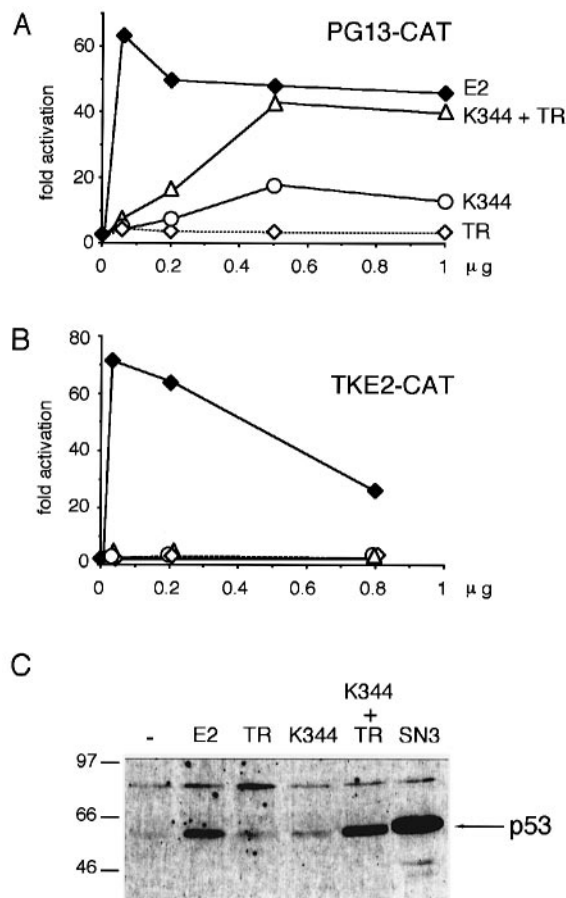
Fig. 5. The E2 activation and DNA binding domains contribute synergistically to the activation of p53. HeLa cells were transfected with increasing doses of vectors expressing E2 (◆), E2TR (◇), E2K344, either alone (○) or in combination with 0.2 μg of E2TR (△), along with 2 μg of reporter plasmids PG13-CAT (**A**) or TKE2-CAT (**B**). pCGE1TTL was used as a negative control. CAT assays were performed with cellular extracts from cells harvested 44 h post-transfection. Values expressed relative to E1TTL from one typical experiment are shown. (**C**) Western blot performed with 1/5 of extracts from cells transfected with 0.2 μg of vectors encoding E1TTL, E2, E2TR, human p53 (SN3) or 0.8 μg of pCGE2K344 either alone or in combination with 0.2 μg of pCGE2TR. p53 was detected with Ab1801. Molecular weight markers are indicated on the left in kDa.

with the wild-type E2, the activation obtained by the combination of the two mutated E2 proteins required a higher input of expression plasmid. Activation reached equivalent levels at concentrations >0.5 μg. The loss of DNA binding activity of the E2K344 mutant was confirmed by its inability to activate transcription from TKE2-CAT (a reporter plasmid containing six E2 binding sites upstream of the herpes simplex virus thymidine kinase promoter) under conditions in which wild-type E2 activated transcription up to 70-fold (Figure 5B). Moreover, co-expression of E2TR + E2K344 did not activate TKE2-CAT, indicating that the two proteins did not heterodimerize to reconstitute an active sequence-specific transactivator. The synergistic activation of PG13-CAT by E2K344 and E2TR was correlated with an increase in the level of the p53 protein (Figure 5C). Indeed the steady-state level of p53 was much higher in cells expressing both E2TR and E2K344 together, as compared with cells transfected with the negative control vector or any one of the two

expression plasmids alone. p53 protein was lower than that accumulating in cells transfected with a plasmid encoding a human wild-type p53 protein (SN3). These results further support our assumption that E2-induced activation of p53 is mediated by two independent pathways, one of which is independent of the down-regulation of E6/E7 transcription.

We next looked at whether E2 could modulate p53 activity in HPV-negative cell lines (Table I). We used seven different cell lines which can be divided into two categories according to their p53 status. HeLa, HepG2 and NIH3T3 cells contain wild-type p53 alleles, while C33, SW13 and HaCaT cells express a mutated form of p53, and SAOS cells do not express any p53. The presence of transcriptionally active endogenous p53 was confirmed by comparing the activity of PG13-CAT with that of MG13-CAT (similar to PG13-CAT except that the p53 binding elements have been mutated). Expression of E2 in HepG2 and NIH3T3 cells resulted in a 2- and 2.4-fold increased activity of the PG13-CAT p53 reporter plasmid, respectively. By contrast, E2 did not induce any activation of PG13-CAT in the p53-negative cells (data not shown), in conditions in which exogenous p53 induced a weak (SW13), moderate (C33) or strong (HaCaT, SAOS) activation of PG13-CAT. We therefore introduced exogenous wild-type human p53 to monitor the effect of E2 on PG13-CAT activity. Under these conditions, E2 could activate PG13-CAT transcriptional activity reproducibly by 2.2- to 4.5-fold in the four different cell lines. In all cases, we checked that E2 expression induced at least a 50-fold activation of the E2 reporter plasmid TKE2-CAT. We deduced from these experiments that E2 could potentiate p53 transcriptional activity in HPV-negative cell lines, albeit at a much lower level than that achieved in HeLa cells.

E2 induces apoptosis
We had noted previously the impossibility of generating HeLa clones stably expressing E2 (Thierry and Yaniv, 1987). In addition, at early times after transfection, E2 appeared to exert some toxic effect on the cells (unpublished observations). Since under certain circumstances the activation of p53 has been shown to induce apoptosis (Diller *et al.*, 1990; Yonish-Rouach *et al.*, 1991), we therefore examined whether E2 expression would trigger cell death.

Forty hours after transfection, successfully transfected HeLa cells were gated with the H2Kd fluorescein isothiocyanate (FITC)-coupled antibody, and analysed for their DNA content by flow cytometry. Figure 6A shows that the sub-2n population of E2-expressing cells was 3-fold greater than that of cells transfected with the negative control plasmid (E1TTL) or a vector expressing E2TR. This population presumably corresponds to cells which have lost genomic material following cell death. The effect of E2 on the cell and nuclear morphologies was examined by immunofluorescence (Figure 6B). In panels 1–3, successfully transfected cells appear green as a consequence of their labelling with the anti-H2Kd antibody conjugated with FITC. A sub-fraction (shown by arrows) of the transfected cells expressing full-length E2 (panel 3) or the mutant E2K344 (panel 2) exhibited a dramatic reduction in the size of their cytoplasm and nuclei. In addition,
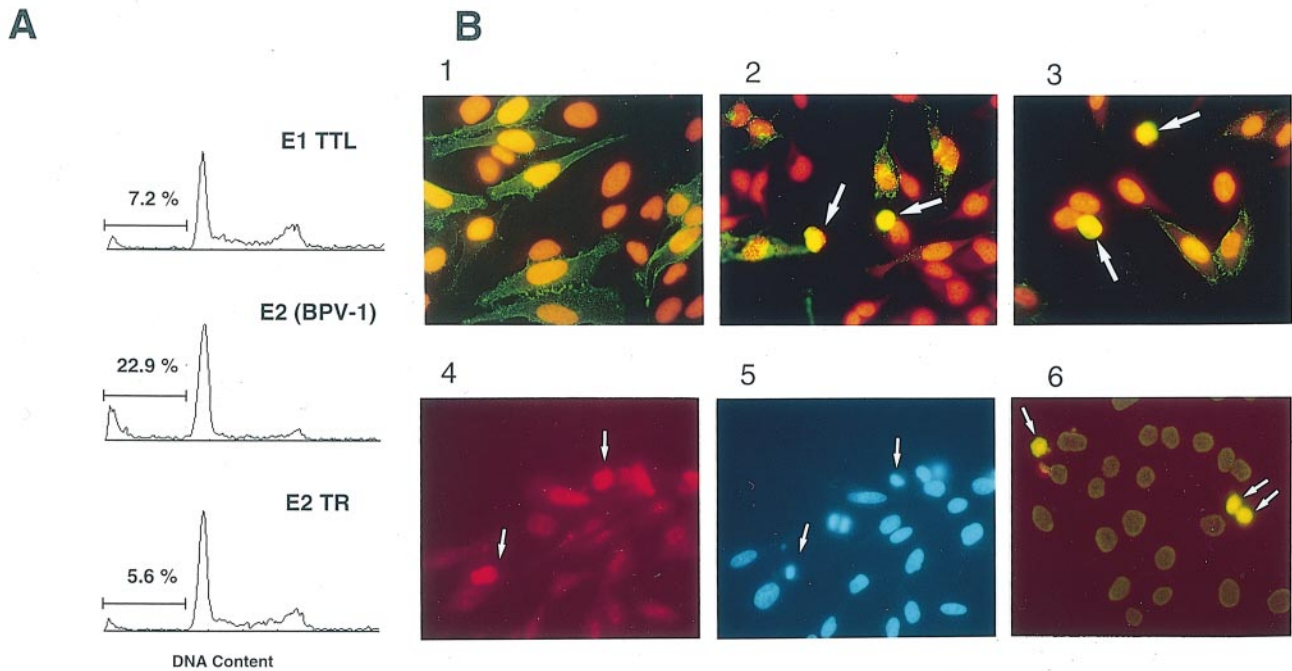
Fig. 6. Role of E2 in cell death. HeLa cells grown on 10 cm dishes (A) or on coverslips (B) were transfected with 1.5 µg of pCGE1TTL, pCGE2 or pCGE2TR, and 0.5 µg of H2Kd expression plasmid. (**A**) Transfected cells were gated for analysis of their DNA content by flow cytometry. The proportion of sub-2n DNA is indicated on the left. (**B**) Representative images of cells expressing E2TR (panel 1), E2K344 (panel 2) or E2 (panels 3–6). The nuclei were stained with propidium iodide (panels 1–3). H2Kd-positive cells were stained in green by the anti-H2Kd antibody coupled to FITC (panels 1–3). Panels 4 and 5 represent the same section of cells stained either with a Texas red-coupled antibody detecting the E2 protein (panel 4) or with DAPI (panel 5). An example of TUNEL-positive nuclei is shown in panel 6. Arrows point to transfected cells which show abnormal figures.

these cells were detaching from the surface of the coverslip, and often appeared in a different focal plane from that in which the normal neighbouring cells lie. These abnormalities were not observed in cells transfected with E2TR (panel 1). E2 expression was also visualized by direct staining with an anti-E2 antibody coupled to Texas red (panel 4). The corresponding staining of these cells with 4,6-diamidino-2-phenylindole (DAPI) shows two E2-positive cells with condensed chromatin (arrows in panel 5). In addition, cells transfected with E2 expression vectors contained DNA breaks which were labelled by the TUNEL assay (panel 6). TUNEL-positive cells represented 7.5, 12 and 50% of the proportion of the H2Kd-positive cells expressing E1TTL, E2TR and E2, respectively. Expression of the HPV18 E2 protein also led to apoptosis, as judged by chromatin condensation, sub-2n DNA content and TUNEL reaction (data not shown). These observations suggest that E2 induces cell death through apoptosis. Cell death was not caused non-specifically by overexpression of a nuclear protein, since the characteristic features of apoptosis (cell death, DNA fragmentation and chromatin condensation) were not above the background level in E2TR-expressing cells.

p53 transcriptional activity is required for E2-induced G_1 growth arrest, but is dispensable for E2-mediated apoptosis

Elevation of p53 levels either blocks G_1 progression (El-Deiry et al., 1993; Xiong et al., 1993; Harper et al., 1995) or induces apoptosis (Diller et al., 1990; Yonish-Rouach et al., 1991). It has been shown previously that E2 expression arrests HeLa and HT3 cells in G_1 (Hwang et al., 1993; Dowhanick et al., 1995). To explore further the role of p53 in both E2-induced apoptosis and G_1 growth arrest, we inhibited the transcriptional activity of p53 by co-expressing c-p53 (trans-dominant-negative mutant of p53) in the presence of E2.

Forty hours after transfection, cells were labelled with propidium iodide and analysed by flow cytometry for their DNA content. Successfully transfected cells were gated with a FITC-conjugated antibody specific for a surface marker (H2Kd) encoded by a co-transfected plasmid. As shown in Figure 7, transient expression of both HPV18 and BPV-1 E2 proteins (upper panel) increased the proportion of cells which are blocked in G_1 phase (70 and 73.5% for HPV18 and BPV-1 E2, respectively, as compared with 55.1% for cells transfected with the negative control plasmid). Co-expression of the dominant-negative c-p53 (hatched bars) almost completely relieved the E2-induced G_1 block, under conditions in which it completely abolished the transcriptional activity of p53 (centre panel). E2-mediated apoptosis was monitored by the proportion of cells having a DNA content <2n. HPV18 and BPV-1 E2-expressing cells had a sub-2n population of 15.8 and 19.4%, respectively, as compared with 7% for cells expressing the negative control (lower panel). However, this E2-mediated increase of apoptosis was not reverted by co-expression of the dominant-negative c-p53 (hatched bars), but on the contrary was slightly enhanced. These data suggest that apoptosis and G_1 cell cycle arrest observed in E2-transfected cells occur through distinct pathways, and that only the latter involves transcriptionally active p53.

Since E2-mediated apoptosis did not require transcrip-

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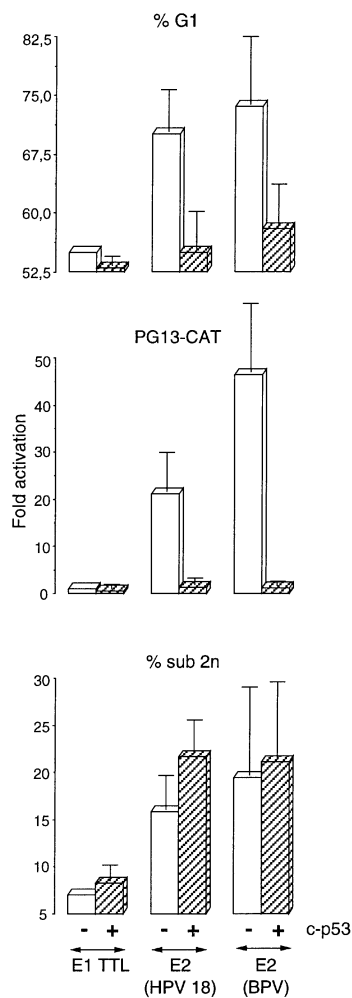
Fig. 7. Effect of a *trans*-dominant-negative mutant of p53 on the E2-induced apoptosis and G_1 growth arrest. HeLa cells were transiently transfected with 5 μg of PG13-CAT and 0.5 μg of vector encoding H2Kd, 1.5 μg of E1TTL, HPV18 E2 or BPV-1 E2 expression vectors, along with 1.5 μg of a plasmid encoding c-p53 (hatched bars) or vector alone (open bars). At 40 h after transfection, the H2Kd-positive cells were gated and their DNA content analysed by flow cytometry. The percentage of the cells in the G_1 phase is plotted schematically in the upper panel. The lower panel represents the proportion of cells having a DNA content <2n. A tenth of the cell population was assayed for CAT activity (central panel).

Table II. E2 does not induce apoptosis in p53-negative cell lines

Cells	E1TTL	E2	p53
HaCaT ($n = 3$)	17	16	27
C33 ($n = 4$)	18	17	23
SAOS ($n = 3$)	17	17	47

Cells were transfected with vectors encoding E1TTL, E2 or p53 together with the green fluorescent protein. The numbers represent the percentage of transfected cells which underwent apoptosis as determined morphologically by counting the number of green dead cells. These values are means calculated from several (*n*) independent transfection experiments.

tionally active p53, we addressed whether E2 could induce cell death in p53-defective cells (Table II). C33 (derived from an HPV-negative cervical carcinoma), HaCaT or SAOS cells, all lacking functional p53, were co-transfected with expression vectors for E1TTL, E2 or human wild-

type p53, together with plasmids coding for the green fluorescent protein and H2Kd. Twenty to forty hours after transfection, apoptosis was determined by counting the number of dead green cells. E2 did not increase the percentage of dead cells in comparison with the negative control E1TTL in any of the three cell lines, whereas p53 significantly increased the level of apoptosis in SAOS and HaCaT cells and, to a lesser extent, in C33 cells. These results were confirmed by FACS analysis and TUNEL labelling (data not shown). These experiments demonstrate that E2 alone is not sufficient to trigger cell death, and support the theory that E2-mediated apoptosis requires wild-type p53.

Discussion

Inactivation of the viral E2 protein appears to play a crucial role in the development of cervical cancer. Indeed, the progression of HPV18-associated dysplasia to a malignant state correlates with the disruption of the E2 ORF as a consequence of integration of the viral DNA into the cellular genome (Berumen *et al.*, 1994). Such an event has occurred in HeLa cells which are derived from an HPV18-positive cervical carcinoma (Schwarz *et al.*, 1985). Their transformed phenotype is dependent upon the continuous expression of the viral E6 and E7 proteins (Bosch *et al.*, 1990). These genes are transcribed from the unique P_{105} promoter which contains binding sites for the E2 protein (Thierry *et al.*, 1987b). We previously have shown in co-transfection experiments that E2 represses the P_{105} promoter by specifically binding to its cognate DNA sequences located close to the TATA box (Thierry and Howley, 1991; Demeret *et al.*, 1994). In this study, we showed that reintroduction of both HPV18 and BPV-1 E2 into HeLa cells repressed transcription of the endogenous E6/E7 genes, whose products inactivate the cellular tumour suppressor genes p53 and Rb, respectively (Dyson *et al.*, 1989; Scheffner *et al.*, 1990). It has been reported previously that the BPV-1 E2 protein is able to increase the level of the p53 protein in HeLa cells ~8- to 20-fold (Hwang *et al.*, 1993, 1996; Dowhanick *et al.*, 1995). It was hypothesized that this elevation of p53 by E2 might result from the down-regulation of E6 expression through transcriptional repression of the P_{105} promoter. However, our results indicated that down-regulation of E6 transcription was not sufficient to activate p53, since E2TR (a deleted version of E2 lacking most of the activation domain) did not increase the steady-state level of p53 protein under conditions in which it efficiently repressed endogenous E6/E7 transcription.

The distinct properties of the full-length and truncated E2 proteins suggest the existence of an alternative pathway leading to p53 activation. To gain insight into such a mechanism, we applied a more sensitive assay for measuring variations in p53 levels. Since p53 function is tightly dependent on its ability to activate specific target genes (Pietenpol *et al.*, 1994), we showed that the BPV-1 and HPV18 E2 proteins expressed in HeLa cells could activate two p53-responsive reporter plasmids (PG13-CAT and MDM2-CAT). In contrast, the short E2 versions induced only a moderate activation of p53 transcriptional activity (not exceeding 10% of that obtained with the full-length proteins). These experiments indicate a clear discrepancy

in the abilities of both forms of E2 to activate p53, while they both repressed endogenous E6/E7 transcription. However, subtle variations in the levels of repression of E6 transcription might exist between full-length and truncated E2 proteins. It is not excluded, therefore, that E2TR-expressing cells continue to produce enough E6 protein to degrade p53. We have attempted to follow the level of the E6 protein in HeLa cells. However, as previously reported, it is rather difficult to detect overexpressed E6 protein, and almost impossible to detect the endogenous E6 with the antisera currently available (data not shown, and see discussion in Crook et al., 1994; Butz et al., 1995). Nevertheless, the existence of an alternative pathway of p53 activation independent of E6 repression was confirmed by the ability of the E2K344 mutant protein to induce p53 activity, despite the fact that it is defective for DNA binding, repression of HPV transcription and activation of E2-responsive reporter plasmids. These observations indicate that E2 activates p53 independently of the repression of E6 transcription. E2-induced activation of p53 transcriptional activity appears to be mediated through two independent pathways which do cooperate. Indeed, co-expression of both E2TR and E2K344 conferred a level of p53 activation close to that observed with the full-length protein. Although we cannot completely rule out that E2K344 and E2TR mutant proteins might form heterodimers, they did not reconstitute a functional site-specific transactivator. Our data thus indicate that E2-mediated sequence-specific activation and p53 activation are independent phenomena. Several hypotheses can be envisaged to explain the underlying mechanism. First, E2 could interact with p53, leading to the stabilization of the protein. If such an interaction exists, it would be likely to involve an auxiliary factor, as suggested by our failure to co-immunoprecipitate E2 and p53 synthesized in vitro. Second, E2 could interfere with factors which enhance the DNA binding activity of p53, such as casein kinase II (Hupp et al., 1992), protein kinase C (Hupp and Lane, 1994; Takenaka et al., 1995) or cyclin-dependent kinases (Wang and Prives, 1995). Alternatively, E2 could interact with a factor that inhibits the transcriptional activity of p53, e.g. MDM2 (Momand et al., 1992). These points are currently being investigated.

If E2 activates p53 independently of the repression of E6 transcription, we would expect E2 to activate p53 transcriptional activity in HPV-negative cell lines. We indeed observed that E2 induced a moderate but consistent activation of the PG13-CAT reporter plasmid in HPV-negative cells containing wild-type p53, such as HepG2 or NIH3T3. In p53-null SAOS cells or in cells containing point mutations in the p53 gene (SW13, C33 or HaCaT), E2 was also able to enhance PG13-CAT activation when co-expressed with exogenous p53. However, the level of E2-mediated activation of p53 in HPV-negative cells remained 10 times lower than that achieved in HeLa cells. This observation raises the possibility that activation of p53 might be amplified in cells expressing the viral proteins. To address this hypothesis, we looked at whether E2 could interact directly with E6 synthesized in vitro or interfere with the ubiquitin-dependent degradation of p53. We have tested this possibility and found that E2 did not protect p53 from E6-mediated degradation in vitro (data not shown). In addition, expression of a dominant-negative

mutant of E6 in HeLa cells did not interfere with the E2-mediated activation of p53, suggesting that E2 and E6 do not interact in vivo (data not shown). We currently are testing the interaction of E2 with the E7 viral oncoprotein.

BPV-1 E2 has been shown previously to induce a G_1 growth arrest in HeLa cells, supposedly (although not demonstrated) through an increase in the level of p53 (Hwang et al., 1993; Dowhanick et al., 1995). In the present study, we confirmed these observations with BPV-1 E2, and showed that the homologous HPV18 E2 protein behaved similarly. In addition, we evaluated the importance of p53 in this process by co-expressing a trans-dominant-negative mutant (c-p53) which completely inactivated p53 transcriptional activity. We showed that expression of c-p53 overcame the E2-mediated block of the cell cycle, providing convincing evidence that the sequence-specific transactivation function of p53 was responsible for this effect.

More strikingly, and for the first time, we have shown that both BPV-1 and HPV18 E2 proteins could trigger cell death when transiently expressed in HeLa cells. E2-mediated cell death shares many characteristic features of apoptosis, e.g. chromatin condensation, DNA content <2n and double-stranded DNA breaks. The pathway leading to E2-mediated cell death seems to diverge, at least partly, from the one involved in E2-induced G_1 growth arrest, since it did not require the p53 transcriptional activity. However, the role of p53 as a regulator of transcription cannot be ruled out completely. Firstly, expression of the dominant-negative p53 mutant might block only a subset of p53-activated genes including the ones participating in the G_1 growth arrest, without affecting those involved in apoptosis. Secondly, p53 might repress transcription of cell survival genes. Alternatively, one cannot exclude a direct p53-independent effect of E2 on apoptosis. We do not favour such a hypothesis, since expression of E2 in three p53-negative cell lines did not induce apoptotis. In addition, our data are in agreement with several recent reports which have shown that p53-induced apoptosis is independent of the transcriptional activity of p53 (Caelles et al., 1994; Wagner et al., 1994; Haupt et al., 1995, 1996; Rowan et al., 1996).

E2-mediated cell death appears independent of the repression of E6/E7 transcription, since E2TR was unable to induce an abnormal cellular phenotype, whereas E2K344 could cause cell death to some extent. The lack of effect observed with E2TR can be explained in two different ways. Firstly, E2TR not only represses E6 synthesis, but also that of E7. Down-modulation of E7 activates the retinoblastoma protein, which has been shown to protect cells from apoptosis (Almasan et al., 1995; Haas-Kogan et al., 1995). Secondly, the level of p53 generated by the E2TR pathway would not be high enough to trigger cell death. Alternatively, it would require an additional modification of p53 induced by the transactivation domain of E2.

In conclusion, as depicted in the model presented in Figure 8, we found that E2 induces p53 in HeLa cells by at least two pathways, one of which is mediated through the repression of endogenous E6/E7 transcription. In the second pathway, E2 activates p53 either through a direct interaction, or through an auxiliary factor (x). It has been shown that in normal cells the p53 protein can adopt two
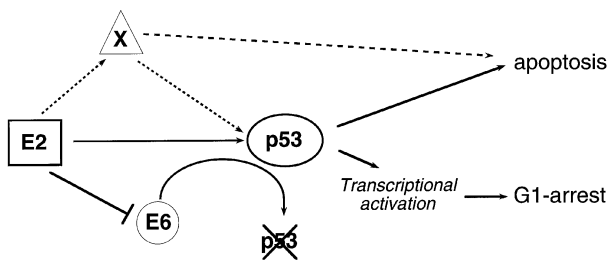
Fig. 8. E2 induces apoptosis and G_1 growth arrest through two different pathways. In this model, E2 activates p53 by at least two mechanisms. The major one involves an interaction between E2 and p53, which is either direct (plain arrow) or mediated through an auxiliary factor x (dotted arrow). In the second pathway, E2 represses the P_{105} transcription of the E6 gene, inhibiting the degradation of p53. The transcriptionally active p53 will induce a G_1 growth arrest, while the transactivation-deficient p53 will lead to apoptosis.

distinct conformations differing in their sequence-specific transactivation function (Hupp *et al.*, 1995). The latent state can be activated by a post-translational modification of the carboxy-terminal end of the protein. One can hypothesize that in HeLa cells, E2 increases both conformations of the p53 protein. The transcriptionally active state of p53 would cause a G_1 growth arrest, while the other conformation of the protein might trigger apoptosis. Alternatively, E2 could induce apoptosis independently of p53. The question raised by our experiments is how the balance between the two effects, G_1 growth arrest versus cell death, is determined, and what conditions influence the choice between these fates. We think that both phenomena are independent, and that a G_1 growth arrest will protect cells from E2-mediated apoptosis. This hypothesis is supported by the two following observations. First, cell cycle inhibitors could protect cells from apoptosis (data not shown). Second, E2 did not kill every transfected cell, and apoptosis plateaued ~24 h after transfection, corresponding roughly to one cell cycle (data not shown). Analysis of the DNA content of E2-transfected cells after this time revealed that they were arrested mostly in G_1.

The ability of E2 to induce p53 activation, G_1 growth arrest and cell death raises some questions about the relevance of these effects to the viral life cycle. Since E2 counteracts the effects of the HPV transforming proteins, its deletion in most HPV18-associated cancers appears a necessary step towards carcinogenic progression. One can speculate that, as long as E2 is expressed, it modulates the transforming activity of the virus, allowing the productive viral cycle. HPV gene expression is tightly regulated by the differentiation programme of the epithelium (Dürst *et al.*, 1992). The expression of the E6 and E7 proteins in the cells of the basal layers presumably keeps these cells cycling. The E2 protein, which might be produced in the intermediate layers, would shut off HPV transcription and divert the cellular transcription/replication factors towards the viral DNA replication process. Expression of the capsid genes takes place only in the terminally differentiated layers of the epithelium. Reactivation of p53 at this stage could help the cells to differentiate. The mechanism of this differentiation is not known, but it involves a nuclear destruction step which might resemble apoptosis. In that sense, E2 would help a final stage of epithelial differentiation that allows the release of the virus into the environment.

Materials and methods

Plasmids

The CAT reporter plasmids used in this study include the following: P_{105}-CAT, previously referred to as p18-4321 (Thierry and Howley, 1991), contains sequences of the HPV18 LCR between nucleotides 6930 and 120; TKE2-CAT (previously referred to as TK-E2BS) contains six E2 binding sites upstream of the herpes simplex virus thymidine kinase promoter (Thierry *et al.*, 1990); PG13-CAT contains 13 binding sites for p53 upstream of the polyomavirus promoter, while in MG13-CAT the p53 binding sites have been mutated (Kern *et al.*, 1992). MDM2-CAT containing the p53-responsive mdm2 promoter was kindly provided by Moshe Oren. pC53-SN3 expressing human wild-type p53 (Baker *et al.*, 1990) is a kind gift from Bert Vogelstein. The BPV-1 E2 expression vectors pCGE2 (Ustav and Stenlund, 1991) and pCGE2TR (Demeret *et al.*, 1994) have been described previously. pCGE2K344 expresses a BPV-1 E2 mutant protein which contains a lysine instead of an arginine at position 344. It was constructed by sub-cloning a *Bam*HI fragment containing this mutated ORF from the C59 kz E2 K344 (kindly provided by Alison McBride) into the *Bam*HI-restricted pCG vector (Tanaka and Herr, 1990). HPV18 E2 was expressed from the pCGE2(18) vector previously described (Demeret *et al.*, 1994). A shorter version of HPV18 E2 (DBD: DNA binding domain) corresponding to E2TR was expressed from pCGE2.DBD. It was constructed by sub-cloning a 934 bp *Apa*I–*Bam*HI fragment from pGEM-E2.C.L. (Thierry and Howley, 1991) into pCG. As negative controls, pCGE1.B TTL (Le Moal *et al.*, 1994) or pGGE1.18 TTL were used. This latter plasmid was constructed by inserting the TTAGTTAACTAA translation termination linker into the *Taq–Bst*NI 2160 bp HPV18 E1 ORF cloned in pCG. The plasmid PKC.Kd.wt (kindly provided by Jean-Pierre Abastado) expresses the MHC class I H2Kd molecule from the SV40 early promoter. CMVgfp (kindly provided by Alexandro Garcia-Carranca) expresses the green fluorescent protein from the cytomegalovirus promoter.

Cell culture, transfections and CAT assays

Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 7% fetal calf serum (FCS), penicillin (500 IU/ml) and streptomycin (125 μg/ml). At 24 h before transfection, 2×10^5 or 5×10^5 HeLa cells were plated in 6 or 10 cm dishes, respectively. The other cells were seeded at a higher density (5×10^5/6 cm dishes for NIH3T3, SAOS-2, HaCaT and SW13, and 7.5×10^5/6 cm dishes for C33 and HepG2). Transfections were performed by the calcium phosphate co-precipitation method as described previously (Desaintes *et al.*, 1995). DNA was prepared by the Qiagen procedure and adjusted to 6 μg (6 cm plates) or 12 μg (10 cm plates) per transfection with Blue Scribe plasmid. CAT assays were performed with 1/10 to 1/2 of total cell extract as described earlier (Desaintes *et al.*, 1992). Quantification of the acetylated [^{14}C]chloramphenicol was determined with a PhosphorImager (Molecular Dynamics).

Western blots

HeLa cells in 10 cm dishes were harvested 40–44 h post-transfection and resuspended in 200 μl of Laemmli buffer. Fifteen μl were loaded on a 10% SDS–polyacrylamide gel. After transfer, the nitrocellulose membranes were incubated with primary and secondary horseradish peroxidase-conjugated antibodies, using the ECL detection kit (Boehringer Mannheim) according to the manufacturer's recommendations. Antiserum specific for BPV-1 E2 has been described earlier (Dostatni *et al.*, 1991). The p53 monoclonal antibody Ab1801 was purchased from Santa Cruz Biotechnology.

Flow cytometry

HeLa cells in 10 cm dishes were detached from the plate by incubation for 10 min in phosphate-buffered saline (PBS), 0.1% EDTA. For experiments measuring cell death, the floating cells were also collected. Cells were incubated for 45 min at 4°C in PBS, 10^{-4}% azide, 2% FCS with the H2Kd-specific monoclonal antibody (SF111) 1/500 (a kind gift from Jean-Pierre Abastado). After washing, the cells were incubated with an anti-mouse antibody coupled to FITC 1/500 (Amersham) for an additional 45 min, followed by fixation in 80% ethanol. The cells were then rehydrated in the PBS buffer, and incubated for 30 min at 37°C with propidium iodide (PI) (10 μg/ml) and RNase (10 μg/ml). Cells were analysed with a flow cytometer (EPICS XL from Coulter). The FITC-positive cells were gated for analysis of their DNA content after exclusion of the doublets. The cell cycle was analysed with the Multi-Cycle software (Phoenix Flow Systems, Inc.).

Immunofluorescence

HeLa cells grown on coverslips were rinsed with PBS 24–75 h after transfection before being incubated with the SF111 antibody (1/500 in PBS + 2% FCS), followed by an additional incubation with a secondary anti-mouse FITC-coupled antibody. At this step, cells were fixed in ethanol:acetic acid 95:5. After rehydration, cells were stained with PI (10 μg/ml) or incubated with a rabbit polyclonal antibody against BPV-1 E2 (Dostatni *et al.*, 1991) followed by an anti-rabbit antibody coupled to Texas red (Amersham) and DAPI 0.15 μg/ml.

TUNEL assay

Forty hours after transfection, HeLa cells on coverslips were fixed in 4% paraformaldehyde for 30 min, and permeabilized for 2 min in 0.1% Triton X-100, 0.1% sodium citrate. The TUNEL reaction was carried out using the *in situ* cell death detection kit (Boehringer Mannheim) as specified by the manufacturer.

RNA extraction, primer extension and Northern blot

HeLa cells were transfected in 10 cm plates with 1 μg of the H2Kd expression vector and 1 μg of either pCGE2(18), pCGE2, pCGE2TR or pCGE1TTL plasmids. The transfected cell population was enriched by sorting cells recognized by the anti-H2Kd antibody with a flow cytometer FACS Star Plus (Becton-Dickinson). Total RNA was prepared with the Qiagen RNeasy kit. Five μg of RNA were annealed with ^{32}P-labelled primers for 10 min at 68°C and then allowed to cool down slowly to room temperature. Two specific primers were used: a 24 base oligonucleotide (CTGTAAGTTCCAATACTGTCTTGC) complementary to the 5′ HPV18 E6 ORF and a 25 base oligonucleotide (ATCCATGGTG-AGCTGGCGGCGGGTG) complementary to the human β-actin RNA. Reverse transcription was performed with annealed RNAs at 37°C for 1 h with the MuLV reverse transcriptase in standard conditions (Ham *et al.*, 1991a). The products of the reactions were resolved on a denaturing gel. For Northern blot analysis, 5 μg of RNA were loaded on a 1% MOPS–agarose gel. After transfer, the nitrocellulose membranes were hybridized with a ^{32}P-labelled p53 probe. The probe was generated by 10 PCR cycles performed on a human p53 cDNA fragment with a single primer (TCAGTCTGAGTCAGGCCCTTCTG) hybridizing to the 3′-end of the p53 ORF.

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