Disruption of the human papillomavirus type 16 E2 gene protects cervical carcinoma cells from E2F-induced apoptosis

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Human papillomavirus type 16 (HPV-16) is a DNA tumour virus that has been implicated in the development of cervical cancer. In non-transformed HPV-infected cells, the HPV E2 protein regulates transcription of the viral E6 and E7 oncogenes. Malignant transformation is usually accompanied by disruption of the E2 gene and consequent deregulated expression of E6 and E7. Here we show that re-introduction of the HPV-16 E2 protein into an HPV-16-transformed cervical carcinoma cell line results in a decrease in growth rate and, in the absence of serum growth factors, cell death via apoptosis. E2 expression increases E6/E7 mRNA levels. This brings about an increase in E7 protein levels, which in turn leads to an increase in free E2F, a condition that has previously been shown to induce apoptotic cell death. Despite the increase in E6 mRNA there is no detectable E6 protein in these cells and E2 expression does not reduce the activity of a p53-responsive promoter. Our data suggest that disruption of the E2 gene produces HPV-transformed cells that are less liable to undergo apoptosis and, therefore, more likely to form cervical tumours.

Introduction

The human papillomaviruses (HPV) are a family of DNA tumour viruses that infect epithelial cells and generally produce benign hyperproliferative lesions (or warts). It is now generally accepted that some so-called high risk virus types produce lesions which have the potential to undergo malignant transformation. HPV-16 and HPV-18, for example, are thought to play a primary role in the development of invasive cervical cancer and its precursor lesions (reviewed by zur Hausen, 1991). Malignant transformation is dependent upon expression of the viral E6 and E7 oncogenes. The E6 and E7 proteins form complexes with the products of the cellular tumour suppressor genes p53 (Werness et al., 1990) and Rb (Dyson et al., 1989), respectively, to modulate the cell cycle and promote cell immortalization (Hawley-Nelson et al., 1989; Münger et al., 1989; Watanabe et al., 1989).

In HPV-16-transformed cervical carcinoma cells, transcription of the E6 and E7 oncogenes is under the control of a single promoter located upstream of E6 known as P97 (Smotkin & Wettstein, 1986). Transcription from the P97 promoter is controlled by a number of cellular transcription factors and by the product of the viral E2 gene (Phelps & Howley, 1987; Cripe et al., 1987). The E2 protein binds as a dimer to four palindromic sites found upstream of P97 (Chong et al., 1990). Although the full-length E2 protein from bovine papillomavirus (BPV) has been shown to activate transcription from the BPV-1 early promoter (Spalholz et al., 1985), the precise role of the HPV E2 protein in the regulation of HPV-16 transcription has been difficult to establish. The HPV E2 protein has been shown to activate transcription from some P97 promoter derivatives linked to reporter genes (Phelps & Howley, 1987; Cripe et al., 1987; Ushikai et al., 1994; Bouvard et al., 1994). However, HPV E2 has also been reported to repress the activity of other promoter derivatives (Romanzuk et al., 1990; Dostatni et al., 1991; Tan et al., 1992). In addition to its role in transcriptional regulation, the E2 protein is also important in viral DNA replication. The E2 protein forms a complex with the viral replication initiation factor E1 and recruits this protein to the viral origin of replication (Mohr et al., 1990; Frattini & Laimins, 1994).

The HPV genome exists as an extrachromosomal element in pre-neoplastic cells but is often integrated into the host genome in malignant cervical lesions (Yee et al., 1985; Schwarz et al., 1985; Dürst et al., 1985). Chromosomal integration frequently results in disruption of the E2 open reading frame and consequent loss of the E2 protein (Baker et al., 1987). It has been argued that disruption of the E2 gene would lead to deregulation of the cell cycle and the cell differentiation pathway, and finally, to the transformed phenotype. Although the BPV E2 protein has been shown to suppress the growth of HPV-
transformed cells (Hwang et al., 1993, 1996; Dowhanick et al., 1995), the effect of HPV-16 E2 on growth of HPV-16-transformed cells has not been examined in detail. We placed the HPV-16 E2 gene under the control of an inducible promoter and stably transfected this construct into cells containing integrated HPV-16 DNA with a disrupted E2 gene. The induction of E2 expression in the transfected cells resulted in the inhibition of cell growth and, in the absence of serum growth factors, the initiation of cell death via apoptosis. We discuss the implications of these findings in terms of the biological role of the E2 protein in HPV-infected cells and in cervical carcinogenesis.

**Methods**

- **DNAs used in this study.** The HPV-16 E2 gene was amplified by PCR (94 °C for 1 min, 50 °C for 2 min and 72 °C for 2-5 min, for 25 cycles in standard buffer) from HPV-16 DNA template using the oligonucleotide primers 5′-GGGGTACCCCAAAACGATGGAGACT-3′ and 5′-GGGAAGCTTTCATATAGACATAAATCCAG-3′. These primers placed KpnI and HindIII restriction sites at the 5′ and 3′ ends of the E2 coding sequence, respectively. The 1 kb PCR product was cloned between the KpnI and HindIII sites of the eukaryotic expression vector pMEP-4 (Invitrogen), immediately downstream of the metallothionein promoter. The resulting pMEP-E2 expression vector was sequenced using a panel of E2-specific sequencing primers to check for the occurrence of any point mutations.

- **Cell culture and transfections.** SiHa cells, and the stably transfected clones derived from them, were cultured in RPMI 1640 (GIBCO) supplemented with 10% foetal bovine serum (GIBCO), 100 μg/ml streptomycin (Evans Medical) and 60 μg/ml penicillin (GIBCO). pMEP-E2 was linearized using the restriction enzyme ClaI then transfected into SiHa cells by electroporation (960 μF/280 V). Stably transfected clones were selected using hygromycin (400 μg/ml). The hygromycin-resistant clones were transferred to individual plates and grown in the presence of 200 μg/ml hygromycin (Boehringer-Mannheim).

- **Antibodies.** E2-specific antibodies were raised in rabbits using a truncated E2 protein (E2Ct) consisting of the C-terminal 68 amino acids of HPV-16 E2 (kindly donated by S. Billet, University of Bristol, Bristol, UK). Two rabbits received monthly injections of 50 μg E2Ct subcutaneously and intramuscularly with Freund’s Adjuvant. Bleeds were collected each month and tested for antibody titration by ELISA. All the work described here was performed using the sixth bleed antisera. Mouse monoclonal and goat polyclonal E7-specific antibodies, mouse monoclonal E6-specific antibodies, and the p53-specific monoclonal antibody Ab 1801 were obtained from Santa Cruz Biotechnology.

- **Immunofluorescence.** Cells were grown on cover-slips then incubated with 10 μM CdCl₂ overnight to induce E2 expression. The cells were fixed in 3% paraformaldehyde for 15 min, then washed three times in PBS (15 min each wash) and permeabilized by washing in 0.1% Triton X-100 (v/v) for 4 min. After washing in PBS/0.2% BSA, cells were incubated for 1 h with the polyclonal E2 antisera described above (1:300 in PBS/1% BSA). Following three washes with PBS, cells were incubated with FITC-conjugated donkey anti-rabbit antibodies (Sigma) (1:1000 in PBS/1% BSA) for 45 min. After two washes in PBS, the cells were mounted on glass slides in Moviol (Calbiochem) and E2 staining was observed using an epifluorescence microscope (Zeiss) at 60×, 1.4 oil immersion objective. In the competition experiments, E2-antiserum was pre-incubated with 200 μg of either GST or a GST–E2 fusion protein (provided by A. Thain, University of Bristol, Bristol, UK) for 30 min at 4 °C.

- **Immunoprecipitation and Western blotting.** Around 2×10^7 cells were collected by trypsinization, washed in PBS, and then lysed in PBS containing 0.3% Triton X-100, 0.1% Na₂₃PO₄, 0.3 μg/ml Leupeptin, 0.3 mM NH₄VO₃, and 2 mM NaF at 4 °C for 20 min. The detergent-soluble fraction was recovered by centrifugation and pre-cleared by adding 100 μl pre-immune rabbit serum and 25 μl protein A-agarose beads (Sigma) and mixing for 1 h at 4 °C. After centrifugation, E7 was immunoprecipitated from the supernatant using a mixture of E7-specific monoclonal and polyclonal antibodies and 30 μl protein A-agarose beads mixed for 2 h at 4 °C. The E7 complexes were collected by centrifugation and washed three times in lysis buffer. E7 was visualized by Western blotting using E7-specific monoclonal antibodies and the enhanced chemiluminescence ECL reaction (Amersham).

- **Northern analysis.** Total RNA was extracted from around 3×10^7 cells using Qiagen columns. The mRNA was subsequently isolated using Qiagen oligo(dT) resin, according to the manufacturer’s instructions. mRNA was separated on 1% agarose–formaldehyde gels then transferred to GeneScreen Plus nylon membrane as above. Blots were pre-hybridized at 42 °C in formamide buffer (Sambrook et al., 1989) then probed for either E2 expression using a radiolabelled E2 cDNA or E6/E7 expression using a radiolabelled E6/E7 ORF. After 16 h hybridization at 42 °C, the blots were washed as recommended by the membrane manufacturer. Northern blots were stripped and re-hybridized using an actin probe to control for differences in loading. In each experiment, the levels of E2 or E6/E7 mRNA were quantified using a PhosphorImager and standardized using actin mRNA as an internal control.

- **Cell viability assays.** Cell viability was measured using the MTT [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide] assay. After removal of the medium, cells were incubated with 50 μg/ml MTT for 2 h at 37 °C. Blue formazan crystals were extracted and dissolved in DMSO. Absorbance was measured at a wavelength of 560 nm in an ELISA plate reader (Mossman, 1983). Chromatin condensation was visualized after staining with acridine orange. Cells were collected by centrifugation (3000 r.p.m. for 10 min in an Eppendorf microcentrifuge) and then resuspended in 200 μl 0.0005% acridine orange (Sigma) made up in PBS. A 20 μl drop was placed in a porta glass, covered with a coverslip, and viewed using an epifluorescence microscope (Zeiss).

- **Flow cytometry.** Cells were trypsinized, then collected by centrifugation. After washing with 1 ml PBS, the cells were pelleted in an
Results

Expression of E2 in HPV-16-transformed cells

The BPV E2 protein has been shown to inhibit the growth of HeLa cells, an HPV-18-transformed cell line (Hwang et al., 1993). Since the BPV E2 protein and the HPV E2 protein have significantly different effects on the activity of HPV promoters (Bouvard et al., 1994), we decided to express the HPV-16 E2 protein in HPV-16-transformed cells. To avoid any growth inhibition during the establishment of stably transfected cell lines, we placed the HPV-16 E2 gene under the control of an inducible promoter. The E2 gene was cloned into the eukaryotic expression vector pMEP-4 immediately downstream of the heavy metal-inducible metallothionein promoter. The resulting E2 expression vector was used to stably transfect SiHa cells, a cervical carcinoma-derived cell line that contains an integrated copy of the HPV-16 genome in which the viral E2 gene has been disrupted (Baker et al., 1987). Southern and Northern analysis showed that two stably transfected SiHa-E2 cell lines contained E2 DNA and expressed E2 mRNA upon cadmium induction (data not shown). The presence of the E2 protein in these SiHa-E2 cell lines was demonstrated using immunofluorescence and Western blotting; SiHa and SiHa-E2 cells were grown in the presence of 6 μM cadmium overnight then processed for immunofluorescence using polyclonal antisera raised against the isolated C terminus of the HPV-16 E2 protein (Figs 1a and 1b, respectively). The E2 antibodies stained the nuclei of cadmium-treated SiHa-E2 cells (Fig. 1b) but did not stain cadmium-treated parental SiHa cells (Fig. 1a). Immunostaining was blocked by pre-incubation of the E2 antibodies with a GST–E2 fusion protein prior to immunofluorescence (Fig. 1c), whereas pre-incubation with GST alone had no effect (Fig. 1d). The presence of the E2 protein in SiHa-E2 cells, but not in SiHa cells or SiHa cells stably transfected with pMEP alone (SiHa-pMEP), was confirmed by Western blotting using the same polyclonal antisera (data not shown). Low levels of the E2 protein were detected in SiHa-E2 cells prior to the addition of cadmium; this is probably a consequence of leaky control of the metallothionein promoter.

E2 alters cell growth rate

Living cells reduce the tetrazolium salt MTT resulting in a colour change that can be measured accurately using an ELISA plate reader (Mosmann, 1983). To determine the effect of HPV-16 E2 on the growth rate of SiHa cells, we used the MTT assay to count live cells. In the absence of serum growth factors, the viability of SiHa-E2 cells declined rapidly whereas SiHa cells continued to grow (Fig. 2). The addition of 6 μM cadmium resulted in a further decrease in the viability of SiHa-
E2 cells. These data indicate that the HPV-16 E2 protein has a negative effect on the growth rate of HPV-16-transformed cells and that, in the absence of serum growth factors, expression of the E2 protein induces cell death. These differences between SiHa cells and SiHa-E2 cells are not due to clonal selection; both SiHa-E2 stable cell lines showed a similar reduction in growth rate and SiHa cells stably transfected with empty pMEP vector were indistinguishable from untransfected SiHa cells (data not shown). The low growth rate and loss of viability shown by SiHa-E2 cells in the absence of cadmium is probably due to leaky control of the metallothionein promoter. This suggests that in the absence of growth factors, the small amount of E2 present in ‘uninduced’ cells is sufficient to bring about significant levels of cell death.

**E2 induces apoptosis**

Having shown that the HPV-16 E2 protein induces cell death in HPV-16-transformed SiHa cells, we set out to determine whether death was occurring via the apoptotic or necrotic pathways. Apoptotic cell death has several characteristic features including DNA fragmentation, blebbing of the plasma membrane, chromatin condensation, and the appearance of cell fragments with subG0 DNA content (reviewed by Earnshaw, 1995). In contrast, necrotic cell death is characterized by cell swelling followed by rupture of the cell membrane. Populations of SiHa and SiHa-E2 cells were grown in either the presence or absence of 6 µM cadmium and then examined by flow cytometry (Fig. 3). In the absence of serum, SiHa cells show a typical cell cycle distribution (Fig. 3a, day 1) which does not change over time (Fig. 3a, day 3). In contrast, SiHa-E2 cell lines grown in the absence of serum for 24 h contain a subG0 population that rises in number over time (Fig. 3c). After three days in the presence of 6 µM cadmium, some subG0 cells are apparent in the SiHa population, possibly due to the toxicity of this heavy metal (Fig. 3b, day 3). However, the addition of cadmium to SiHa-E2 cells results in the appearance of a subG0 population within 24 h (Fig. 3d). These data suggest that E2-induced cell death occurs via apoptosis. In order to verify this conclusion we next examined the morphology of E2-expressing cells.

In apoptotic cell death the chromatin is often seen to collapse against the nuclear periphery. In many cases the entire nucleus condenses into a single dense ball, while in others the chromatin forms smaller balls, each surrounded by nuclear envelope (Earnshaw, 1995). Fig. 4 shows SiHa and SiHa-E2 cells stained for nucleic acids using acridine orange. Cadmium-treated SiHa cells show diffuse nuclear staining (Fig. 4a), whereas in cadmium-treated SiHa-E2 cells the chromatin is condensed into either a single ball or several smaller balls (Figs 4b and 4c, respectively). Taken together with the results of the flow cytometry experiments described above, these data show that the presence of the E2 protein induces cell death in HPV-16-transformed SiHa cells via the apoptotic pathway.

**HPV-16 E2 activates transcription of E6 and E7**

The E2 protein could induce apoptosis by either of two mechanisms. Firstly, E2 could repress transcription of the E6 and E7 genes. This would be expected to result in an increase in the level of p53 which could in turn mediate apoptosis in response to growth factor withdrawal. Secondly, E2 could
activate transcription of the E6 and E7 genes. One result of this activation would be an increase in the level of free E2F caused by the binding of E7 to Rb and p107; over-expression of E2F-1 has previously been shown to induce apoptosis in serum-starved cells (Wu & Levine, 1994; Qin et al., 1994). To distinguish between these mechanisms, we determined the effect of E2 expression on the steady-state level of E6/E7 mRNA and the amount of E6 and E7 protein present in SiHa cells.

mRNA was extracted from SiHa and SiHa-E2 cells, grown in either the presence or absence of cadmium. Northern hybridization using a probe corresponding to the E6/E7 region of HPV-16 showed that the addition of cadmium had little or no effect on the level of the E6/E7 mRNA present in SiHa cells (Fig. 5a, lanes 1 and 2). In contrast, SiHa-E2 cells contain higher levels of E6/E7 mRNA than SiHa cells and this difference is augmented by the addition of cadmium (Fig. 5a, lanes 3 and 4). Fig. 5(b) shows the results of four independent experiments; in each case E6/E7 mRNA levels were standardized using actin mRNA as an internal control.
Having shown that E6/E7 mRNA levels in SiHa-E2 cells are increased in response to cadmium induction we next looked at the E7 and E6 proteins. SiHa and SiHa-E2 cells were grown in the absence of foetal calf serum and assayed for E7 protein using immunoprecipitation. E7-specific antibodies precipitated an approximately 18 kDa protein (the apparent molecular mass of HPV-16 E7) from cadmium-treated SiHa-E2 cells (Fig. 6, lane 4) but not from untreated SiHa-E2 cells (lane 3) or the parental SiHa cells (lanes 1 and 2). Western analysis using the same E7-specific antibodies detected E7 protein in both SiHa and SiHa-E2 cell extracts but not in HaCat cell extracts (a non-HPV-transformed cervical carcinoma cell line). However, the presence of several non-specific bands prevented quantification (data not shown). In contrast, E6-specific antibodies failed to detect any proteins corresponding to the apparent molecular mass of the E6 protein in either cadmium-treated or untreated SiHa-E2 cells or in the parental cells (not shown). Taken together with the results of the Northern analysis, these data suggest that the HPV-16 E2 protein activates transcription of the E6 and E7 oncogenes resulting in an increase in E6/E7 mRNA and a corresponding increase in E7 protein. However, the increase in E6/E7 mRNA does not produce easily detectable levels of the E6 protein.

E2 expression leads to increased E2F activity

E7 binds to Rb and p107 resulting in the displacement of E2F (Dyson et al., 1989). The increase in E7 levels caused by expression of the E2 protein would therefore be expected to result in increased E2F activity. To test this prediction, we assayed E2F activity in SiHa and SiHa-E2 cells using reporter plasmids that carry the luciferase gene under the control of either wild-type or mutated E2F-binding sites (shown in Fig. 7a as Wt and Mut, respectively). Transient transfection of the reporter plasmid carrying wild-type E2F-binding sites into SiHa cells grown in either the absence or presence of cadmium resulted in low levels of luciferase activity (Fig. 7b, lanes 1 and 2). In contrast, transient transfection of this reporter plasmid into both SiHa-E2 cell lines resulted in significant luciferase activity in the absence of cadmium and high levels of luciferase activity in the presence of cadmium (Fig. 7b, lanes 5 and 6, respectively). The reporter plasmid carrying mutated E2F-binding sites produced only low levels of luciferase activity in both cell lines. The high levels of E2F-dependent transcription seen in SiHa-E2 cells grown in the presence of cadmium suggest that the E2-induced increase in E7 protein results in a consequent increase in E2F activity. Over-expression of the HPV-31 E2 protein in HPV-negative cells has recently been shown to increase E2F-1 mRNA levels (Frattini et al., 1997). However, Northern analysis using an E2F-1-specific probe showed that induction of E2 expression has little or no effect on E2F-1 mRNA levels in SiHa-E2 cells (data not shown). The lower levels of E2F-dependent transcription seen in SiHa-E2 cells grown in the absence of cadmium are probably caused by the leaky expression of E2 from the metallothionein promoter.
E2 expression and p53 activity

The E6 protein binds to p53 and targets this protein for degradation (Scheffner et al., 1990). Any increase in E6 levels might therefore be expected to lower p53 activity within the cell. As previous studies have indicated that E2F-induced cell death occurs via a p53-dependent pathway (Qin et al., 1994; Wu & Levine, 1994), we next assayed p53 transcriptional activity in SiHa and SiHa-E2 cells. A p53-dependent mdm2-luciferase reporter plasmid was transiently transfected into SiHa-pMEP and SiHa-E2 cells grown, in either the presence or absence of cadmium, and luciferase activity was determined as described previously. As can be seen from the data in Fig. 8(a), addition of cadmium did not reduce the activity of the mdm2 promoter in either cell line. In Each cell line, the presence of cadmium resulted in a slight increase in mdm2 promoter activity, possibly due to the toxicity of this heavy metal. In contrast, over-expression of wild-type p53 strongly stimulated the mdm2 promoter (Fig. 8b). Taken together with the results of Western analyses, these data suggest that E2 expression does not result in the production of E6 protein and does not reduce the transcriptional activity of p53.

Discussion

This study has shown that the HPV-16 E2 protein induces apoptotic cell death in HPV-16-transformed cervical carcinoma cells. Tumours and cervical carcinoma cell lines often contain integrated copies of the HPV genome in which the viral E2 gene has been disrupted (Dürst et al., 1985; Baker et al., 1987). This observation led to the hypothesis that deletion or disruption of the E2 gene could result in de-regulated expression of the virus-transforming proteins E6 and E7 and that this could be a prerequisite for cell immortalization (Romanczuk & Howley, 1992, and references therein). The HPV E6 and E7 proteins are known to interact with p53 and Rb, respectively (Werness et al., 1990; Dyson et al., 1989). Although these cellular proteins have been shown to be important in the regulation of apoptosis, surprisingly little work has been done on the induction of apoptosis by HPV (for a review, see Razvi & Welsh, 1995). Our results suggest that disruption of the E2 gene might prevent the apoptotic death of HPV-transformed cells.

The E6 protein binds to p53, blocking p53-mediated transcriptional repression (Lechner et al., 1992) and promoting p53 degradation (Scheffner et al., 1990; Hubbert et al., 1992). The normal role of p53 is to induce apoptosis in response to a variety of signals including growth factor withdrawal and DNA damage. However, both p53-dependent and p53-independent apoptotic pathways exist; thus for example, thymocytes that lack p53 no longer enter apoptosis in response to DNA damage but do enter apoptosis in response to antigen (Lowe et al., 1993). The E7 protein binds to Rb and the Rb-related protein p107. Binding of E7 to these cellular proteins promotes the release of E2F, which in turn promotes cell cycle progression. Over-expression of E2F-1 in serum-starved cells results in p53-dependent apoptosis (Qin et al., 1994; Wu & Levine, 1994) as does expression of the HPV E7 protein in the lens of transgenic mice (Howes et al., 1994; Pan & Griep, 1994). As the E2 protein regulates transcription of the E6 and E7 genes, any change in the expression of E2 might be expected to have a direct effect on cell viability.

The BPV-1 E2 protein has been shown to inhibit the growth of HPV-transformed cells but to have no effect on HPV-negative cells unless expressed at very high levels (Hwang et al., 1993, 1996; Dowhanick et al., 1995). This suppressive effect of the BPV E2 protein on cell growth was found to correlate with a decrease in E6/E7 mRNA levels (Hwang et al., 1993; Dowhanick et al., 1995). Recently, over-expression of the HPV-18 E2 protein has been shown to repress transcription of the E6 and E7 genes and induce cell death in HeLa cells, an HPV-18-transformed cell line (Desaintes et al., 1997). We have shown that the HPV-16 E2 protein inhibits the growth of HPV-16-transformed cells. However,
our data suggest that the HPV-16 E2 protein activates transcription from the P97 promoter resulting in increased expression of the E6 and E7 genes. There are a number of possible explanations for the different effects of E2 from these different virus species. The BPV and HPV-16 E2 proteins have previously been shown to have opposite effects on HPV promoter activity (Bouvard et al., 1994). Over-expression of the BPV E2 protein has been shown to repress transcription from the HPV-16 P97 promoter, whereas under identical conditions, the HPV-16 E2 protein has been shown to activate transcription from the same promoter (Bouvard et al., 1994). Furthermore, the BPV-1 E2 ORF produces three products; one product corresponds to the full-length E2 protein and contains both the DNA-binding domain and the transcription activation domain, whereas, two shorter products contain only the DNA-binding domain (Chin et al., 1988; Chiang et al., 1991). These short BPV E2 gene products repress transcription of the E6 and E7 genes. Although short forms of the HPV E2 proteins have been reported (Chin et al., 1988; Doorbar et al., 1990), the importance of these proteins in the regulation of HPV-16 gene expression is not clearly understood. Another possibility for the different effects of E2 is that the level of E2 expression plays an important role in determining whether these proteins activate or repress promoter activity. The HPV-18 P105 promoter and the HPV-16 P97 promoter may be activated at low levels of E2, such as those seen in our stable cell lines, whereas these promoters could be repressed by the high levels of E2 produced by other expression systems. Finally, a further possibility is that the BPV E2 protein, the HPV-18 E2 protein and the HPV-16 E2 protein may interact differently with cellular transcription factors.

In our experimental system, transcription activation by the HPV-16 E2 protein resulted in an increase in E6/E7 mRNA, an increase in E2F activity and, ultimately, in the induction of cell death in two independent stable cell lines. A variety of studies have shown that, in the absence of serum growth factors, over-expression of E2F-1 can induce cell death via a p53-dependent pathway (Wu & Levine, 1994; Qin et al., 1994). Any increase in E6 expression caused by the re-introduction of E2 would be predicted to down-regulate p53 activity and this might be expected to block E2F-induced cell death. However, we did not detect an increase in E6 levels in cells expressing the E2 protein. Furthermore, the presence of the E2 protein did not decrease the activity of the p53-dependent mdm2 promoter. These data are in agreement with previous studies that detected functional p53 protein in several HPV-positive cell lines (Butz et al., 1995). The apparent absence of E6 protein in these cells might be a consequence of the location of the P97 transcription start site. The major HPV-16 transcript in SiHa cells and HPV-16-positive cervical neoplasms is initiated within the E6 ORF and encodes only the E7 protein (Hemström Nilsson et al., 1996). Taken together, these data suggest the following model: E2 activates transcription of the E6 and E7 oncogenes resulting in increased E7 levels. This in turn results in the release of E2F and, as little or no E6 protein is produced, the induction of apoptosis via a p53-dependent pathway. An alternative model proposed by Desaintes et al. (1997) suggests that the E2 protein represses transcription of E6, thereby stabilizing p53 and inducing apoptosis. However, a truncated E2 protein that lacks the transcription activation domain, but which is capable of repressing transcription of E6 in transient transfection experiments, does not stabilize p53 or induce cell death (Desaintes et al., 1997). In contrast, over-expression of the HPV-31 E2 protein has been reported to decrease p53 levels in cells that do not contain the E6 gene (Frattini et al., 1997). The opposite effects of E2 over-expression on p53 levels observed by these groups could be due to the particular experimental systems used in each case, or could be further evidence of differences in the transcriptional activities of E2 proteins from different HPV species.

In conclusion, we have shown that the HPV-16 E2 protein activates transcription of the E6 and E7 oncogenes and induces apoptosis in HPV-16-transformed cervical carcinoma cells. These data might help to explain the high frequency of E2 gene disruption seen in cervical carcinoma cells. Presumably, disruption of the E2 gene produces HPV-transformed cells that are less liable to undergo apoptosis and, therefore, more likely to form cervical tumours. The dramatic effect of the E2 protein on the survival of HPV-transformed cells opens up the possibility that this protein could be used in gene therapy for virus-transformed carcinomas.

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