Transformation of primary BRK cells by human papillomavirus type 16 and EJ-ras is increased by overexpression of the viral E2 protein

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The close association between human papillomavirus type 16 (HPV-16) and cervical cancer implies some role for the virus in the development of this disease. Recent studies have shown that HPV-16, under the control of strong heterologous promoters, can cooperate with the activated ras oncogene to transform primary baby rat kidney cells. Virus types associated with benign lesions, e.g. HPV-6 and -11, do not function in this system. The discrimination between virus types associated with benign and tumorigenic lesions by this assay implicate it as a useful system for the study of transformation in vitro. The studies reported here investigate the activity of the HPV-16 early gene product E2 in transformation. In the presence of exogenous E2, endogenous viral promoters are stimulated sufficiently to give a high efficiency of transformation in primary epithelial cells. This transactivation by E2 obviates the need for heterologous promoters, and implicates increased viral gene expression as a prerequisite for transformation. The stimulatory effect of E2 appears to be mediated through increased levels of expression of the E7 protein, which has been shown in similar assays to be sufficient to give transformation in cooperation with ras. CAT assays confirm that HPV-16 E2 can transactivate the HPV-16 early promoters. These studies demonstrate some of the elements in a complex series of events likely to be involved in the development of cervical carcinomas.

Introduction

Human papillomaviruses (HPVs) are a group of small DNA tumour viruses that are closely linked with genital lesions. HPV types 6 and 11 (HPV-6 and -11) DNAs are found predominantly in benign anogenital lesions (condylomas) whereas types 16, 18, 31 and 33 are associated more commonly with high-grade dysplasia, carcinoma in situ and cervical carcinomas. Southern blot analysis has shown that over 90% of cervical cancer biopsies contain HPV DNA, strongly implying a role for HPV in genital cancer (Dürst et al., 1983; Gissmann et al., 1983). More recent studies have revealed the presence of HPV-16 DNA in up to 100% of cervical cancers analysed but also in a high percentage (> 60%) of normal cervical smears (de Villiers et al., 1987; Gergely et al., 1987; Young et al., 1989), indicating a much higher rate of infection in the general population than was thought previously. These data, in conjunction with additional epidemiological evidence, suggest that other factors must be involved for progression from a benign lesion to cervical carcinoma. Early studies on skin warts induced by the cottontail rabbit papillomavirus (CRPV) revealed the basic characteristics of papillomavirus oncogenesis. CRPV is only weakly oncogenic by itself, but the progression of induced carcinomas can be accelerated by the application of chemical carcinogens to the skin of the animals (Rous & Friedewald, 1944), establishing the role of cofactors in papillomavirus-associated carcinogenesis.

HPV-16 is the most prevalent virus type associated with high-grade genital lesions. DNA from HPV-16 has been shown to cooperate with an activated Ha-ras oncogene to transform primary baby rat kidney (BRK) cells in vitro (Matlashewski et al., 1987). A number of reports have shown that morphological transformation of primary cells in this type of assay requires two cooperating oncogenes or transforming agents, one from the establishment class and one from the transforming class (Land et al., 1983; Ruley, 1983). For example the adenovirus E1A gene alone is capable of the establishment of primary cells, but in the presence of the activated ras gene complete morphological transformation can be achieved. By this criterion HPV-16 contains at least one oncogene of the establishment class. The genome
contains eight potential open reading frames (ORFs) and there is evidence that splicing events generate further virus-encoded proteins (Ahola et al., 1987). Deletion mutant analysis strongly implicated a protein (or proteins) derived from the E6–E7 region to be required for cooperation with the ras gene in the BRK assay (Matlashewski et al., 1987). More detailed analysis has shown that in this system the transforming activity can be localized to the E7 ORF (Storey et al., 1988; Phelps et al., 1988), and that sustained expression of E7 is required for the maintenance of the transformed phenotype in vitro (Crook et al., 1989). Analysis of cell lines derived from cervical cancer biopsies show that the E7 protein is still expressed after many passages (Smotkin & Wettstein, 1986), further emphasizing the need for E7 in transformed cells. However the means by which E7 expression is controlled within the cell are as yet unknown.

In the co-transformation assays described thus far, transcription of the viral genes was controlled by a strong heterologous promoter. Endogenous papillomavirus promoters alone were not sufficiently active in the BRK assay and transformation was not observed (Matlashewski et al., 1987). Some of these endogenous promoters are contained within the upstream regulatory region (URR) of the viral genome. This region immediately precedes the viral early genes, is approximately 1000 base pairs long, and contains cis regulatory elements involved in plasmid DNA replication, virus transcription and its control (Rosi et al., 1983; Lusky & Botchan, 1984; Sarver et al., 1984; Waldeck et al., 1984; Spalholz et al., 1985). The early promoter of HPV-16 (P97), located upstream of the second ATG codon of E6, is known to be one of the major sources of mRNA in transformed cells and all E6–E7 transcripts in HPV-16-containing tumours originate from this site (Smotkin et al., 1989). Genetic analysis of bovine papillomavirus type 1 (BPV-1) has shown that an important mechanism for transcriptional control involves the transactivation of an enhancer in the URR by the viral E2 gene product. The BPV-1 E2 protein binds DNA, recognizing the sequence ACCGNN4CGGT which is conserved in all the known papillomavirus URRs (Dartmann et al., 1986; Hawley-Nelson et al., 1988). Transactivation of virus transcription is clearly important in the viral life cycle as the level of transcription from most papillomavirus promoters is rather low in the absence of the E2 protein (Giri et al., 1985; Thierry & Yaniv, 1987). In studies using the HPV promoter elements to direct the transcription of the early region, it was shown that deletion mutations affecting the E2 ORF of BPV-1 and premature termination codons located in the E2 ORF dramatically decrease the efficiency of focus formation in C127 cells (Sarver et al., 1984; Lusky & Botchan, 1984). Sequence homology within the terminal regions of the E2 protein (Giri & Yaniv, 1988), and its ability to transactivate across species barriers, suggest similar functions for the E2 protein in the different papillomaviruses.

More recently a repressor activity has been associated with the E2 ORF in some virus types (Lambert et al., 1987; Thierry & Yaniv, 1987). Thierry & Yaniv (1987) show that transcription from the early promoter of HPV-18 is repressed by the presence of the BPV-1 E2 protein. It therefore appears that the E2 protein can function either positively or negatively on transcription in vitro. The viral regulatory region contains a number of other potential control sites (Gloss et al., 1989), including a keratinocyte-dependent enhancer (Cripe et al., 1987), and a glucocorticoid receptor-binding element (Gloss et al., 1987) which may also be important in transcriptional control.

The experiments described here examine the role of E2 and of glucocorticoid hormones in regulating the expression of the early genes of HPV-16 from endogenous HPV promoters. The BRK cells used in the cotransformation assays are of a primary epithelial type and therefore provide a good model system for studying gene expression in this virus. Our results show conclusively that overexpression of the HPV-16 E2 protein increases transcription from the HPV-16 promoter elements, leads to increased levels of the E7 protein within the cell, and gives rise to a higher efficiency of transformation of BRK cells in cooperation with the Haras oncogene. This demonstrates that the HPV-16 E2 protein acts as a transactivator of early gene expression in this system, suggesting a possible role for E2 in cell transformation in vivo.

**Methods**

**Construction of HPV-16 expression plasmids.** The HPV-16 (W12) DNA used in these studies was the episomal virus genome cloned from a cervical wart as previously described (Crook et al., 1988). Briefly, the entire virus DNA was cloned at the BamHI site of pSP64, preserving the early region but interrupting L1. To generate the E2 constructs HPV-16 DNA was digested using AvaII, to yield a 2.7 kb fragment containing sequences from nucleotide (nt) 2713 to 5471. A stop codon just upstream from the putative start codon for the E2 gene was removed by digestion using nuclease Bal 31, and the fragment was subsequently digested with FokI to generate a 1 kb fragment containing the entire E2 ORF and only 8 nt of the E5 gene (nt 2755 to 3638). This was blunt-ended with T4 DNA polymerase, followed by the addition of EcoRI linkers, and then inserted into the EcoRI site of the vector pJ4LI, to form the plasmid pJ4LE2. The vector pJ4LI was a gift from J. Morgenstern. To create the N-terminal domain of E2, the FokI fragment was digested with NdeI and a 371 bp fragment was isolated which contains nt 2755 to 3126. The fragment was blunt-ended and EcoRI linkers were added, then inserted into the pJ4LI vector as described above to form the plasmid pJ4LI2*2.
**Transfection and selection.** Cultures of primary BRK cells were prepared and transfected by the DNA–calcium phosphate coprecipitation method (Wigler et al., 1979). Aliquots of the DNA–calcium phosphate precipitate (0.4 ml) containing 10 μg of each of the indicated plasmids were added to 90 mm dishes of sub-confluent primary BRK cells. After glycerol treatment the cells were split to two 90 mm dishes and cultured in Dulbecco’s modified Eagle’s medium containing 10% foetal calf serum and 200 μg/ml G418, in the presence or absence of 10^{-4}M-dexamethasone. Three weeks after transfection, colonies were either isolated with cloning rings and propagated as cell lines, or the dishes were fixed with formal saline and stained with Giemsa.

**Nucleic acid hybridization.** Total genomic DNA was isolated from transformed BRK colonies which had been transfected with the various HPV DNA-containing plasmids and E-J ras oncongene. DNA samples (10 μg) were digested with BamHI, KpnI or PstI, electrophoresed in 1% agarose gels and subjected to Southern blot analysis (Southern, 1975) using Hybond-N filters (Amersham). Hybridizations were performed using a nick-translated 32P-labelled 1' 1 kb EcoRI–EcoRI fragment (nt 7458 to 112) was blunt-ended using the Klenow fragment of DNA polymerase I, and BamHI linkers were added. This was cloned in the sense orientation, into the BamHI site of pBLCAT3 to generate the plasmid pBLCAT3603.

**Transient transfections and CAT assays.** DNA transfections were performed by DEAE–dextran precipitation using 5 μg of the CAT plasmid plus various amounts of plasmids expressing E2. At 4 h post-transfection, the cells were treated with 10% DMSO for 2 min. The monolayer was washed once with phosphate-buffered saline (PBS) and medium containing 10% serum was then added back. The cells were harvested 48 h after transfection.

CAT assays were performed as described previously (Gorman et al., 1982) with modifications. Briefly, cell extracts were incubated with 0.1 μCi [35S]methionine in 250 mM-Tris–HCl pH 7.8, at 37°C for 1 h. The products of the acetylation reaction were quantified by liquid scintillation.

**Immunoprecipitations.** For analysis of the E7 protein, cells were detached from the beads by boiling in SDS–PAGE disruption buffer (50 mM-Tris–HCl pH 7.4, 2% SDS, 0.1% bromphenol blue, 5% glycerol, 0.1 M-EDTA and 1% aprotinin) and analysed by fluorography (Bonner & Laskey, 1974). For E6 protein detection, cells were labelled with 500 μCi [3H]thymidine per 90 mm dish for 18 h. Cells were lysed in 1 ml tris–HCl–EDTA (20 mM-Tris–HCl pH 7.4, 10 mM-MgCl2, 100 mM-NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1% aprotinin). Protein was immunoprecipitated using AB-2 rat monoclonal antibody (Oncogene Sciences) and analysed by PAGE as described above.

**Results**

**E2 enhances cotransformation by HPV-16 and E-J ras**

Transfection experiments were performed using HPV-16 DNA (W12) cloned into pSP64 as described in Methods. The HPV-16 DNA was derived from a cervical wart containing episomal copies of the HPV DNA (Stanley et al., 1989). The entire virus genome is present in this construct including the non-coding region (URR) which contains sequences essential for transcription in vivo. This plasmid was transfected into BRK cells alone or together with the activated ras oncongene. The plasmid pSV2-neo was also included in the transfections and the cells were subsequently placed under G418 selection (Southern & Berg, 1982). Transformation was monitored by the appearance of dense colonies 3 to 4 weeks post-transfection. As shown in Fig. 1, HPV-16 (W12) will cooperate only weakly with the activated ras oncongene to give generally less than five G418-resistant colonies per dish (see Table 1). In experiments (Matlashewski et al., 1987; Storey et al., 1988) in which HPV-16 DNA was cloned behind heterologous promoters, transformation in cooperation with ras was approximately 10-fold higher than in the experiments described here where only the endogenous HPV-16 promoter was present.

The E2 gene of the papillomavirus encodes a regulator of viral gene expression (Spalholz et al., 1985; Haugen et al., 1987; Hirochika et al., 1987). The cotransformation assay used here provides a method for studying this regulation further by assaying the effects of E2 on the weak endogenous viral promoters, to deduce possible roles E2 may play in transcriptional control and transformation. For these studies the E2 gene from HPV-16 was cloned behind a strong heterologous promoter (Moloney murine leukaemia virus long terminal repeat; MoMuLV LTR) in the vector pJ4Q, as shown in Fig. 2. This construct was transfected into BRK cells together with HPV-16 (W12) ras and pSV2-neo as described above. Fig. 1 shows that in the presence of the pJ4QE2 construct, HPV-16 (W12) will cooperate with the ras oncongene to give 30 to 40 G418-resistant colonies per dish. This is an approximately five- to 10-fold increase in the number of colonies obtained when cells are transfected with HPV-16 (W12) and ras alone, indicating that E2 can increase the activity of the viral promoters.
Fig. 1. Transformation of primary BRK cells by HPV DNA. All dishes of cells were transfected with pSV2-neo, pEJ6.6 (containing the Ha-ras oncogene isolated from the EJ/T24 human bladder carcinoma cell line) and HPV-16 (W12, derived from a cervical wart), pJ40E2 (c and d) and pJ40E2*2 (e and f) plasmids were transfected also; (a) and (b) were transfected with HPV-16 and ras, only. Cultures were grown in the presence of 200 µg/ml of G418 and with 10^{-6} M-dexamethasone (b, d and f) or without (a, c and e) as shown. Dishes were fixed and stained 3 weeks after transfection.

Table 1. Number of G418-resistant colonies obtained from plasmid treatment of BRK cells

<table>
<thead>
<tr>
<th>Transfected plasmids</th>
<th>No hormone</th>
<th>Dexamethasone (10^{-6} M)</th>
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<tbody>
<tr>
<td></td>
<td>1*</td>
<td>2 3 4</td>
</tr>
<tr>
<td>W12 + ras</td>
<td>2 0</td>
<td>ND § 10</td>
</tr>
<tr>
<td>W12 + ras + pJ40E2</td>
<td>46 27 43</td>
<td>15 23 1 32 28</td>
</tr>
<tr>
<td>W12 + ras + pJ40E2*2</td>
<td>24 40 25</td>
<td>50 15 24 31 30</td>
</tr>
<tr>
<td>W12 + pJ40E2</td>
<td>0 0 3 0</td>
<td>2 0 3 0</td>
</tr>
<tr>
<td>W12 + pJ40E2*2</td>
<td>0 0 0 0</td>
<td>1 0 4 0</td>
</tr>
<tr>
<td>ras + pJ40E2</td>
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<td>0 0 0 0</td>
</tr>
<tr>
<td>ras</td>
<td>0 0 0 0</td>
<td>0 0 0 0</td>
</tr>
</tbody>
</table>

* Each experiment contained, in addition to the indicated plasmids, pSV2-neo encoding resistance to G418. The numbers of foci are those obtained when 10 µg of each HPV-16-containing plasmid, and 5 µg of pEJ6.6 and pSV2-neo, were added to a 90 mm dish of primary subconfluent BRK cells. Where indicated, dexamethasone was added to the plates after transfection. The numbers indicate that four experiments were done without hormone, and four with hormone.

† HPV-16 DNA from the W12 cell line cloned at the BamHI site of pSP64.
‡ pEJ6.6 plasmid containing the Ha-ras oncogene derived from the human EJ/T24 bladder carcinoma cell line.
§ ND, Not determined.
‖ E2 region of HPV-16 (nt 2755 to 3868) expressed from the MoMuLV LTRs as shown in Fig. 2.
¶ E2 region of HPV-16 (nt 2755 to 3126) expressed from the MoMuLV LTRs as shown in Fig. 2.

It is believed that the DNA-binding and transactivation functions of E2 are encoded by separate domains of the protein. To investigate whether DNA binding is important for these observations the 5' region of the gene (nt 2755 to 3126), encoding the transactivation domain of
the protein and lacking the region encoding the DNA-binding function (McBride et al., 1988; Giri & Yaniv, 1988), was inserted into the pJ4Ω vector to give pJ4ΩE2*2 (Fig. 2). This construct was then transfected into BRK cells as described above. The results show that the pJ4ΩE2*2 construct functioned as efficiently as the full-length E2 in this assay, to increase the number of foci obtained with HPV-16 (W12) and ras (Fig. 1 and Table 1). EJ-ras or HPV-16 DNA alone gave no colonies.

In order to verify that the transformed colonies contained the transfected DNA, cells from these colonies were expanded and analysed for HPV-16 DNA by Southern blot analysis (Southern, 1975). Total cellular DNA from monoclonal cell lines was digested with BamHI, KpnI or PstI, and probed with nick-translated HPV-16 E2 DNA (nt 2755 to 3868). Fig. 3 shows that integrated HPV-16 DNA was present at a high copy number in the cotransfected cell lines. Next we analysed the RNA from these clones to verify that the transfected DNAs were expressed. Total RNA was isolated and analysed by Northern blotting using an 810 bp DNA probe derived from between the multiple cloning site and the polyadenylation site of the pJ4Ω vector (BglII-PstI fragment). Fig. 4(a) shows that those cells transfected with the pJ4Ω constructs continue to express high levels of pJ4ΩE2 (lanes 3 and 4), and pJ4ΩE2*2 (lanes 5 and 6). Fig. 4(b) shows a Northern blot of the same RNA samples, probed with the E2 DNA probe used in the Southern blots. This shows that a number of HPV-16 transcripts are produced in all the cell lines, ranging in size from about 1 to 5 kb.

**Glucocorticoid effects on HPV-16 transformation**

Gloss et al. (1987) made the observation that there was a potential glucocorticoid receptor-binding element in the non-coding region of the virus. To examine the response of HPV-16 to hormones we performed cotransformation assays as described, but the cells were treated with 10⁻⁶ M-dexamethasone (a synthetic glucocorticoid hormone) after transfection. In the presence of dexamethasone the efficiency by which HPV-16 (W12) will cooperate with ras in this assay was increased approximately threefold (Table 1 and Fig. 1), giving 15 to 20 G418-resistant colonies per plate. This implies that the non-coding region is responsive to dexamethasone, leading to an enhanced ability to cooperate with ras in the transformation of BRK cells. Studies by Pater et al. (1988) and Crook et al. (1988) also have shown glucocorticoid responsiveness with HPV-16 and HPV-18 in similar cotransformation assays.

These results indicate that both E2 and dexamethasone are capable of increasing the frequency of cotransformation which can be obtained using the endogenous HPV-16 promoter alone. The substantial difference in the observed number of colonies (Fig. 1) indicates that E2 has a more dramatic effect than glucocorticoids in this assay. When dexamethasone was added to the cells

![Fig. 3. Southern blot analysis of HPV-16 DNA sequences in BRK cells which had been cotransfected with HPV-16 (W12), pSV2-neo, the EJ-ras oncogene and E2 sequences (pJ4ΩE2 or pJ4ΩE2*2). Each lane contains 10 µg of genomic DNA isolated from a monoclonal cell line, digested with either BamHI, KpnI or PstI. Lanes 1 to 3 contain DNA isolated from cells transformed with HPV-16 (W12) and ras (lanes 1 to 3 represent digests with BamHI, KpnI or PstI respectively). Lanes 4 to 6 contain DNA derived from cells transformed by HPV-16 (W12), ras + pJ4ΩE2 cut with BamHI, KpnI and PstI, respectively. Lanes 7 to 9 contain pJ4Ω DNA cut with the same enzymes, lanes 10 to 12 are similar digests of DNA derived from cells transformed with HPV-16 (W12), ras + pJ4ΩE2*2.](image-url)
Fig. 4. Northern blot analysis of RNA obtained from a number of monoclonal cell lines derived from transformed BRK colonies. Lanes 1 and 2, HPV-16 (W12) + ras grown in the presence of 10^{-6}m-dexamethasone; lanes 3 and 4, HPV-16 (W12) + ras + pJ4\(\Omega\)E2; lane 6, HPV-16 (W12) + ras + pJ4\(\Omega\)E2*2. Lane 5 contains RNA derived from a polyclonal mixture of BRK cells transfected with HPV-16 (W12) + ras + pJ4\(\Omega\)E2. (a) The probe used was an 810 bp fragment (BglII-PstI) derived from the simian virus 40 polyadenylation region 3' of the multiple cloning site of pJ4\(\Omega\). The DNA probe used in (b) was the 1-1 kb FokI insert of pJ4f2E2 (nt 2755 to 3868).

Fig. 5. Tumorigenicity of cloned cell lines in syngeneic rats. Animals were injected with 5 × 10^6 cells per flank and monitored for 6 weeks for tumour development. The growth curves represent the average of six tumours per cell line. Tumour size was calculated as \((W^1 \times W^2)/(W^1 + W^2)\), where \(W^1\) and \(W^2\) are diameters (in cm) measured in two dimensions. (■) HPV-16 (W12) + pEJ6.6 + pJ4\(\Omega\)E2; (○) HPV-16 (W12) + pEJ6.6 + dexamethasone; (▲) HPV-16 (W12) + pEJ6.6 + pJ4\(\Omega\)E2*2. The symbol † indicates that the animals were sacrificed.

Transfected with pJ4\(\Omega\)E2 no increase in colony number was seen; indeed the number was frequently reduced (Table 1). We suspect that the reduction observed was either due to toxicity effects of the dexamethasone itself, or possibly due to excess and therefore lethal levels of virus protein(s) produced as a result of stimulation by both dexamethasone and E2.

### Tumour production

Acquisition of a transformed phenotype in vitro is not always accompanied by an increase in tumorigenic potential. We therefore tested the cell lines for their ability to produce tumours in syngeneic rats. Five million cells from each of the transformed cell lines were injected into each flank of three rats per cell line, and monitored for 6 weeks for tumour development. The cells transfected with HPV-16 (W12), ras and pJ4\(\Omega\)E2 gave large tumours (six out of six) within 2 weeks (Fig. 5). Initially all cells transfected with HPV-16 (W12) and ras, and grown in the presence of dexamethasone, gave tumours but these were smaller in size and began to regress after 2 weeks. The tumours stopped regressing by week 4 and recommenced growth, albeit at a slightly reduced rate. Cells transfected with HPV-16 (W12), ras and pJ4\(\Omega\)E2*2 gave smaller tumours in comparison to those with the full-length E2 construct, and by week 6 four of six of the tumours had regressed completely. The more rapid tumour growth observed in the presence of full-length E2, in comparison to the truncated form, suggests that the DNA-binding domain confers a greater tumorigenic potency. From the growth characteristics in vitro it was expected that the cell lines derived in the presence of dexamethasone would grow more slowly in the absence of administered hormone and this was reflected by the partial regression of all tumours formed after an initial 2 week growth period. Animals injected with normal BRK cells failed to give tumours.

### Activation by E2 of promoters in HPV-16 URR

Previous data by Thierry & Yaniv (1987) and Smits et al. (1988) have indicated that although E2 proteins from human virus strains are capable of transactivation, the arrangement of E2-binding sites close to the P97 promoter in these virus types actually leads to the repression of transcription. Therefore we performed CAT assays with the p14\(\Omega\) E2 constructs and a region of the URR containing P97 and two potential E2 sites (located around nt 35 and 50), to confirm that transactivation could occur. The 3' portion of the HPV-16 URR, from the EcoRI site at nt 7454 to the AvaII site at nt 112, was cloned into the plasmid pBLCAT3 to give pBLCATA603 as depicted in Fig. 6. pBLCAT3 contains the coding region of the CAT gene but lacks any promoter
E2 in HPV-16 transformation

Fig. 6. Schematic diagram of the CAT plasmid construction. The EcoRI–AvalI fragment, containing the 3' region of the HPV-16 URR, was cloned into the BamHI site of the MCS of pBLCAT, in front of the CAT encoding sequence. The diagram is not to scale. pBLCATΔ603 was transfected into NIH 3T3 cells together with the E2-expressing plasmids pJ4ΩE2 or pJ4ΩE2*2, and assayed for CAT activity. No significant levels of CAT activity above those obtained with pBLCAT were detected with pBLCATΔ603 alone. However in the presence of pJ4ΩE2 a three- to four-fold induction of CAT activity from pBLCATΔ603 was observed, indicating that the HPV-16 URR contains promoter elements responsive to E2 (Fig. 6). In the presence of pJ4ΩE2*2 a twofold induction of CAT expression was observed. The level of induction increased with the amount of E2-expressing plasmid transfected. The reduced ability of the truncated protein to induce CAT activity is consistent with our findings in the cotransformation assay, where the truncated form of the protein seems slightly less active. These results are in accordance with findings by Cripe et al. (1987) and confirm that with the constructs used in our experiments, HPV-16 is capable of transactivation.

Protein expression

To demonstrate further that the E2 protein was acting on the viral early promoters, the levels of expression of E6 and E7 proteins were examined within our transformed cell lines. All E6–E7 transcripts in HPV-16-containing tumours (so far studied) originate from P97; therefore stimulation of this promoter should be directly reflected by changes in the amounts of these proteins within the cell. Cells were labelled for 1 h with [35S]cysteine and immunoprecipitated with antibodies towards the HPV-16 early gene products E6 (kindly provided by Dr D. Lowy and Dr E. Androphy) and E7 (kindly provided by Dr F. Wettstein). As shown in Fig. 7(b) the levels of E6 protein were the same whether the cells had been transfected with an E2-containing plasmid, or had received glucocorticoids. Immunoprecipitations of E7 from a number of individual clones (Fig. 7a) show that E7 levels were consistently higher in lines transfected with pJ4ΩE2 or pJ4ΩE2*2. In addition levels did appear slightly higher in cells receiving dexamethasone. The cells were also labelled with [35S]methionine and were precipitated with a rat monoclonal antibody specific to the ras oncogene product. Fig. 7(c) shows that the ras protein was expressed in all these cell lines and that the levels remained constant, regardless of the presence of E2 protein or dexamethasone. This demonstrates that the activities of E2 and of dexamethasone, in the cotransformation assays, are not mediated by having any influence on ras protein levels.

We conclude that HPV-16 E2 can transactivate the viral promoters, and as a result increases the level of the E7 protein expressed within the cell, allowing the virus to cooperate more efficiently with the ras oncogene in transforming BRK cells. The E6 and E7 genes are transcribed from the same promoter so it is expected that E6 transcription would be affected also. However, in all cell lines examined, the E6 protein levels remained constant, irrespective of conditions. This may indicate that expression of the E6 protein is closely controlled at the translational level.

Discussion

The results presented here indicate that the E2 protein of HPV-16 is able to transactivate viral gene expression in primary epithelial cells, resulting in an increased frequency of transformation. The BRK cotransformation assay provides a good model system in which to study the regulation of the papillomavirus genes, because HPVs are strictly epitheliotropic in vivo. All papillomavirus types, to date, exhibit a control mechanism mediated by the product of the E2 ORF, suggesting that

<table>
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<th>Construct</th>
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<tr>
<td>pBLCAT3</td>
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<td>0</td>
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<tr>
<td>pBLCATΔ603</td>
<td>473, 373, 548</td>
<td>0</td>
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<td>pBLCATΔ603 + pJ4Ω</td>
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<tr>
<td>pBLCATΔ603 + pJ4ΩE2 (5 pg)</td>
<td>1079, 1498</td>
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<td>(10 pg)</td>
<td>2156, 2212, 2223</td>
<td>4.4</td>
</tr>
<tr>
<td>pBLCATΔ603 + pJ4ΩE2*2 (5 pg)</td>
<td>1105, 640, 773, 722</td>
<td>1.7</td>
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<td>(10 pg)</td>
<td>899, 1196, 1224</td>
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Fig. 7. Immunoprecipitation analysis of protein expression. Cells were labelled with [35S]cysteine as described in Methods and proteins were immunoprecipitated with an anti-E7 rabbit polyclonal antibody in (a) (Smotkin & Wettstein, 1986), or an anti-E6 polyclonal antibody in (b) (Androphy et al., 1987). (a) Lane 1, HPV-16 (W12) + ras; lanes 2 and 6, HPV-16 (W12) + ras + dexamethasone; lane 3, HPV-16 (W12) + ras + pJ4OE2; lanes 4 and 5, HPV-16 (W12) + ras + pJ4OE2*2; lane 7, untransfected BRK cells. (b) Lane 1, HPV-16 (W12) + ras + pJ4OE2; lane 2, HPV-16 (W12) + ras + pJ4OE2; lane 3, HPV-16 (W12) + ras + pJ4OE2*2; lane 4, untransfected BRK cells. (c) Cells were labelled with [35S]methionine for 18 h and proteins were immunoprecipitated with a rat anti-Ha-ras monoclonal antibody (Oncogene Sciences) in lanes 1 to 4 or a rat preimmune serum in lane 5. Lane 1, HPV-16 (W12) + ras + pJ4OE2; lane 2, HPV-16 (W12) + ras + pJ4OE2*2; lane 3, HPV-16 (W12) + ras + dexamethasone; lanes 4 and 5, 14/2 cells (HPV-16 E7 + ras + dexamethasone).

It must play a critical role in transcriptional regulation during the life cycle of the virus. CAT assays show clearly that the HPV-16 E2 gene products can transactivate the URR, and the demonstration of elevated levels of the E7 protein in the presence of E2 suggests that it is the E6–E7 promoter which is stimulated. The increase in levels of E7, which in HPV-16 has been shown to have transforming activities (Storey et al., 1988; Phelps et al., 1988), results in higher frequencies of cotransformation in cooperation with ras and an increased tumorigenic potential in immunocompetent animals. The extreme tumorigenicity of the E2-containing cell lines suggests that we may be observing E2-mediated effects on cellular mechanisms as well. Previous data (Spalholz et al., 1987) demonstrate that control by the E2 protein is at the level of transcription rather than through an increased mRNA stability. Data show that dexamethasone can also affect viral transcription but to a lesser extent than E2 in this assay.

In terms of the life cycle of the virus, the expression of the full-length E2 gene is likely to occur at an early stage of infection when the DNA is episomal. This would lead to the activation of the viral promoters and in turn to the expression of the early genes. There is genetic evidence (DiMaio & Settleman, 1988) that continued E2 activity is required for the maintenance of BPV-1-induced transformation and for normal viral DNA replication. In BPV-1 E5 is necessary for transformation and its expression is directly regulated by E2 (Hermonat et al., 1988; Prakash et al., 1988). Our data indicate that the expression of E7 in HPV-16, the major transforming protein in this virus type, is also controlled by E2 and it has been shown by Crook et al. (1989) that sustained expression of E7 is required for the maintenance of the transformed phenotype in vitro. It is possible that E2, in addition, may modulate host gene expression, and that perturbation in virus or host gene expression is necessary for carcinogenic progression from benign papillomas to carcinomas.

It has been suggested that virus integration into the host genome is a critical event for oncogenic transformation. Unfortunately integration is still poorly understood, largely due to the non-permissiveness of tissue culture cells to papillomaviruses. Detailed analyses of DNA and RNA derived from transformed cell lines have shown that integration into the host genome often occurs within the E1 or E2 ORFs, leading to the disruption of the E2 gene. The presence of mRNA, corresponding to the whole of the E2 gene, within some cervical cancer cell lines implies that disruption of the E2 gene is not essential for transformation (Baker et al., 1987). However its frequent interruption in malignant cells does...
suggest that the sustained expression of E2 is not absolutely required for the transformed phenotype. To maintain expression of the transforming proteins following integration other factors must be present within the cell to increase the activity of the apparently weak viral promoters when E2 is absent. The integration event itself may put the viral early genes in juxtaposition to cellular genes, whose control mechanisms could then assume control of viral gene expression. Alternatively trans-acting factors may be provided by the host cell or from the external environment. Results presented here show that HPV-16 is responsive to glucocorticoids; these may be examples of such factors.

The E2 protein, in addition to its ability to transactivate, also appears capable of repressing transcription. Thierry & Yaniv (1987) showed that the BPV-1 E2 protein could repress the HPV-18 promoter in CAT assays. It was postulated that this repression arises through steric interference of a transcription complex being formed on the early P105 promoter (P97 in HPV-16), since there are two E2 binding sites located between the putative CAAT and TATA boxes of this promoter. However the homologous E2 gene product of HPV-18 has a very limited effect on both the HPV-18 enhancer and promoter constructions. In BPV-1 the E2 binding sites are found more than 130 bp upstream of the major promoter P98; therefore any steric interference is unlikely and transactivation is observed with the full-length protein. In a study by Smits et al. (1988) it was reported that the interruption of the E2 ORF increases the frequency of transformation of human fibroblasts by HPV-16. The fibroblasts used in this system were heterozygous and had a deletion in the short arm of one chromosome 11. Normal diploid cells were not susceptible to transformation. Despite the abnormality of this cell line, repression by E2 was clearly observed. Our studies in contrast show clearly that E2 can transactivate the HPV-16 URR, and there was no evidence of repression. Cripe et al. (1987) have shown also that the URR of HPV-16 is positively responsive to E2. We must therefore conclude that the E2 protein can both transactivate and repress transcription, with the particular activity exhibited being dependent on the virus type and the assay system (e.g. cell line) used. These differences must be resolved to understand how the protein acts in vivo. This dependence on the assay system used has also been found in other transformation studies; in epithelial cells E7 is sufficient to cause transformation (Storey et al., 1988), whereas in keratinocytes both E6 and E7 appear necessary (Schlegel et al., 1988).

In addition to the importance of E2 to the virus itself, it serves as a good model for the action of transactivating proteins in general. E2 has transactivating activity, the specificity of which is determined by direct binding to a known palindromic sequence repeated several times in the URR of all known papillomaviruses. DNA-binding and transactivation functions are encoded by two separate domains of the protein. Several transactivating proteins have been isolated (Brent & Ptashne, 1985; Hope & Struhl, 1986; Keegan et al., 1986) that have separate domains for DNA binding and transactivation. It has been shown that these domains are often interchangeable between proteins. For example in the GAL4 transactivator protein of yeast, the DNA-binding domain can be replaced by a prokaryotic DNA-binding domain without losing the ability to transactivate (Keegan et al., 1986). The functional interchangeability between these two proteins supports the notion that DNA binding is not directly involved in transactivation, as suggested by the existence of a whole family of transactivators that do not appear to bind DNA, such as the adenovirus E1A protein (Petterson & Roberts, 1986). DNA binding appears important either directly, or through cofactors, for the localization of the transactivator close to its target promoter. Previous studies have suggested that the DNA-binding region of the E2 protein is essential for specific viral transactivation. Results shown here indicate that this is not always the case; the transactivation domain of the protein, alone, can replace the full-length protein in this assay. Haugen et al. (1988) have reported similar observations when using the truncated protein in CAT assays. They show that if sufficiently high levels of the truncated protein are used, the DNA-binding domain is not necessary to achieve transactivation. Our CAT assays are in agreement, showing a twofold induction of CAT activity in the presence of pJ4ΩE2*2. These data imply that the DNA-binding activity of E2 serves to localize the protein at the cognate cis attachment sites, and thus enhances its activity on the specific promoter.

Experiments are in progress to examine the activity of the DNA-binding domain of the E2 protein in this assay, particularly with respect to its role in tumour formation. Previous evidence suggests that this portion of the protein is capable of repressing the activity of the full-length protein, where the transactivation domain is still intact (Cripe et al., 1987; Lambert et al., 1987; Chin et al., 1988). This ability to repress is most likely the result of competition for the specific E2-binding sequences in the URR. The importance of this activity in vivo is unclear. The E2 ORF in BPV-1 appears to encode a second E2 protein of 31K (full-length E2 is 43K), encoded by the C-terminal part of the E2 ORF, which has repressor activity (Hubbert et al., 1988; Choe et al., 1989). Therefore it is possible that expression from the E2-responsive promoters in the URR is controlled by the relative concentrations of the two forms of the protein. However the transcriptional map of HPV-16 early genes...
is incomplete and no cDNAs corresponding to HPV-16 'short' E2 proteins have so far been identified in infected cells.

It is clear that the products of the E2 ORF play a vital role in the viral life cycle. A better knowledge of how these positive and negative regulators of transcription are interrelated in the control of gene expression is an essential element in our understanding of the role of the papillomaviruses in carcinogenesis.

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References


