Epidermal growth factor induction of human papillomavirus type 16 E6/E7 mRNA in tumour cells involves two AP-1 binding sites in the viral enhancer

Martina Peto, Ines Tolle-Ersü, Hans Georg Kreysch and Gerd Klock*

* Institut für Biochemie, Technische Hochschule Darmstadt, Petersenstrasse 22, 64287 Darmstadt and 2 E. Merck, Frankfurter Strasse 250, 64293 Darmstadt, Germany

The early genes E6 and E7 from human papillomaviruses (HPVs) play a key role in the development of cervical cancer. Modulation of E6 and E7 gene expression may alter tumour progression; therefore, modifiers of viral transcription such as hormones or growth factors are potential risk factors in cancer development. We have analysed the effects of epidermal growth factor (EGF) on E6/E7 mRNA from human papillomavirus type 16 (HPV-16) by Northern blot in two cell lines, SiHa cervical carcinoma cells, and HPK IA, an HPV-16-immortalized keratinocyte cell line. E6/E7 mRNA is EGF-inducible in SiHa cells, with the earliest response after 2 h. In contrast, in HPK IA cells no increase in E6/E7 RNA is observed, suggesting a differential EGF response of viral transcription in tumour cells compared with keratinocytes. We demonstrate that the cell type-specific HPV-16 enhancer is a target of EGF-induced signals, as its activity is amplified by EGF in SiHa cell transfections. However, when transfected into HPK IA keratinocytes, the viral enhancer shows no EGF response. The enhancer contains two binding sites for the transcription factor AP-1, a potential mediator of the EGF signalling cascade. Enhancer subfragments with single AP-1 binding sites are also EGF-responsive in SiHa cells. Mutating either AP-1 site in the complete enhancer decreases the EGF response, whereas a double mutation causes a complete loss of EGF regulation, suggesting that the EGF induction of HPV-16 early transcription requires AP-1 activation. We conclude that alterations of EGF responsiveness that increase viral oncogene expression may contribute to cervical cancer progression.

Introduction

It is now well established that certain types of human papillomaviruses are involved in the development of anogenital cancer (Galloway & McDougall, 1989; zur Hausen, 1991). HPV-16 and HPV-18 belong to a group of genital HPVs which are found at high frequency in cervical cancers (zur Hausen, 1991). In their normal life cycle, these HPVs infect keratinocytes of mucosal skin, where viral reproduction is linked to cellular differentiation (Galloway & McDougall, 1989; Meyers et al., 1992). The viral early genes E6 and E7 from HPV-16 and -18 DNA have cell immortalizing and transforming activities (Dürst et al., 1987; Matlashewski et al., 1987; Sedman et al., 1991) which are believed to result from the ability of E6 and E7 proteins to inactivate tumour suppressor proteins (Dyson et al., 1989; Scheffner et al., 1990). Since E6 and E7 proteins appear to play a key role in cervical cancer development, all events that modulate E6/E7 gene expression, like mutations of cellular and viral regulatory genes, or biochemical signals regulating transcription, may also influence tumour growth and progression. One such event could be the integration of HPV DNA into the cellular genome (Schneider-Maunouri et al., 1987; Schwarz et al., 1985), as it frequently results in inactivation of the viral E2 open reading frame (ORF) thereby relieving HPV E6 and E7 transcriptional repression by E2 protein (Bernard et al., 1989; Romanczuk, 1990). In contrast, a large proportion of cervical cancers containing HPV-16 DNA still retain an episomal viral genome (Fuchs et al., 1989; Matsukura et al., 1989), suggesting that other events such as mutations of cellular genes may contribute to tumour progression by causing a deregulation of viral gene expression (Bosch et al., 1990; Smits et al., 1990; zur Hausen, 1991).

HPV-16 E6 and E7 transcription is regulated by elements in the long control region (LCR) including the promoter P97, which is under control of an upstream enhancer that shows specificity for epithelial cell types (Cripe et al., 1987; Gloss et al., 1987). The enhancer has
a complex structure and contains numerous binding sites for nuclear proteins (Chong et al., 1991; Gloss et al., 1989; Ishijii et al., 1992; List et al., 1994; May et al., 1994; Sibbet & Campo, 1990). Steroid hormones, namely glucocorticoids and progesterone, induce HPV-16 early transcription through a glucocorticoid/progesterone response element (GRE/PRE) in the enhancer (Chan et al., 1989; Gloss et al., 1987). In addition, HPV-16 E6/E7 mRNA is inducible by oestrogens (Mitrani-Rosenbaum et al., 1989), and by the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) through protein kinase C (PK-C) activation (Chan et al., 1990). The TPA regulation is mediated by two binding sites for the transcription factor AP-1 in the HPV-16 enhancer (Chan et al., 1990; Cripe et al., 1990). AP-1 is a heterodimer consisting of c-Jun and c-Fos proto-oncoproteins, or other members of the Fos and Jun families (Angel et al., 1987; Curran & Franza, 1988). TPA activates AP-1 in a variety of cell types; however, it is not known how HPV-16 is naturally regulated via the AP-1 pathway in cervical epithelial cells.

Epidermal growth factor (EGF) and transforming growth factor-alpha (TGF-α), which serve as mitogens for epithelial cells and several other cell types (Carpenter & Cohen, 1990; Derynck, 1992), are potential natural inducers of AP-1 in epithelial cells. Binding of EGF or TGF-α to the EGF receptor (EGFR) elicits a variety of intracellular signals, including activation of the intrinsic EGFR tyrosine kinase (for review, see Carpenter & Cohen, 1990; Ullrich & Schlessinger, 1990; Davis, 1993). Recent evidence suggests that activation of a phosphorylation cascade plays a key role in growth factor receptor signal transduction (for review, see Pelech & Sanghera, 1992; Davis, 1993). Following p21^{ras} activation, a protein kinase cascade is initiated that includes Raf-1 kinase, MAP kinase kinase (also called MEK), MAP kinase and ribosomal protein S6 kinase (rsk) (Ahn et al., 1990; Chung et al., 1991; Kolch et al., 1993; McCormick, 1993; Wood et al., 1992). The family of MAP kinases (mitogen-activated protein kinases), also called extra-cellular signal-regulated protein kinases (ERKs), have serine/threonine kinase activity and are themselves phosphorylated during activation (Ahn et al., 1990; for review, see Pelech & Sanghera, 1992). MAP kinases are believed to play a key role in conveying the signal evoked by growth factors like EGF to the nucleus: they have been shown to phosphorylate transcription factors, like c-Jun and c-Fos, thereby presumably contributing to AP-1 activation (Pulverer et al., 1991; Smeal et al., 1992; Davis, 1993).

A link between the EGF response and papillomavirus proteins was suggested by the finding that bovine papillomavirus type 1 (BPV-1) protein E5 (Martin et al., 1989) or HPV-16 E5 can cooperate with EGFR (Leechanachai et al., 1992; Pim et al., 1992) or with human T cell leukaemia/lymphotrophic virus p12 protein (Franchini et al., 1993) in cell transformation. Integrated HPV genomes have often deleted both the E2 and E5 ORFs, suggesting that effects of HPV E5 protein could play a role in growth factor signal transduction and cell transformation at an early stage in tumour development, when the HPV genome is still episomally replicated. These findings and the linkage of EGF signalling pathways to the proto-oncogenes ras and raf have prompted us to investigate the possible effects of EGF on HPV-16 oncogene expression. In a recent study, HPV-16 E6/E7 transcription was shown to be repressed by long-term incubation with EGF in an HPV-16 immortalized cell line (Yasumoto et al., 1991). We have shown here that in the cervical carcinoma cell line SiHa, E6/E7 mRNA is induced by EGF treatment. The HPV-16 enhancer functions as an EGF-inducible element in SiHa cells. Two AP-1 binding sites in the enhancer are essential for the EGF response, suggesting an involvement of the EGFR/MAP kinase signalling cascade. Because no EGF-dependent increase in viral early transcription is observed in HPV-16 immortalized keratinocytes, we conclude that changes in growth factor signalling pathways may play a role in tumour progression involving HPV-16.

**Methods**

Oligonucleotides and plasmids. Oligonucleotide synthesis was performed on an Applied Biosystems 380A DNA synthesizer by the phosphoramidite method. The oligonucleotides were purified by HPLC before use. In the CAT expression vector pBLCAT5, a part of the pUC sequence containing several fortuitous protein binding sites was replaced by a fragment with poly(A) sites to prevent readthrough into the CAT gene (Boshart et al., 1992). pH16EN1 contains the 400 bp HPV-16 enhancer fragment (position 7455–7583; exact length 399 bp) in syn orientation upstream of the herpes simplex virus tk promoter and the CAT gene in pBLCAT5 (List et al., 1994). The following plasmids were constructed based on pBLCAT5 and contain enhancer subfragments upstream of the tk promoter in syn-orientation (except for pH16EN189-O, anti-orientation). pH16EN189-O contains a 189 bp fragment (position 7454–7643) which was inserted as a BamHI–Rsal fragment into BamHI–SalI (filled-in) sites of pBLCAT5. Similarly, to construct pH16EN210-U, an Rsal (partial)–BamHI fragment (position 7644–7853) from pH16EN1–189-O was inserted into pBLCAT5/BamHI/SalI (filled-in). A 156 bp fragment (SphI–DraIII; HPV-16 position 7465–7620) was cut out from pH16-SAI-15 (List et al., 1994) with HindIII and SpeI and inserted into pBLCAT5 (HindIII/XbaI), resulting in pH16EN156-W. To construct pH16EN112-Z, overlapping oligonucleotides covering the enhancer core from fpse to fp66 (Gloss et al., 1989) were inserted into pBluescribe cut with SphI and SalI, resulting in pH16ENbl112-Z, and recloned as a SalI–XbaI fragment into the SalI site of pBLCAT5 to give pH16EN112-Z. The sequence of the insert consisting of eight oligonucleotides (interrupted by ‘,’') is given in Fig. 1. The construction was confirmed by sequencing the insert.

**Cell culture and Northern analysis.** SiHa cells were grown in DMEM (Seromed) or DMEM–F12 (1:1) (Gibco) provided with 9% fetal calf...
EGF regulation of HPV-16 E6/E7 in tumour cells

5' cgagTACACTGCACTATGTGCAA, CTACTGAATCACTATGTACATTGTGTCATATAAATA, 
3'tcgagctcATGTGACGTGATACACGTTGATGAC, TTAGTGATACATGTAACACAGTATTTTATTTAGTG, 
AATCACTATGCGCCAACGCCTACATACCGCT, GTTAGGCACATATTTTTGGCTTGTg, -3' 
ATACGCGGTTGCGGAATGTATGGCGACAATC¢, GTGTATAAAAACCGAACAcagc t -5'

Fig. 1. Oligonucleotides used to construct the 112 bp enhancer core fragment. Eight oligonucleotides are shown which were used to construct the enhancer core covering footprints fp4e to fp6e (Gloss et al., 1989), resulting in the plasmid pH16EN-112-Z. Complementary sticky ends are underlined; details of the cloning strategy are given in Methods.

(a) 
(b) 

Fig. 2. EGF induces E6/E7 mRNA in the cervical carcinoma cell line SiHa. Autoradiograms of Northern blots performed with total RNA isolated from EGF-treated or untreated SiHa cells. The EGF-dependent increase of E6/E7 mRNA is first observed 2 h after EGF addition. The two E6/E7 mRNAs, 4.1 and 4.4 kb in length, were not separated due to a compression by the ribosomal 28S RNA; these mRNAs are thought to originate from P97 transcripts (Chan et al., 1989); the bands at 1.6/1.4 kb and possibly other minor species (Baker et al., 1987; Mitrani-Rosenbaum et al., 1989) are barely visible since total RNA instead of poly(A)+ RNA was used. The probes used for hybridization were an RNA probe complementary to the HPV-16 E6 and E7 genes (Dürst et al., 1987), or a DNA probe for the GAPDH gene (Stewart et al., 1990). SiHa cells cultivated in DMEM containing 9% FCS were put on medium containing 0.5% FCS 24 h before the time course experiment, which was performed up to 48 h (a) or 4 h (b), and was started without medium change. The cells in two parallel plates were treated either with EGF (10 ng/ml) dissolved in PBS, or with PBS alone as a control. After the indicated times, total RNA was isolated and 10 μg RNA from each sample was analysed by gel electrophoresis and blotted on gene screen filters. The filters were first hybridized with the E6/E7 probe (panels i) and secondly with the GAPDH probe (panels ii) to control for RNA loading.

Northern analysis was performed with total RNA by the glyoxal denaturation method, and after electrophoresis, the RNA was blotted to GeneScreen filters (Du Pont). The filters were hybridized with an RNA probe covering the HPV-16 E6 and E7 reading frames (position 7454-875; Dürst et al., 1987). The probe was synthesized by T3 RNA polymerase (Boehringer Mannheim) in the presence of \([\alpha-32P]UTP\). Rehybridization of the filters was performed with a DNA probe of the GAPDH gene (Stewart et al., 1990) that was synthesized by random priming with DNA polymerase I Klenow fragment in the presence of \([\alpha-32P]dATP\). Autoradiography was done with Kodak XAR film for 3 to 14 days.
EGF receptor assay by immunofluorescence. The cell lines A431 (Haigler et al., 1978), Jurkat, HPK IA or SiHa cells were washed with BSA-PBS (PBS without Ca\(^{2+}\) and Mg\(^{2+}\) containing 1% BSA and 0.03% NaN\(_3\)) and adjusted to \(1 \times 10^6\) cells/ml. Aliquots (1 ml) of the cell suspensions were distributed into 12 x 75 mm polypropylene tubes. After centrifugation (5 min at 300 g), supernatants were removed and the cells were resuspended in 100 \(\mu\)l BSA–PBS containing an FITC-conjugated monoclonal antibody directed against the ligand binding domain of the human EGF receptor (MAb 425–FITC, E. Merck; isotype, mouse IgG2a; staining concentration, 10 \(\mu\)g/ml; Murthy et al., 1987). Parallel samples of each cell line were incubated with BSA–PBS, or a solution of FITC-conjugated mouse IgG2a antibody, which served as a control for nonspecific binding. After 15 min incubation on ice, the cells were washed twice with BSA–PBS and counterstained with propidium iodide for dead cell exclusion. Fluorescence distributions of cells were analysed by flow cytometry (FACScan, Beckton-Dickinson). All cells were negative with the IgG2a control antibody. Jurkat cells, as well as two insect cell lines tested, were EGFR negative and gave relative fluorescence intensities (MAb425–FITC/IgG2a–FITC) of less than 1:3.

Adaption of SiHa cells to serum-free medium. SiHa cells grown in DMEM with 10% FCS, penicillin and streptomycin (see above) were shifted stepwise to media with lower serum concentrations. The protocol included 4 days on 5% FCS, 3 to 4 days on 2.5% FCS, and 4 days on 0.5% FCS. Next, the cells were adapted for 4 to 5 days to a serum-free defined medium (sf medium): DMEM–F12 (1:1) supplemented with 15 mm-HEPES, BSA (final concentration 1 mg/ml), insulin (8 \(\mu\)g/ml) (both from Boehringer Mannheim), transferrin (5 \(\mu\)g/ml), sodium selenite (4 \(\mu\)g/ml), phosphoethanolamine (14 \(\mu\)g/ml) (all from Serva), hydrocortisone (10 \(\mu\)g/ml; Sigma), kanamycin (50 \(\mu\)g/ml) and amphotericin B (2.5 \(\mu\)g/ml). The cells were split every 3 to 4 days, after which the trypsin used was complexed with soybean trypsin inhibitor (Serva). The adapted cell line, called ‘SiHa-sf’, was grown for a further two to three passages in sf medium and stored in liquid nitrogen. Aliquots of the SiHa-sf cells were thawed and cultured for two to three passages before the transfections.

DNA transfections and CAT assays. SiHa cells were transfected with plasmid DNA using the DEAE-dextran procedure, as described (List et al., 1994). Two different protocols were used, both with SiHa cells in normal medium and with the adapted cells (SiHa-sf) in the serum-free, defined sf medium. In the first protocol, the cells were plated at a density of 0.4 to 2 x 10^6 per 9 cm dish 18 h prior to the transfection, which was done with 10 \(\mu\)g plasmid DNA per dish. Next, the cells were washed twice with Tris-buffered saline (TBS), and incubated with 25% DMSO (in DMEM) for 3 min. The cells were put on 5% serum for 16 to 18 h, followed by 24 h on low serum (0.5%) for SiHa cells grown in serum, or 18 h on sf medium, followed by sf medium without insulin and hydrocortisone for SiHa-sf cells. Parallel culture dishes were subsequently treated with EGF (10 or 50 ng/ml, as indicated) for 6, 8 or 24 h, or left untreated as controls. In the second protocol, 2 x 10^6 cells were seeded 3 days before the transfection (or 5 x 10^6 cells 1 day before transfection) in 15 cm dishes and were transfected with 25 \(\mu\)g DNA. After the DMSO shock, the cells were kept in medium with 5% serum for 4 h, split on 5 cm dishes and kept in medium containing 5% serum for 14 h, before they were put on low serum and treated with or without EGF, as above. HPK IA cells were transfected by lipofection as described by Teifel & Friedli (1995). The two lipids dimethyl-dioctadecylammonium bromide (DDAB; Rose et al., 1991), purchased from Fluka, and azolectin (phosphatidylcholine II resp. IV; Sigma) were dissolved in water to final concentrations of 0.4 mg/ml (DDAB) and 1 mg/ml (azolectin), respectively. The suspension was sonicated (50 W/5 min), vortexed, sonicated again for 5 min, and stored on ice before use. A 16 \(\mu\)l volume of the lipid mixture was mixed with 4 \(\mu\)g plasmid DNA (30 to 60 \(\mu\)l volume) in 0.4 ml (final volume) DMEM–F12 medium (without additives) and incubated for 30 min at 20 °C, resulting in the lipofection mix used per 6 cm diameter culture dish. HPK IA cells were plated at 10^5 cells/cm^2 on 6 cm dishes 1 day before the lipofection and were grown in DMEM–F12 medium with 9% FCS. The medium was changed to low serum (0.5% FCS) for 24 h after the lipofection reaction and the cells were incubated for 24 h. The cells were then transferred to medium containing low serum (0.1% NCS, 24 h) and treated with EGF (50 ng/ml) for 24 h. Cellular extracts were prepared by freezing and thawing, and CAT enzyme activity tests were performed with 30 to 100 \(\mu\)g protein (List et al., 1994). The CAT activities shown are mean values from three to six independent transfections.

Site-directed mutagenesis. The HPV-16 enhancer in pH16EPH-wt was mutated by the method of Kunkel et al. (1987), as described (U-
EGF regulation of HPV-16 E6/E7 in tumour cells

1949

DNA mutagenesis kit, Boehringer Mannheim; List et al., 1994). DNA primers used for mutagenesis were as follows: (mutant bases are underlined, the resulting plasmids are given in parentheses) mAPlfp4, 5’ GTGCAACTACTGAAATCTATGTACATTGCTG 3’ (pH16EPH-AP1-4e); mAPlfp9, 5’ GAACTGTGTAAAGGTAGTACTTACATT-GTTCAATTGTG 3’ (pH16EPH-AP1-9e); both oligonucleotides were used together to construct the double mutant pH16EPH-AP1-4e9e. For mutagenesis, the oligonucleotides were hybridized to the single-stranded template and incubated with T4 DNA polymerase and DNA ligase and subsequently transfected into E. coli BMH 71-18 mutS. Positive clones were identified by restriction digestion or by sequencing; plasmid DNA for transfections was prepared from E. coli DH5α using Qiagen columns. Two independent clones were isolated for each mutation, and were confirmed by sequencing of the complete enhancer and by restriction digestion. The two independent clones were tested in transfections, and CAT activities are given as mean values of transfections with both isolates.

Results

HPV-16 E6/E7 mRNA is inducible by EGF in SiHa cells, but not in HPK IA keratinocytes

The human cervical carcinoma cell line SiHa was chosen to examine possible effects of EGF on HPV-16 E6 and E7 transcription. SiHa cells contain the HPV-16 genome as a single incomplete copy integrated in the cellular genome, with inactivated E2 and E50RFs (Baker et al., 1987). SiHa cells grown in the presence of 9% FCS were put on low serum (0.5% FCS) for 24 h. Subsequently, the cells were treated with 10 ng/ml EGF for various times, or left untreated as a control (Fig. 2a). Total RNA was isolated and analysed by Northern blot using an E6/E7 probe. As can be seen from Fig. 2, EGF treatment causes an increase in E6/E7 mRNA (4.9/4.1 kb). The EGF-dependent induction of the viral RNA is seen throughout the kinetics, starting from 2 h up to 48 h. The GAPDH mRNA, which served as a control, is not inducible by EGF (Fig. 2a). The observed EGF regulation was confirmed by repeating the EGF induction and Northern analysis (data not shown). In addition to the growth factor response, a gradual increase in the basal E6/E7 expression is observed during the time course, independently of EGF treatment. Growth factor induced transcription of immediate-early genes, such as c-jun and c-fos occurs within minutes, whereas induction of the ‘delayed early response’ genes may require the formation of Jun and Fos proteins (Williams & Lau, 1993). To find out when the EGF response initiates, we performed a time course with 0.5 h, 1 h, 2 h and 4 h EGF treatment. The earliest EGF-dependent increase in E6/E7 mRNA is seen after 2 h, whereas no EGF induction but rather a slight repression was observed between 0.5 and 1 h EGF treatment (Fig. 2b). The time course suggests that the observed EGF induction of the viral RNA directly follows the cellular immediate and delayed early genes.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>F(EGFR)</th>
<th>F(control)</th>
<th>R</th>
<th>ΔF</th>
</tr>
</thead>
<tbody>
<tr>
<td>A431</td>
<td>1640.0</td>
<td>14.3</td>
<td>114.4</td>
<td>1625.7</td>
</tr>
<tr>
<td>Jurkat</td>
<td>3.1</td>
<td>2.8</td>
<td>1.1</td>
<td>0.3</td>
</tr>
<tr>
<td>HPK IA</td>
<td>342.9</td>
<td>5.3</td>
<td>65.6</td>
<td>337.6</td>
</tr>
<tr>
<td>SiHa</td>
<td>79.2</td>
<td>8.2</td>
<td>9.7</td>
<td>71.6</td>
</tr>
</tbody>
</table>

Fig. 4. Presence of the EGF receptor in SiHa and HPK IA cell lines. The cell lines were incubated with FITC-conjugated antibodies: MAb 425–FITC is directed against the human EGF receptor, and FITC-conjugated mouse IgG2a served as a negative control, since specific binding of this antibody to the cells was not observed. Fluorescence intensities of the cells (F), resulting from binding of the fluorescent antibodies to the cell surface, were analysed by flow cytometry. F(control) results from the control antibody, IgG2a; F(EGFR) is from MAb 425; R is the relative intensity of the specific and the control (nonspecific) fluorescences, which was below 1.3 for Jurkat cells and for two additional insect cell lines tested in parallel (not shown); ΔF, the specific fluorescence, is the difference between the two fluorescence intensities (EGFR and control antibodies) and is a measure of the amount of EGF receptor.

Next we wanted to determine whether in the keratinocyte cell line HPK IA, E6/E7 mRNA is also regulated by EGF. HPK IA cells are human keratinocytes immortalized with HPV-16 DNA (Dürst et al., 1987). For EGF regulation, HPK IA cells were put on low serum medium and were subsequently induced with EGF, after which total cellular RNA was isolated and analysed by Northern blot (Fig. 3). The amount of
E6/E7 mRNA is virtually unchanged when HPK IA cells are treated with EGF. As with SiHa cells, the GAPDH mRNA served as a control and did not respond to EGF treatment in HPK IA cells.

To exclude the possibility that the missing EGF response in HPK IA keratinocytes was due to the absence of EGF receptor (EGFR), we tested HPK IA cells and SiHa cells by immunofluorescence staining. The EGFR-overexpressing cell line A431 served as a positive control, and cells without EGFR were also included (Fig. 4). We used the fluorescent antibody MAb 425–FITC, which is directed against the ligand binding domain of EGFR and recognizes this receptor domain on the cell surface. Following incubation of the cells with the EGFR antibody, fluorescent cells were analysed by a fluorescence-activated cell sorter (FACS). Both cell lines, HPK IA and SiHa, express the EGFR receptor on the cell surface, with higher fluorescence intensity resulting from HPK IA keratinocytes (Fig. 4). A431 cells give the strongest signal, which is consistent with previous findings of 10 to 50 times higher EGFR expression in these cells compared to normal cells (Fabricant et al., 1977; Haigler et al., 1978). This demonstrates that the EGF receptor is present in HPK IA cells, suggesting that E6/E7 mRNA production is regulated differently in the tumour cell line SiHa, where EGF induces the viral genes, compared with HPV-16-immortalized keratinocytes that showed no EGF response.

**EGF increases the HPV-16 enhancer activity in SiHa cells**

The transcriptional enhancer from HPV-16 responds to various signals: steroid hormones and phorbol ester TPA modulate viral early gene expression by stimulating the enhancer activity. To find out whether the EGF regulation described above is also conferred by the enhancer, we performed transient transfections in SiHa cells with the plasmid pH16EN-1 containing the 400 bp enhancer fragment upstream of the tk promoter and
CAT gene. The vector pBLCAT5 was transfected in parallel and served as a control. The transfected cells were treated either with EGF or with 10% FCS, or left untreated for 8 h, after which the cells were harvested and CAT enzyme activity was determined in cellular extracts. Fig. 5(a) shows that the vector pBLCAT5 is not inducible by EGF or FCS (‘Serum’). In contrast, the plasmid pH16EN-1 containing the HPV-16 enhancer responds to EGF treatment with a 2-5-fold increase in CAT activity (Fig. 5a). Interestingly FCS, which contains a number of growth factors, causes only a slight increase in CAT expression. We performed several transfections with EGF treatment for 8 or 24 h to determine the EGF inducibility of pH16EN-1 under both conditions. The EGF response varied between 2-3- and 2-9-fold (on average 2-6-fold) with 8 h EGF treatment, and 2-7- to 4-8-fold (average induction 3-7-fold; see also below) when induced for 24 h (Fig. 5b).

In order to exclude effects of serum growth factors on the EGF response, we wished to transfect SiHa cells in a serum-free, defined medium. Therefore, SiHa cells adapted to serum-free medium (sf medium; see Methods), termed SiHa-sf, were transiently transfected with pH16EN-1 or pBLCAT5. The cells were subsequently kept in sf medium supplemented with EGF, insulin or FCS, or without inducers, respectively and CAT activities were determined in cellular extracts. The results depicted in Fig. 5(c) show that, while the vector pBLCAT5 did not respond to any of the growth factors, the HPV-16 enhancer plasmid pH16EN-1 showed a 4-5-fold induction of CAT expression by EGF treatment in SiHa-sf cells. Insulin and FCS gave a weaker increase in CAT activity (1-3- and 2-2-fold, respectively; Fig. 5c). These findings demonstrate that in SiHa cells the HPV-16 enhancer is EGF-responsive both under standard conditions (with serum) or when cultivated in serum-free defined medium.

To find out whether the missing EGF-inducibility of E6/E7 mRNA in HPK IA keratinocytes correlates with non-responsiveness of the viral enhancer to EGF in these cells, we transfected HPK IA cells using a lipofection protocol (see Methods). CAT enzyme activity expressed from the enhancer plasmid pH16EN-1 is not EGF-inducible in HPK IA cells (Fig. 6), while the vector pBLCAT5 shows a weak repression in these cells. The 400 bp complete HPV-16 enhancer only weakly stimulates the tk promoter under these conditions; in independent HPK IA transfections, we observed up to 10-fold enhancing effects but no EGF response of the viral enhancer (data not shown). Taken together, these findings support the idea that the enhancer is required for the EGF response in SiHa cervical carcinoma cells, whereas in keratinocytes EGF induction of the enhancer is not permitted.

**EGF responsiveness of HPV-16 enhancer subfragments**

The 400 bp HPV-16 enhancer contains two AP-1 binding sites that allow a transcriptional response to TPA, presumably via activation of PK-C (Chan et al., 1990; Cripe et al., 1990). In epithelial cell types, EGF is a potential natural inducer of AP-1, whose activation probably requires the ras/MAP kinase signalling cascade. In order to localize EGF responsive elements in the cell type-specific enhancer, we constructed subclones of pH16EN-1 containing enhancer subfragments with or without the two AP-1 binding sites (Fig. 7a). The subclones were transfected into SiHa cells and the average EGF responses are given in Fig. 7(a). The EGF-inducibility of the complete 400 bp enhancer in pH16EN-1 varied between 2-5- and 12-fold, with an average induction of 5-5-fold (Fig. 7a and data not shown). Similarly, CAT expression of pH16EN-189-O, containing the distal 189 bp subfragment with the fp4e AP-1 site, was EGF-responsive (2-7-fold; Fig. 7a). In contrast, a related fragment lacking the AP-1 site in pH16EN-156-W gave no significant EGF response compared to pBLCAT5, suggesting that the fp4e AP-1 site on the
Fig. 7. Enhancer subfragments containing a single AP-1 binding site are EGF responsive. (a) Location of subfragments of the HPV-16 400 bp enhancer; the AP-1 binding sites ([]) within fp4e and fp9e are marked. The plasmids with the enhancer fragments were transfected into SiHa cells. Four independent experiments with triplicate cell cultures kept in the presence or absence of EGF, respectively, were performed; the cultures were then treated with EGF for 24 h. The relative CAT activities from EGF-treated and -untreated cells from the four experiments are given as ‘EGF induction’ (average values; see text). (b) High cell density prevents the EGF response of the enhancer core fragment. Two parallel series of SiHa cell cultures plated at two different densities were transfected in
**EGF regulation of HPV-16 E6/E7 in tumour cells**

1953

---

**Fig. 8.** Two AP-1 binding sites in the complete enhancer are required for the EGF response. Mutations were introduced by site-directed mutagenesis into the wild-type 400 bp enhancer in pH16EPH-wt, as described (List et al., 1994). In two single mutants and a double mutant, respectively, the AP-1 binding sites in fp4e or fp9e in the viral enhancer have been destroyed, as indicated. SiHa cells were transfected and tested for EGF-induced CAT expression (see Fig. 7). Both the basal expression (without EGF) and the EGF inducibility of the two single mutants are lower than with the wild-type; the double mutation completely prevents EGF induction.

---

The distal fragment is important for EGF regulation of the 189 bp fragment (Fig. 7a). When the fp4e AP-1 site was tested as part of the central core enhancer in pH16EN-112-Z, it gave on average a 2.5-fold EGF response (Fig. 7a). Interestingly, in repeated experiments we observed a large variation (1.1- to 4.2-fold) in the EGF response of the enhancer core when comparing independent transfections. This variation was not due to a complete lack of EGF response of the transfected cells, since the wild-type enhancer and subfragments were still inducible (data not shown). This suggested that the EGF responsiveness of the enhancer core fragment was subject to additional regulation (see below). Finally, we tested the EGF response of the proximal 210 bp enhancer fragment in pH16EN-210-U which contains the fp9e AP-1 binding site. The proximal fragment gave an EGF-regulated CAT expression with a 3-fold induction on average (Fig. 7a). This suggests that the AP-1 site in fp9e may also be involved in the EGF response of the viral enhancer.

The results with the central enhancer core indicated that an additional regulation could dominate the function of this enhancer domain, which either prevents or permits the EGF response. We had observed that the EGF response of the enhancer core on pH16EN-112-Z was lost when the cells had reached about 90% confluency, while 40% confluent cells gave good EGF induction (data not shown). Therefore we performed two parallel transfection experiments with different cell densities (Fig. 7b). At the lower cell density (70% confluency), the core enhancer fragment still gave a significant EGF response (pH16EN-112-Z, 1.7-fold induction); in contrast, the high cell density (100% confluency) prevented EGF-inducibility of the core fragment (1.1-fold induction). Remarkably, the complete 400 bp enhancer was EGF responsive under both conditions, but showed a weaker induction at the higher cell density. At high density, the proximal enhancer fragment (pH16EN-210-U) gave a better induction (4.3-fold; compared to the lower density 2-fold induction; Fig. 7b) that was identical to the complete enhancer (4.3-fold). This confirms the assumption that the contribution of the enhancer core to the EGF response is minimal at high cell density. Furthermore, these results suggest that parallel ('high' or 'low'; cell numbers plated are shown also). The subfragment-containing plasmids were tested for EGF responsiveness by 24 h EGF treatment, followed by CAT assays (see a). At the lower cell density (70% confluency; ■), a weak response of the enhancer core fragment on pH16EN-112-Z is still observed (1.7-fold inducible), whereas the parallel cell culture at high cell density (100% confluency; □) does not give an EGF response.
the HPV-16 enhancer contains two EGF responsive elements covering the fp4e and fp9e AP-1 binding sites.

Both AP-1 sites in the HPV-16 enhancer are essential for maximal EGF responsiveness

To determine the importance of the two AP-1 sites for the EGF response of the complete enhancer, we constructed AP-1 site mutants in the 400 bp HPV-16 enhancer in pH16EPH-wt by site-directed mutagenesis. In the mutant pH16EPH-AP1-4e, the fp4e AP-1 site is exchanged from 5' CTGAATCA to CTGAACAT; in the second mutant, pH16EPH-AP1-9e, the fp9e AP-1 site is destroyed by the change from 5' GTTAGTCA to GTAGTACT. In addition, we constructed a double mutant, pH16EPH-AP1-4e9e, carrying mutations in both AP-1 binding sites. The mutants were transfected into SiHa cells and tested for EGF regulation of CAT expression. As can be seen from Fig. 8, destroying the fp4e AP-1 site in pH16EPH-AP1-4e causes a strong reduction of the EGF response (1.8-fold inducible) compared with the wild-type 400 bp enhancer (3.4-fold). Similarly, mutating the AP-1 site in fp9e reduces the EGF regulation to 1.7-fold. Finally, the double mutant pH16EPH-AP1-4e9e with no functional AP-1 site has completely lost EGF-inducibility (Fig. 8). Furthermore, when comparing the basal CAT activity (without EGF) of the AP-1 site mutants, one sees a strong reduction of transcriptional activity compared to the wild-type. These results clearly demonstrate that the two AP-1 binding sites are important both for the basal activity and for the EGF responsiveness of the complete HPV-16 enhancer.

Discussion

The expression of the HPV E6 and E7 proteins plays a central role in growth and development of cervical carcinomas. Therefore, we are interested in identifying agents that function as modulators of HPV-16 gene expression. In the cervical carcinoma cell line SiHa, Northern analysis of total cellular RNA showed that viral E6/E7 mRNA is inducible by EGF (Fig. 2). The time course of the EGF regulation revealed that E6/E7 mRNA is induced following the immediate-early response genes, such as c-fos and c-jun (Bravo, 1990). With an earliest response at about 2 h, HPV-16 E6 and E7 genes have EGF-induction kinetics similar to the cellular genes, for vinculin and β1-integrin (Bellas et al., 1991). The observed time course is also consistent with an EGF-induced synthesis and subsequent activation of AP-1 proteins, followed by activation of the enhancer (see below).

The increase in E6/E7 mRNA during the time course, which occurred independently of EGF treatment (Fig. 2), may be explained from the growth behaviour of SiHa cells: these cells show optimal proliferation when they have reached at least 30 to 40% confluency (our unpublished results); also, they can easily be adapted within a few days to grow in serum-free medium. These findings indicate that an autocrine growth factor, rather than serum growth factors may be required for optimal SiHa cell proliferation. We speculate that such a factor may accumulate in the culture medium during the time course and exert inducing effects similar to EGF.

In contrast to SiHa cervical carcinoma cells, in HPK IA keratinocytes we did not observe an EGF induction of HPV-16 E6/E7 mRNA. This is consistent with published results, where in the HPV-16-immortalized cell line PHK160b, E6/E7 mRNA was not inducible but was repressed after 48 h EGF treatment (Yasumoto et al., 1991). Similar observations were recently reported in laryngeal papilloma cells, which harbour HPV types 6 and 11, where EGF caused a reduction in HPV RNA and induced abnormal differentiation (Vambutas et al., 1993). Changes in growth factor response during tumour development also appear to play a role in other systems: the inhibitory effect of TGF-β on cell proliferation usually observed in normal cells was frequently absent in tumour cells (Anzai et al., 1992). We propose that the differential EGF response of HPV-16 E6/E7 mRNA in tumour cells compared with immortalized keratinocytes represents different levels of tumour progression of the two cell types. In tumour cells, an EGF-dependent increase in E6 and E7 expression may synergize with the normal growth stimulatory effect of EGF.

The molecular mechanisms underlying the differential EGF regulation of HPV-16 early gene expression are still unknown; however, it is likely that changes in the cellular signal transduction occurring during tumour progression play a role. The large number of signals produced by EGF inside the cell (Carpenter & Cohen, 1990; Davis, 1993) provides many possibilities for changes in signal transduction resulting in an altered transcriptional response. An additional factor possibly involved in tumour progression is the HPV E5 gene product: the E5 protein cooperates with the proliferative and transforming activity of EGF (Leechanachai et al., 1992; Pim et al., 1992) and elicits a down-regulation of the EGF receptor (Straight et al., 1993). Furthermore, E5 also seems to modify the transcriptional response to EGF, as Bouvard et al. (1994) have recently reported effects of E5 plus EGF on the HPV-16 enhancer. Therefore, it seems likely that a loss of E5 expression (by inactivation of the E5 gene during viral integration) modifies the EGF-response of cellular and viral gene expression.

The HPV-16 enhancer plays a key role in viral early transcription and is the target for a number of regulatory
signals, such as steroids, phorbol esters and interleukin 6 (Chan et al., 1989, 1990; Kyo et al., 1993). The data presented here suggest that the enhancer is also responsible for the observed EGF regulation of E6/E7 mRNA in SiHa cells. In contrast, in HPK IA keratinocytes we did not find an EGF induction of the viral enhancer, which is consistent with the missing EGF response of E6/E7 mRNA in these cells. We have also examined the response to insulin and serum growth factors in SiHa cells, where we observed the strongest stimulatory effects with EGF, but no comparable inductions with serum, or with insulin (Fig. 5) under conditions where insulin should activate both the insulin- and the insulin-like growth factor I (IGF-I) receptors (Czech, 1989). This specificity for EGF is probably significant, because most cell types including epithelial cells express IGF-I receptors; additionally, EGF and IGF-I appear to exert their proliferative effects at different points of the cell cycle (Humbel, 1990).

To localize EGF-responsive elements, we tested enhancer subfragments with or without an AP-1 binding site. Enhancer fragments containing the fp4e or fp9e AP-1 sites were EGF-responsive, whereas the distal enhancer fragment without the AP-1 site did not give an EGF response (Fig. 7). Interestingly, we observed a varying EGF-inducibility of the central enhancer core fragment (pH16EN-112-Z), which was not EGF-regulated under certain conditions. Transfections with various cell densities have suggested that this lack of an EGF response correlates with a high cell density. This cell density-dependent repression of the EGF responsiveness appears to be restricted to the core enhancer context, since both the wild-type enhancer and a fragment tested in parallel containing the fp9e AP-1 site still showed an EGF induction (Fig. 6b). Cell density-dependent gene expression has frequently been reported, where high cell density can cause either a decrease (Katayama et al., 1991; Kumatori et al., 1991; Li & Beer, 1993) or an increase (Hajnal et al., 1994; Niimi et al., 1991; Resing et al., 1993) in cellular gene expression. Interestingly, some EGF-regulated processes have been shown to be cell density-dependent (Colige et al., 1992; Gardner & Shimizu, 1994; McAllister et al., 1993). One way to regulate gene expression in a cell density-dependent fashion may involve repressors which are activated at high cell density and exert a dominant negative effect selectively on regulatory domains.

The EGF regulation of the AP-1 site mutants within the complete 400 bp enhancer has confirmed the model that the two AP-1 binding sites could be responsible for the EGF induction: mutating a single AP-1 site reduced the EGF response, whereas the enhancer with the double mutation completely lost EGF regulation (Fig. 8). This demonstrates that the two AP-1 binding sites are absolutely required for the EGF response of the viral enhancer. The finding that the basal activity of the complete 400 bp is also affected by mutating the AP-1 binding sites confirms previous observations that the fp4e AP-1 site is important for the activity of the central enhancer core (Chong et al., 1990; Cripe et al., 1990). This finding also indicates that withdrawal of serum for 24 h, which was done before each EGF induction, does not inactivate AP-1 completely, as would be expected for a cell cycle arrest due to the absence of growth factors. The function of a third potential AP-1 binding site overlapping with the GRE/PRE just downstream of the fp4e AP-1 site in the viral enhancer (Chan et al., 1990; Cripe et al., 1990) is still disputed; however, we have evidence suggesting that this element is bound by a factor distinct from AP-1 and does not show the regulation typical of AP-1 sites (H. J. List, S. Nehrwein, V. Patzel, S. Kiel, V. Senner, K. Bleuel, K. Knöbel & G. Klock, unpublished results).

AP-1 binding sites have been identified in a number of genes; however, in most cases the regulatory pathway used in vivo for AP-1 activation has not been determined. AP-1 is a heterodimer formed between members of the Jun and Fos protein families (Curran & Franz, 1988). Activation of AP-1 by EGF involves both transcriptional activation of jun and fos genes (Gille et al., 1992; Quantin & Breathnach, 1988) and changes in the phosphorylation status of the proteins (Davis, 1993). AP-1 can also be activated by the phorbol ester TPA, which is known to activate the PK-C pathway (Angel et al., 1987; Chan et al., 1990). However, recent evidence suggests that PK-C is not required for at least some EGF-induced processes (Franklin & Kraft, 1992; Klein et al., 1992). Interestingly, PK-C can activate the Raf-1 kinase/MAP kinase pathway by direct phosphorylation of Raf-1 (Kolch et al., 1993), suggesting that phorbol esters require the MAP kinase pathway to activate AP-1. In conclusion, AP-1 appears to play a key role in EGF-regulated transcription, although in other systems transcription factors other than AP-1 may allow EGF regulation: the EGF responsive unit of the gastrin promoter does not contain an AP-1 site (Godley & Brand, 1989), and the enhancer from the mouse retrovirus VL30 contains an AP-1-related sequence 5' TGACTCC which does not bind to AP-1 and is not required for the EGF response (Lenormand et al., 1992).

In addition to HPV-16, other HPVs may also respond to EGF, as, for example, the HPV-18 E6 promoter binds AP-1 protein (Offord & Beard, 1990). Interestingly, in vitro protein binding to AP-1 sites in the HPV-18 control region was observed with all three Jun proteins; however, the members of the Jun family were expressed differentially in keratinocytes and tumour cells (Thierry et al., 1992). Similarly, differential c-jun expression under EGF
control has been observed in transformation-sensitive versus -resistant epidermal cells (Ben-Ari et al., 1992). Therefore it should be worth testing whether variant expression of members of the Jun and Fos families plays a role in the differential EGF regulation of HPV-16 E6 and E7 genes in cervical carcinoma cells compared with keratinocytes.

We thank Drs M. Dürst for the HPK IA cells, P. Friedl, M. Teifel and T. Dittrich for help with the lipofections, J. Stollwerk and C. Schirmer for oligonucleotide synthesis, M. Groot, C. Harders, S. Kiel, T. Knöchel, A. Meyerdiers and A. Reeg for assistance, and H. J. List for discussion. This work was supported by the Deutsche Forschungsgemeinschaft, grant KI 528/2-2.

References


Gardner, D. P. & Shimizu, N. (1994). Loss of cytotoxic effect of epidermal growth factor (EGF) on EGF receptor overexpressing...
cells is associated with attenuation of EGF receptor tyrosine kinase activity. *Journal of Cell Physiology* 158, 245-255.


(Received 25 November 1994; Accepted 14 March 1995)