E5a gene of human papillomavirus type 11 is required for initiation but not for maintenance of transformation in NIH 3T3 cells

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We have previously shown that the E5a gene of human papilloma virus type 11 (HPV-11/HPV-6c) is a transforming oncogene. In order to dissect the biological consequences of E5a gene expression we utilized the lac operator/repressor system to manipulate E5a gene expression. Cells were cotransfected with the lac repressor gene and the E5a gene that had been inserted downstream of a simian virus 40 (SV40) promoter containing the lac operator sequence. The expression of E5a gene could therefore be repressed by binding of lac repressor to the lac operator sequence in proximity to this SV40 regulatory region. The transfected cells were cultured in the presence of the inducer IPTG and under G418 selection. IPTG derepressed E5a gene expression by binding to the repressor and reducing its affinity for the lac operator sequence. In these studies, we found that E5a-transformed cells still maintained the transformed phenotype as judged by growth density, cell morphology and anchorage-independent growth when E5a gene expression was repressed. We also found that c-jun expression was induced 3 h after E5a expression was induced by IPTG and c-jun expression was not shut down after repression of E5a expression. This is the first demonstration that the E5a gene of HPV-11 initiates transformation of NIH 3T3 cells but is dispensable for maintenance of the transformed phenotype.

Introduction

The human papillomaviruses (HPVs) are naturally occurring DNA tumour viruses that induce epithelial cell proliferation during the course of a productive infection. HPV-6 and HPV-11 are the aetiologcal agents of condylomata acuminata and respiratory papillomata (Gissmann et al., 1983; Mounts et al., 1982). The predominant viral transcript of HPV-6 and HPV-11 in genital condylomata and respiratory papillomata encodes El‘E4, E5a and E5b proteins (Chow et al., 1987; Ward & Mounts, 1989). The E5a and E5b proteins are detected in biopsy specimens of HPV-6c (the independent isolate of HPV-11; Chen et al., 1993) by immunoperoxidase assay (Chen & Mounts, 1989). Chen & Mounts (1990) showed that the E5a gene of HPV-11 (HPV-6c) is a transforming oncogene. However, the biochemical activity of E5a of HPV-11 is not clearly understood.

Bovine papillomavirus (BPV) is the prototype virus for studying the role of papillomaviruses in cell transformation. There are similarities between the HPV-11 E5a and BPV E5 open reading frames (ORFs) (Chen & Mounts, 1989, 1990). The ability of the product of the BPV-1 E5 ORF to transform NIH 3T3 mouse cells and C127 cells has been observed (Lowy & Schiller, 1989; Schlegel et al., 1986; Settlen et al., 1989). Proteins encoded by several DNA tumour virus oncogenes, including those of papillomaviruses, have been shown to form stable complexes with host cell proteins (Dyson et al., 1987; Whyte et al., 1988) and there is evidence that BPV-1 E5 interacts with the 16K component of vacuolar ATPases (Goldstein et al., 1991). It was recently reported that the BPV-1 E5 gene product can interact with the cellular epidermal growth factor receptor (EGFR) resulting in increased colony formation in soft-agar assays in the presence of EGF (Martin et al., 1989). In addition, its interaction with the platelet-derived growth factor receptor mimics ligand binding to the receptor because of homology between the ligand and the C-terminal domain of the E5 protein (Petti et al., 1991). The mechanisms by which the E5a protein of HPV-11 performs its functions are not totally understood.

Deregulation of some genes, such as c-jun, c-myc and c-fos is characteristic of many tumours and tumour cell lines (Craig Hooper et al., 1991; Sistonen et al., 1989; de Groot et al., 1991). These genes belong to the group of immediate early genes that are involved in the regulation of cell growth (Schonthal et al., 1992). Jun proteins can form homodimers or heterodimers with proteins of the Fos family (Halazonetis et al., 1988). These dimers bind to the AP-1 recognition site present in the promoters of...
various genes, thereby regulating their expression. Recent evidence indicates that the expression of c-jun is induced by the E1A protein of adenovirus (de Groot et al., 1991), the X protein of hepatitis B virus (Twu et al., 1993), polyoma virus middle tumour antigen (Schonthal et al., 1992) and human T-lymphotropic virus types I and II (Craig Hooper et al., 1991). These studies demonstrate that c-jun gene expression is correlated with cellular proliferation. Induction of c-jun expression by the effect of the E5a gene of HPV-11 has not been reported previously.

Investigation of the biological consequences of transforming oncogenes is usually performed by comparing the transformed cell line produced by an oncogene with its untransformed parental counterpart (Schwartz et al., 1988). Such a comparison, however, is limited to the analysis of the start and end points of the transformation process. The signal transduction pathway between oncogene participation and transformation events can be complicated by secondary events during the development of stably transformed cell lines (Schwartz et al., 1988). One solution is to study the biochemical activity of purified oncogenic protein; the other is to induce gene expression selectively; this can provide important information in addressing the function of oncogenes. In these studies, we utilized the lac operator/repressor system to manipulate E5a gene expression to dissect the biological consequences of E5a gene expression. This system has been used for selective induction of the expression of a human Ha-ras transgene which leads to the transformation of NIH 3T3 cells (Liu et al., 1992). In our work, the E5a gene of HPV-11 directed by a simian virus 40 (SV40) promoter within which a lac operator sequence had been embedded and the lacI gene, encoding the lac repressor, were used to cotransfect NIH 3T3 cells. Cells assumed the transformed phenotype upon induction of E5a gene expression by IPTG. However, the transformed phenotype was not lost upon repression of the E5a gene of HPV-11 by the subsequent removal of IPTG; during this constitutive expression of c-jun was observed. This result suggests that expression of the E5a gene of HPV-11 is essential for the initiation but not for the maintenance of the transformed phenotype in NIH 3T3 cells.

Methods

**Plasmid construction.** The plasmid pSLa4 contains the E5a, E5b and L2 ORFs of HPV-11 (corresponding to nucleotides 3775 to 5820; Chen, 1990). The 310 bp EcoRI–HpaI fragment was cut from pSLa4 and subcloned into the vector pBluescript KS+ (Stratagene) at the EcoRI and Smal sites to generate pSL13 (Fig. 1). The 310 bp HindIII–BamHI fragment was then transferred to the 6·7 kb plasmid pMt.neo.1 (Chen & Mounts, 1990) to generate pMt(E5a), thus placing the E5a ORF under the control of the mouse metallothionein (Mt) promoter. The plasmid pSVlacOE5a was constructed by transferring a 1·1 kb HindIII fragment derived from pSVlacORas (Liu et al., 1992) containing the SV40 promoter and enhancer elements with a synthetic lac operator inserted between the TATA box and the transcription initiation site, into the HindIII site of pMtE5a2. The orientation of the insert was established by double digestion with EcoRV which cleaves near the 5' end of the SV40 promoter fragment and BamHI within the pMtE5a2 polylinker. The recombinant plasmid pSVlacOE5a carries a G418 resistance marker and utilizes the SV40 promoter to direct transcription of E5a gene.

**Cell culture and DNA transfection.** NIH 3T3 cells and their transgenic derivatives were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% calf serum (DMEM-10). Cells were routinely split 1:3 and not allowed to grow to confluence unless otherwise indicated. Transfections by calcium phosphate precipitation were carried out as previously described (Chen & Mounts, 1990). Approximately 5 × 10⁶ cells were cotransfected in 60 mm dishes with 5 μg of the plasmid pSVlacOE5a (Fig. 1) and 3 μg of pHBlacINLS (Liu et al., 1992), and split 1:4 after 24 h. At 24 h after replating, the cells were selected for resistance to G418 (500 μg/ml; Gibco). To generate E5a-transformed cells, IPTG (20 mm) was added to allow E5a expression in transgenic cells. Colonies with a transformed phenotype were picked and grown in the presence or absence of IPTG for growth density measurement, observation of cell morphology by phase contrast microscopy and anchorage-independent growth in soft agarose. For control experiments the transfected cells were not treated with IPTG, but were subjected to G418 selection. After 3 weeks, the G418-resistant cells were pooled and then cultured with or without IPTG for the measurement of transformation as described above.

**Anchorage-independent growth in soft agarose.** For colony formation in suspension, 60 mm dishes were prepared with a bottom layer of 0·6% agarose in DMEM-10. Duplicate dishes were seeded with 2 × 10⁵ cells suspended in 0·3% agarose in DMEM-10. Plates of cells were fed twice weekly and scored for colony formation after 3 to 4 weeks.

**Southern blot hybridization.** Total cellular DNA was extracted from cells (Mounts et al., 1982). DNA samples (10 μg) were digested with EcoRI, size-fractionated by 1% agarose gel electrophoresis and transferred to nitrocellulose membranes. For 32P labelling by nick translation, the E5a and lac sequences were separated from the vector by digestion of pSVlacOE5a with EcoRI/BamHI and pHBlacINLS with HindIII/XhoI, respectively and recovered after agarose gel electrophoresis. Hybridization and washing were done as described previously (Chen & Mounts, 1990).

**Northern blot hybridization.** Samples of 20 μg of total RNA stored in ethanol were precipitated, dried under vacuum, dissolved in 8 μl of 56% formaldehyde buffer (6·4% formaldehyde, 20 mM-MOPS, 5 mM-sodium acetate, 1 mM-EDTA), incubated for 10 min at 63°C, and chilled on ice. Samples were fractionated according to size on a 13% agarose gel containing 7·1% formaldehyde in MOPS buffer (20 mM-MOPS, 5 mM-sodium acetate, 1 mM-EDTA pH 7.0; Ward & Mounts, 1989). After electrophoresis, gels were soaked for 30 min in 20 × SSC (30 mM-sodium chloride, 0·3 M-sodium citrate) and transferred to nitrocellulose. Filters were baked in vacuo at 80°C for 2 h and incubated in 10 mM-Tris–HCl pH 7·2, 1 mM-EDTA, 1 mM-NaCl, 10 × Denhardt’s solution for 4 h at 65°C. Hybridization was performed for 60 h at 68°C with 32P-labelled probes by nick translation in vitro in 0·72 M-NaCl, 0·09 mM-sodium citrate, 0·078 mM-KH₂PO₄, pH 6·8, 0·012 M-EDTA, 0·5% SDS and 10 × Denhardt’s solution. Filters were washed to remove non-specifically bound probes and to obtain defined stringencies. A DNA probe for 28S RNA (courtesy of Dr Chang Ming-Fu) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control for RNA loading. Filters were air-dried and exposed to Kodak XAR film with Du Pont Lightning-plus intensifying screens.
Results

Lac operator/repressor expression system

To investigate the transforming capacity of the E5a gene of HPV-11, we used the lac operator/repressor system to regulate E5a gene expression. In this system, the cells were made transgenic for a constitutively expressed lacI gene encoding the lac repressor and an activated E5a gene of HPV-11 directed by an SV40 promoter within which a lac operator sequence had been embedded. The expression of the E5a gene was repressed by binding of the lac repressor to a lac operator sequence in proximity to the SV40 regulatory region. The inducer, IPTG, derepressed the E5a gene under lac operator control by binding the repressor and reducing its affinity for the operator sequence.

A DNA fragment containing the E5a ORF with only 20 bp preceding the E5a AUG codon was cloned into pMt.neo.1 to generate pMtE5a2 (Fig. 1). A fragment containing the SV40 promoter within which a lac operator sequence had been embedded was transferred to pMtE5a2 to generate pSVlacOE5a whose structure is shown in Fig. 1. The plasmid pHflacINLS contains a human β-actin promoter driving the expression of the lacI gene containing a nuclear localization signal (Liu et al., 1992). Cells transfected with pHflacINLS constitutively sequester the lac repressor almost exclusively within the nucleus (Liu et al., 1992).

Phenotypes of cells with or without removal of IPTG treatment

The plasmids pSVlacOE5a and pHflacINLS were co-transfected into NIH 3T3 cells by a modified calcium phosphate precipitation method (Chen & Mounts, 1990). The transfected cells were grown under G418 selection and in the presence of 20 mM-IPTG. After 3 weeks, G418-resistant colonies were picked and grown. Twelve G418-resistant colonies were picked at random and tested for anchorage-independent growth in the presence or absence of IPTG induction. All of 12 G418-resistant cell lines showed anchorage-independent growth with or without inducer. Then, we selected three NIH 3T3 cell clones for further characterization. These three clonal cell lines were named LE5alF3, LE5alC4 and LE5alC5. Table 1 shows the results of three separate biological assays for cell morphology, growth density and

\[ \text{Table 1. Phenotypes of cells with or without IPTG induction} \]

<table>
<thead>
<tr>
<th>Cell line</th>
<th>+ IPTG</th>
<th>- IPTG</th>
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<tr>
<td></td>
<td>Growth density*</td>
<td>Growth in suspension</td>
</tr>
<tr>
<td>LE5alF3</td>
<td>21.0</td>
<td>21.6</td>
</tr>
<tr>
<td>LE5alC4</td>
<td>11.2</td>
<td>21.1</td>
</tr>
<tr>
<td>LE5alC5</td>
<td>12.5</td>
<td>14.6</td>
</tr>
<tr>
<td>Eneo</td>
<td>9.0</td>
<td>8.0</td>
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* Expressed as cell no. × 10⁴/cm².
† +, Too many colonies to count.
anchorage-independent growth. The growth density of LE5aIF3, LE5aIC4 and LE5aIC5 cells did not change significantly after the removal of IPTG. The transformed phenotype of these three clonal cell lines was still maintained after the removal of IPTG. Fig. 2(a) shows the transformed morphology of LE5aIF3 cells in the presence of IPTG; the transformed phenotype was still maintained even in the absence of IPTG (Fig. 2b). Control transfectants (Eneo) that received the vector pMt.neo.1 alone showed fibroblast cell morphology (Fig. 2c) and lower growth density (Table 1). In addition, LE5aIF3, LE5aIC4 and LE5aIC5 cells were capable of anchorage-independent growth when assayed for the ability to form colonies in soft agarose. Table 1 shows that all of them could form colonies of similar size and number in the presence or absence of IPTG, whereas Eneo cells containing the vector alone did not grow in soft agarose (Fig. 2g). Fig. 2 also shows the colony growth in soft agarose of LE5aIF3 cells in the presence (e) or absence of IPTG (f). These results demonstrated that the transformed phenotype of E5a-transfected cell lines was maintained even after E5a gene expression was repressed by the absence of IPTG.

Transfectants which received the E5a and lacI genes were also cultured in the absence of IPTG and under G418 selection. After 3 weeks, the pooled G418-resistant colonies were collected and named LE5aIC1. These cells showed fibroblast-like morphology (Fig. 2d) and did not grow in soft agarose (Fig. 2h). No E5a gene transcription was detected by Northern hybridization analysis (data not shown). When LE5aIC1 cells were treated with IPTG they assumed the transformed phenotype. Similar results to those described above were obtained (Fig. 2a, b, e and f) showing that the transformed phenotype could not be reversed by the removal of IPTG treatment.

Analysis of E5a and lacI genes in transfected cells

DNA was isolated from the cotransfected cell lines to demonstrate the presence of E5a and lacI DNA sequences. The DNA was digested with EcoRI and Southern blot hybridization with the E5a and lacI gene probes was performed. The DNA was digested with EcoRI, which cuts the plasmid pSVlacOE5a twice and pHs/lacINLS once as shown in Fig. 1. Fig. 3 shows the results of the hybridization analysis. The E5a gene (panel a, lanes 1 to 3) and lacI (panel b, lanes 1 to 3) gene were identified in all three cell lines. In Fig. 3(a) the 1.5 kb hybridization band contained the intact copy of the E5a gene; in Fig. 3(b) multiple hybridization bands were observed. No hybridization band was detected in Eneo cells (lanes 4) or NIH 3T3 cells (lanes 5). The input DNAs were stably maintained under constant conditions of selection.
Induction of E5a mRNA and expression of lacI mRNA in stably transfected clonal cell lines

To confirm that the observed biological responses to IPTG correlated with expression of the E5a and lacI transgenes, the production of the E5a and lacI mRNA in transfected cells with or without IPTG administration was assessed by Northern blot analysis. Fig. 4(a) shows the results of induced and repressed expression of the E5a gene as determined by hybridization analysis. Hybridization of total RNA to E5a probes revealed no detectable transcripts in NIH 3T3 cells (lane 8), Eneo cells (lane 7) and uninduced E5a-transfected cell lines (lanes 2, 4 and 6). An intense band appeared following induction by IPTG administration (lanes 1, 3 and 5) in E5a-transfected cell lines. Constitutive expression of the lacI gene in these three clonal cell lines with or without IPTG administration is shown in Fig. 4(b). No hybridization band was detected in NIH 3T3 cells (lane 8) or Eneo cells (lane 7).

c-jun gene expression is induced by E5a gene product

To investigate the secondary events during the development of stable transformation the expression of cellular oncogenes in the transformed cells was assessed (LE5aIF3 cells). The expression of the c-jun gene in LE5aIF3 cells was found to be higher than that found in parental NIH 3T3 cells (Fig. 5 lanes 1 to 3). No difference in the expression of c-jun mRNA was detected before (lane 2) or after the repression of E5a gene (lane 3) by the removal of IPTG in LE5aIF3 cells. Additional experiments were conducted to determine whether c-jun gene expression could be induced in LE5aIC1 cells which were transfected with E5a and lacI genes but not transformed since these cells were not treated with IPTG. IPTG was added to the culture medium and RNA samples were collected after 3, 6, 9, 12 and 24 h, and analysed. Low levels of c-jun mRNA were expressed in LE5aIC1 cells before IPTG treatment (lane 4) and the level of c-jun mRNA was increased after exposure to IPTG for 3 h (lane 5), 6 h (lane 6), 9 h (lane 7), 12 h (lane 8) and 24 h (lane 9). This result shows that c-jun was induced after E5a gene expression and that c-jun gene expression
activities that it initiated. Cell transformation was increased growth density, altered morphology and loss of measured by three separate biological endpoints: GAPDH control the expression of the E5a gene was to facilitate highly selective induction of E5a gene expression. Since no other endogenous genes should be directly activated by IPTG administration (Liu et al., 1992), the biological endpoints that we monitored should be attributable to the E5a gene product, or to one or more of the metabolic activities that it initiated. Cell transformation was measured by three separate biological endpoints: increased growth density, altered morphology and loss of anchorage dependence (Table 1). The results from these studies show that three clonal cell lines (LE5aIF3, LE5aIC4 and LE5aIC5 cells) still maintained transformed phenotypes after E5a gene expression was repressed owing to the absence of IPTG. However, another explanation for our observation could be that LE5aIF3, LE5aIC4 and LE5aIC5 cells are spontaneous transformants of NIH 3T3 cells and thus have no response to IPTG. LE5aIC1 cells were introduced to settle this argument. LE5aIC1 cells which harboured E5a and lacI transgenes without ever experiencing IPTG treatment showed untransformed phenotypes (Fig. 2d and h). Upon administration of IPTG to LE5aIC1 cells, E5a was induced and transformed phenotypes occurred at a significant frequency which rules out the possibility of spontaneous transformation. Moreover, cells from pooled G418-resistant colonies which were transfected with vector alone could generate only less than 1% of colonies in soft agarose as compared to cells from pooled G418-resistant colonies that had been transfected with E5a (data not shown). In addition, repression of the E5a gene was achieved extensively in this system as confirmed by Northern hybridization analysis (Fig. 4a). The amount of the E5a mRNA was undetectable by Northern hybridization. However, it is still possible that the irreversible transformed phenotype was caused by a minimal level of E5a gene expression. There is no satisfactory E5a antibody that would enable us to show the actual expression of E5a protein in this repression system. Therefore we looked for cellular events induced by E5a gene expression. The results show that E5a gene can activate c-jun gene expression (Fig. 5).

Discussion

In this paper, we report that the E5a gene of HPV-11 is capable of initiating the transformation of NIH 3T3 cells but is dispensable for the maintenance of the transformed phenotype. Previously, we have shown that HPV-11 E5a is a transforming oncogene in NIH 3T3 cells (Chen & Mounts, 1990). We further confirmed the transforming activity of E5a by subcloning the E5a ORF only into the vector pMt.neo.1 and transfecting this construct into NIH 3T3 cells. In addition, HPV-11 E5a gene-transformed cells can induce tumours in nude mice (unpublished data). To study further the transforming effect of the E5a gene, we utilized an inducible gene expression system (lac operator/repressor). The purpose of using the Escherichia coli lactose regulatory system to control the expression of the E5a gene was to facilitate highly selective induction of E5a gene expression. Since no other endogenous genes should be directly activated by IPTG administration (Liu et al., 1992), the biological endpoints that we monitored should be attributable to the E5a gene product, or to one or more of the metabolic activities that it initiated. Cell transformation was measured by three separate biological endpoints: increased growth density, altered morphology and loss of
reported that HPV-16 DNA is stably maintained in transfected C127 cells and in the clonal cell lines derived from single agar colonies, and in tumours induced in nude mice (Morgan et al., 1988). HPV-6 and HPV-11 are predominantly associated with condylomata acuminata and mild cervical dysplasia, whereas HPV-16 is associated with all grades of cervical dysplasia but, unlike HPV-6 and -11, is involved in progressive disease and malignancy (zur Hausen & Schneider, 1987). This different transforming potential in vitro suggests different mechanisms whereby HPV-6/11 and HPV-16 appear to be involved respectively in benign and malignant tumours in vivo (Chasters & McCance, 1989; Howley, 1991; zur Hausen & Schneider, 1987).

Our data support the model that expression of the E5a gene of HPV-11 induces cell transformation by executing one or more early events, and that once the transformed phenotype is attained, the transforming function of E5a is no longer required. Our evidence indicates that this involves the activation of the c-jun gene (Fig. 5). Amplification and rearrangement of cellular DNA sequences specific to BPV-4 transformed cells have been observed (Smith & Campo, 1989). It has been reported that the expression of BPV-1 genes is essential for transformation but not for tumorigenicity, whereas high levels of expression of c-myc oncogene are associated with tumorigenicity (Agrawal et al., 1992). Alterations of the c-myc gene involving amplification and rearrangement are found in DNAs from human cervical carcinomas (Ocadiz et al., 1987). Further study will determine the role of c-jun in the maintenance of transformation induced by E5a of HPV-11.

In summary, E5a of HPV-11 is capable of transforming cells in vitro. Its expression is required for the initiation but not for maintenance of the transformed phenotype. Expression of c-jun, an inducible transcription factor that functions as an intermediate transcription regulator in signal transduction, is induced immediately after E5a gene expression. Our results demonstrate constitutive expression of c-jun after repression of E5a gene expression suggest that it may play a role in E5a-induced transformation.

This research was supported by the National Science Council (NSC-83-0412-B-016-054), Taipei, Taiwan, Republic of China.

References


(Received 3 December 1993; Accepted 7 February 1994)