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Expression of AP1 during cellular differentiation determines human papillomavirus E6/E7 expression in stratified epithelial cells

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E6 and E7 oncoproteins of human papillomavirus (HPV) play significant roles in the pathogenesis of cervical cancer. However, the pattern of E6/E7 expression during the productive virus life cycle in differentiating epithelia of the uterine cervix remains unclear. In addition, little is known about the cellular factors regulating E6/E7 expression in differentiating epithelia. In the present study, using transient expression assays and DNA binding assays, we demonstrated that E6/E7 transcription is critically regulated by the cellular factor AP1, a Jun/Fos heterodimer complex. Immunohistochemical analyses of various uterine cervical lesions showed AP1 expression in lower cell layers of normal cervix and low-grade cervical intraepithelial neoplasia (CIN), while it was detected throughout all layers in high-grade CIN and invasive cancer. *In situ* RNA–RNA hybridization analyses of organotypic raft culture specimens of an HPV-31-containing cell line revealed that E6/E7 transcripts were expressed in most cell layers, with reduced expression in differentiated cells. This pattern of HPV expression correlated with the pattern of AP1 expression detected by immunohistochemical analyses. These findings suggest that E6/E7 expression in differentiating epithelia is dependent on AP1, which appears to be associated with proliferative activity of the cells. Since E6/E7 expression induces cell proliferation, co-expression of AP1 and E6/E7 in undifferentiated cell layers might create a positive regulatory loop, probably contributing to maintenance of initial HPV infection and subsequent activation in basal and suprabasal cell layers.

Introduction

Among over 70 different types of human papillomavirus (HPV) identified, several, including HPV-16, -18, -31, -33, -52 and -58, are associated with cervical cancer (zur Hausen, 1989) and designated as high-risk types. Genetic analyses of high-risk HPVs have revealed two transforming genes (E6 and E7) which remain intact and are actively transcribed in cervical cancers (Baker *et al.*, 1987; Schwartz *et al.*, 1985; Yutsudo *et al.*, 1988). E6 and E7 oncoproteins of high-risk HPVs have been shown to bind tumour suppressor gene products, impairing their normal functions (Scheffner *et al.*, 1990; Werness *et al.*, 1990). These observations strongly suggest that the E6 and E7 genes play significant roles in the pathogenesis of cervical cancer.

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E6 and E7 genes are transcribed from the HPV early promoter, which is designated p97 in HPV-16 and HPV-31 and p105 in HPV-18. The activity of this promoter is regulated by transcriptional control elements in the long control region (LCR), which is located upstream of the start site (Gloss *et al.*, 1987). Several nuclear factors have been shown to bind the LCR at specific sites and regulate the transcriptional activity of the promoter, leading to keratinocyte-specific transcriptional activation of HPV. The factors that regulate expression seem to be different among HPV types, suggesting diverse mechanisms of activation. For HPV-16, the factors AP1, NF1, TEF-1, TEF-2 and Oct1 have all been implicated in transcriptional regulation (Chan *et al.*, 1990; Chong *et al.*, 1991; Ishiji *et al.*, 1992). In contrast, for HPV-18 a keratinocyte-specific factor, KRF-1, has been reported to interact with AP1 to activate transcription (Butz & Hoppe-Seyler, 1993; Mack & Laimins, 1991). No significant activities of NF1 or Oct1 have been demonstrated in this HPV type. We have previously shown

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that AP1 interacts synergistically with two novel factors to achieve full activation of HPV-31b transcription (Kyo *et al.*, 1995). These novel factors were found to be distinct from any of those that regulate either HPV-16 or -18 expression. Taken together, AP1 appears to be a common regulator of expression for various HPV types while additional HPV type-specific cellular factors may be required to cooperate with AP1 to achieve full activation of virus gene expression.

HPVs exhibit a complex pattern of expression during a productive infection in differentiating epithelia. Following infection in stratified epithelial cells, HPV genomes are maintained as low copy number episomes in basal cells. As infected cells migrate from the basal layer and undergo differentiation, late gene expression is activated and virus DNA is amplified (Bedell *et al.*, 1991; Laimins, 1993). Numerous studies have identified L1/L2 late transcripts of HPVs in the differentiated cell layers of the epithelia (Broker *et al.*, 1989; Crum *et al.*, 1988; Higgins *et al.*, 1992). It is likely that increased expression of late transcripts and amplification of HPV genomes result in the assembly of virus particles. While the distribution of late gene expression is well characterized, it is still unclear how early gene expression is modulated during the productive infectious cycle in differentiating epithelia. Using RNA *in situ* hybridization of genital condyloma and cervical intraepithelial neoplasia (CIN) grade I tissues, several studies reported that E6/E7 transcripts were most highly expressed in the superficial layers, suggesting differentiation-dependent expression of E6/E7 (Broker *et al.*, 1989; Chow *et al.*, 1987; Stoler *et al.*, 1989). Other analyses using CIN I tissue demonstrated that E6 transcripts were expressed throughout all layers (Higgins *et al.*, 1992).The actual distribution of E6/E7 expression is therefore unclear. The role of AP1 in E6/E7 expression in differentiating epithelia also remains unclear.

In the present study, we first confirmed that AP1 is a predominant determinant of HPV-31b enhancer activity which directs E6/E7 transcription. All of the Jun family members were found to bind and transactivate HPV expression, probably contributing to the cell-type specificity of HPV transcription. Using *in situ* RNA hybridization analyses of stratified raft cultures of CIN612 cells, E6/E7 transcripts were found to be expressed in most cell layers with a reduced level of expression in differentiated cells. Expression was also shown to correlate with distribution of AP1 factors. These findings suggest that AP1 plays a significant role in the expression of E6/E7 in differentiating epithelia of the uterine cervix.

Methods

■ **Cell culture.** C33A, Hs68 and HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum in the presence of 5% CO_2. SCC13 cells and CIN612 cells were grown in E medium with mitomycin C-treated fibroblast feeders as described previously (McCance *et al.*, 1988). BJAB cells were grown in RPMI-1640 medium supplemented with 10% foetal calf serum. Collagen raft cultures of CIN612 cells for *in vitro* differentiation were prepared as described previously (McCance *et al.*, 1988).

■ **Plasmids.** Reporter plasmids containing the whole LCR of HPV-31b were constructed for use in transient expression assays. HPV-31b LCR (nucleotide 6920–117)-containing p97 early promoter was cloned upstream of the luciferase gene in pGL2-Basic plasmid (Promega). For the construction of reporter plasmids containing substitution mutations in AP1 sites in the HPV-31b LCR, site-specific mutagenesis was performed by a PCR-based protocol (Higuchi, 1990). All the mutated plasmids were sequenced to confirm the presence of the expected mutations. A reporter plasmid containing the whole LCR fragment of HPV-18 was a kind gift from F. Hoppe-Seyler (German Cancer Research Centre, Heidelberg, Germany; Butz & Hoppe-Seyler, 1993). JunB, c-Jun, JunD and c-Fos expression vectors were a kind gift from M. Yaniv (Institut Pasteur, Paris cedex, France; Hirai *et al.*, 1990).

■ **Immunohistochemistry.** Expression of AP1 was determined immunohistochemically by the avidin–biotin–peroxidase method using 10% formalin or 4% paraformaldehyde-fixed and paraffin-embedded sections. Briefly, the tissue sections were deparaffinized, hydrated in graded ethanol and immersed for 20 min in 0·3% hydrogen peroxide to block endogenous peroxidase activity. The slides were washed in PBS pH 7·4 and treated with protease E at a final concentration of 0·1 mg/ml for 20 min at 37 °C. After washing with PBS, slides were treated with 10% normal goat serum for 20 min to inhibit non-specific binding to antisera. Primary antibodies against Jun family members (anti-JunB, SC-#46; anti-c-Jun, SC-#45; anti-JunD, SC-#74 Santa Cruz) were applied to the sections at a final concentration of 0·2 μg/ml for 1 h at room temperature. After rinsing in PBS, the sections were incubated for 30 min with biotin-labelled anti-rabbit IgG (Vector). They were then treated with the avidin–biotin complex (Vector) at room temperature. Negative controls included sections incubated with normal goat serum instead of the primary specific antibody. Sites of peroxidase activity were visualized with DAB substrate kit (Vector).

■ **Transient expression assay.** Five to 10 μg of luciferase reporter plasmids were co-transfected with 2 μg of SV2 *β*-galactosidase plasmid and various amounts of effector plasmids into cultured cells by the calcium phosphate method (for HeLa, C33A cells and HepG2 cells), lipofection (primary foreskin keratinocytes) or electroporation (CIN612 and BJAB) as described previously (Ustav & Stenlund, 1991). At 48 h after transfection, cells were harvested and luciferase assays were performed according to the protocols of the Promega Luciferase Assay system. Luciferase activity was normalized to *β*-galactosidase activity to correct for transcription efficiency in each reaction.

■ **Electrophoretic mobility shift assay (EMSA).** Nuclear extracts were prepared from a variety of cell types as previously described (Schreiber *et al.*, 1989). Five to 10 μg of nuclear extracts were incubated with 2 μg of poly(dI–dC) in the presence or absence of a 100-fold molar excess of unlabelled competitor DNAs on ice for 20 min in a 25 μl reaction volume containing 10% glycerol, 25 mM HEPES pH 7·9, 50 to 100 mM KCl, 4 mM $MgCl_2$ and 0·5 mM PMSF. Following incubation, 10000 c.p.m. of ^{32}P end-labelled oligonucleotide probe was added and the reaction incubated at room temperature for an additional 30 min. The DNA–protein complexes were then separated from free probe by electrophoresis on a 4% polyacrylamide gel at 4 °C. The gel was dried and subjected to autoradiography. For supershift analyses, nuclear extracts were preincubated with specific antibodies for 60 min at 4 °C and then used for EMSA. For competition assays, consensus binding sites for AP1 (5′ CGCTTGATGAGTCAGCCGGAA 3′), Oct1 (5′ TGTCGAATGCAAATCACTA 3′) and TEF-1 (5′ CTAGATGCATGC-TTTGCATACTTCTGCCTAG 3′) (Promega) were used as competitors.

■ **DNase I footprint analysis.** The binding reaction for footprint analysis was the same as that used for EMSA except that the reaction volume was 50 µl. The probe used was a 262 bp fragment (nucleotides 7511–7772) of HPV-31b upstream regulatory region, which was end-labelled with ^{32}P. Following incubation of the probe with the extract, DNase I was added at a final concentration of 30 to 45 U/ml and the samples were further incubated at room temperature for 1 min. The reaction was stopped by adding 100 µl of stop buffer containing 100 mM Tris–HCl pH 8·0, 100 mM NaCl, 1% sodium sarcosyl, 10 mM EDTA and 25 µg sonicated salmon sperm DNA per ml. Following two phenol–chloroform extractions, the DNA was ethanol-precipitated. The pellets were then redissolved, denatured and electrophoresed on denaturing 6 or 8% polyacrylamide gels. The gels were dried and examined by autoradiography. Chemically cleaved DNAs were used as sequence markers.

■ ***In situ* RNA–RNA hybridization.** Raft culture tissue sections embedded in paraffin were dewaxed, rehydrated and denatured in 0·2 M HCl at room temperature for 20 min and in 2 × SSC at 70 °C for 15 min. After washing with PBS, the specimens were fixed with 4% paraformaldehyde followed by blocking with 10 mM DTT, iodoacetamide and *N*-acetylmaleimide, and dehydrated. Using an *in vitro* RNA transcription system (Stratagene), the probes for the E6/E7 region of HPV-31b were synthesized with ^{32}P-labelled UTP from inserts spanning nucleotides 200 to 747 in the E6/E7 ORF, which was cloned into vectors (pBluescript) containing both T3 and T7 polymerase binding sites. Transcription by the T3 polymerase therefore generated anti-sense probes. The probes were added in a hybridization mixture containing 50% formamide, 0·3 M NaCl, 10 mM Tris–HCl pH 8·0, 1 mM EDTA, 1 × Denhardt's solution, 500 µg/ml of yeast tRNA, 500 µg/ml of poly(A) (Pharmacia), 50 mM DTT and 10% PEG (EM Science) to obtain a 0·3 µg/ml final probe concentration. Hybridization was performed by spreading the probe mixture on the specimens and incubating at 45 °C in a moist chamber for 4 h. After hybridization, specimens were washed at 55 °C twice each in wash solution A (50% formamide, 2 × SSC, 20 mM 2-mercaptoethanol), B (50% formamide, 2 × SSC, 20 mM 2-mercaptoethanol, 0·5% Triton X-100) and C (2 × SSC, 20 mM 2-mercaptoethanol). The specimens were then treated with RNase solution containing 40 µg/ml of RNase A (Sigma) and 2 µg/ml of RNase T1 (Sigma), 10 mM Tris–HCl pH 7·5, 5 mM EDTA and 0·3 M NaCl for 15 min at room temperature, followed by washing at 50 °C twice each in wash solution C and A. They were then rinsed with 2 × SSC at room temperature and air-dried. Emulsion autoradiography was performed to detect the signals.

Results

E6/E7 transcription is dependent on AP1 activity in a variety of cell types

AP1 is a significant determinant of enhancer activity of HPV-31. We first confirmed the cell type-specificity of the HPV-31 enhancer and promoter using transient expression assays. Luciferase reporter plasmids containing the enhancer–promoter sequence in its natural context in the HPV-31b LCR were transfected into a variety of cell types and luciferase assays were performed. As expected, LCR activity was found to be the highest in the cell lines derived from keratinocytes such as CIN612, primary foreskin keratinocytes and HeLa cells, whereas reduced expression was observed in non-keratinocyte cell lines such as HepG2 cells (liver cell origin) and BJAB cells (lymphoid cell origin) (Fig. 1*a*), suggesting the keratinocyte-specific transactivation of HPV early genes from the homologous promoter. Surprisingly, transcriptional activity was quite low in C33A cells despite the fact that these cells were of keratinocyte origin. Similar results were obtained using an HPV-18 LCR reporter plasmid. To examine further the reasons

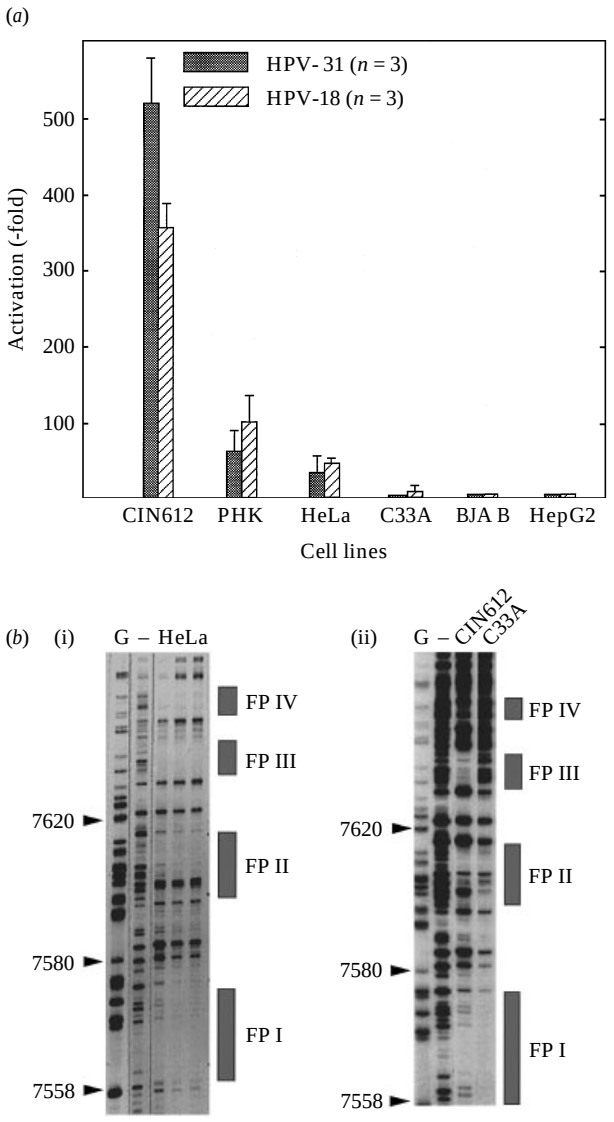

Fig. 1. (*a*) Luciferase assay to examine the cell type-specificity of the HPV promoter. Luciferase reporter plasmids were transfected into a variety of cell types. At 48 h after transfection, cells were collected and luciferase assays were performed. Luciferase activity was normalized by that of a negative control in which cells were transfected with the reporter plasmid containing no enhancer–promoter sequence. PHK, primary foreskin keratinocyte. (*b*) DNase I footprint analysis to identify the factor binding sites within the HPV-31b minimum enhancer. A 262 bp fragment (nucleotides 7511–7772) of the HPV-31 enhancer was end-labelled with ^{32}P and incubated with extracts from HeLa (i), CIN612 or C33A cells (ii) followed by the treatment with DNase I. In (−) lanes, no nuclear extract was added. Five protected sequences were identified and are shown as boxes, designated FPI to V. FPV was on the edge of the enhancer and is not shown.

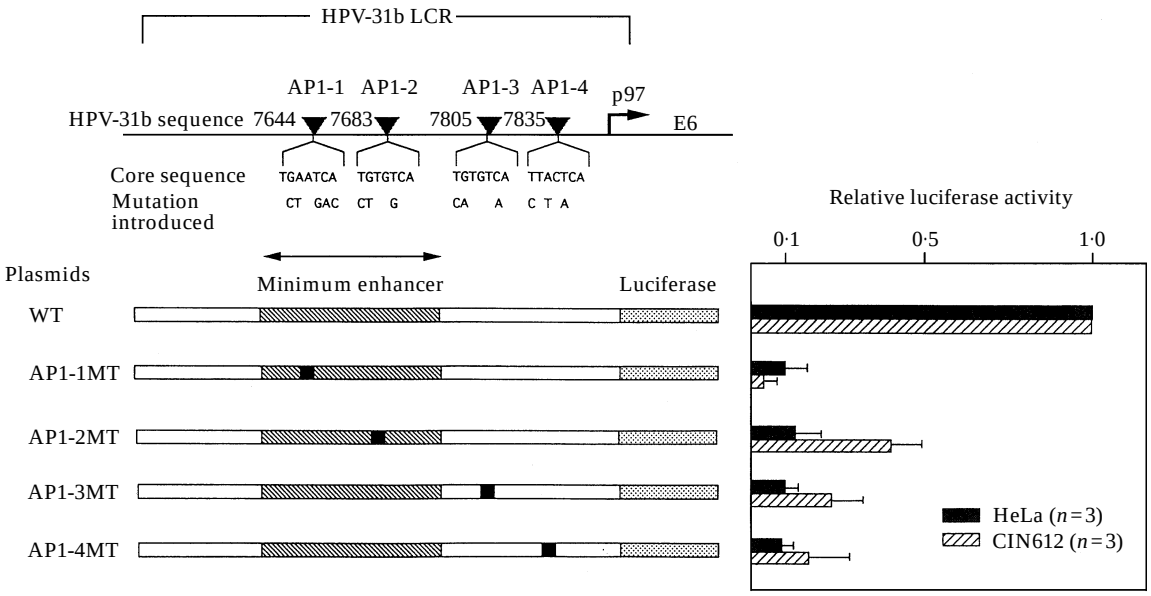

Fig. 2. Luciferase assays to determine the functional significance of AP1 sites in the HPV-31b LCR. Four AP1 sites designated AP1-1, AP1-2, AP1-3 and AP1-4 are shown as well as their core sequences similar to the AP1 consensus motif. Substitution mutations introduced are also shown. Luciferase reporter plasmids containing the whole LCR of HPV-31b with or without mutations in the core sequences of each AP1 site were transfected into HeLa cells or CIN612 cells and luciferase assays were performed. Relative luciferase activity in each plasmid is shown.

for this cell type-dependent expression, footprint analyses were performed in the HPV-31b enhancer region using nuclear extracts from HeLa, CIN612 and C33A cells. As reported previously (Kyo *et al.*, 1995), a series of protected sequences were identified in the minimum enhancer region of HPV-31b with HeLa nuclear extracts, designated FPI to V, respectively. Fig. 1(*b*) shows protected sequences FPI to IV; FPV was located at the end of the probe. In the previous study, we demonstrated that FPI and II, and FPV had similarities to NF1 and KRF-1 sites, respectively, but that they bound novel factors. FPIII was found to bind AP1, and FPIV bound AP1 as well as Oct1. Similar patterns of footprints were also observed using extracts from HPV-31b-positive CIN612 cells. However, when extracts from C33A cells were used, FPIII and IV footprints were diminished while FPI and II (Fig. 1*b*) as well as FPV (data not shown) remained significant. These findings suggested that a reduction in AP1 binding activity in C33A cells might contribute to the decreased activity of the HPV enhancer.

Four AP1 sites in the HPV-31 LCR function synergistically for full activation of the enhancer. Initial analysis of the HPV-31 enhancer region (nucleotides 7511–7772) identified two AP1 sites corresponding to FPIII and IV (designated AP1-1 and AP1-2, respectively; Kyo *et al.*, 1995). Computer-assisted analysis of the entire LCR suggested two other putative sites for AP1 outside the enhancer, designated AP1-3 and AP1-4, both of which contained sequences similar to the AP1 consensus motif (7805–7811 and 7835–7841) (Fig. 2). These sites are located between the enhancer and the start site of

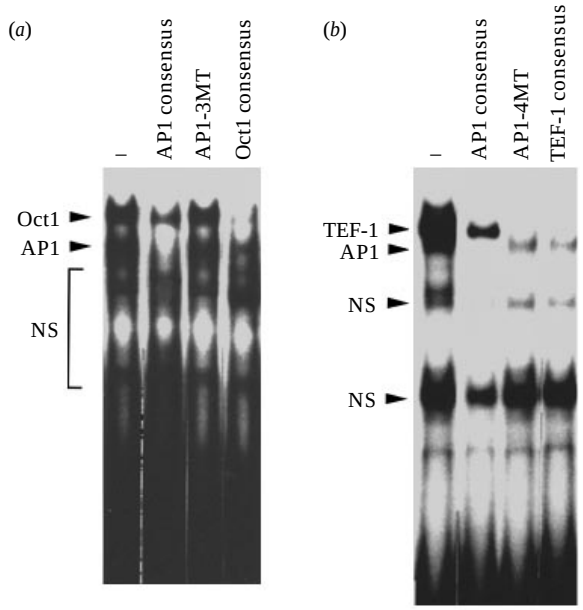

Fig. 3. EMSA to confirm the AP1 binding to the putative AP1 sites outside the enhancer of HPV-31b LCR. Nuclear extracts from HeLa cells were incubated with ^{32}P-labelled probes for AP1-3 (*a*) and AP1-4 (*b*) sites in the presence of the various competitors shown above each lane. NS represents non-specific binding. AP1-3MT and AP1-4MT represent oligonucleotides containing substitution mutations in core sequences of AP1-3 and AP1-4 probes, respectively. The sequences of probes and competitors used are shown in Methods and Table 1.

transcription, indicating that they form part of the LCR promoter. AP1 binding to these sites was confirmed by EMSA. As shown in Fig. 3, specific binding was observed to both sites,

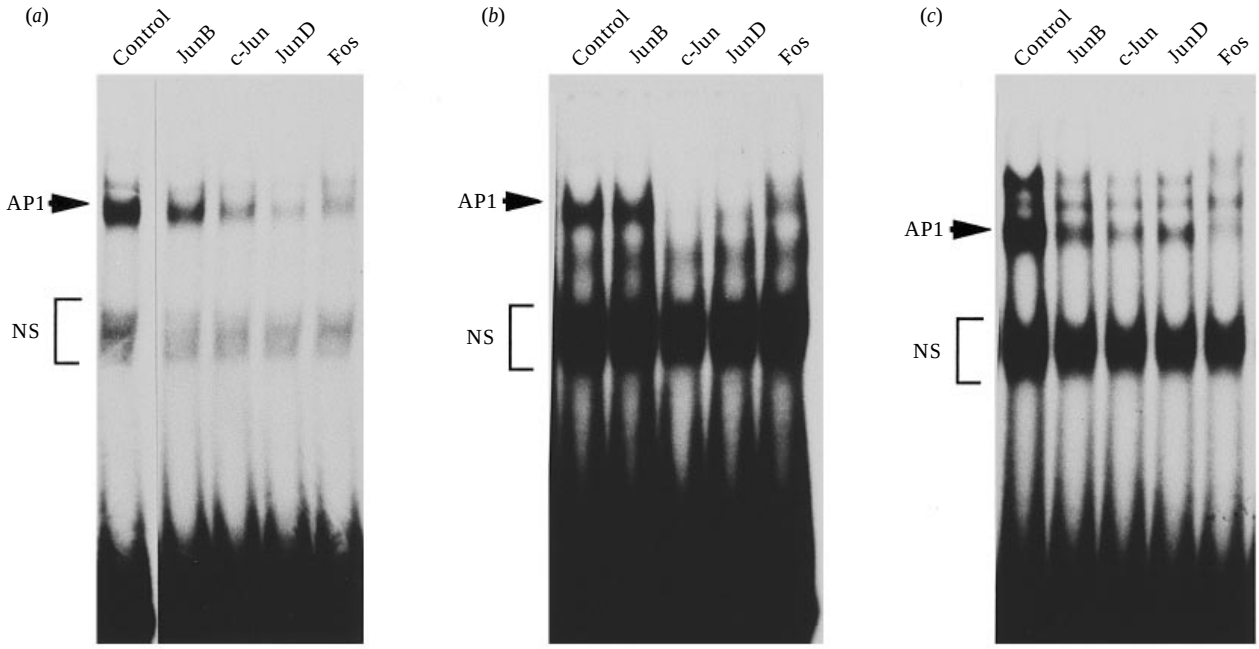

Fig. 4. EMSA showing the binding of Jun members to HPV-31b enhancer in a variety of cell types. Nuclear extracts from CIN612 (*a*), Hs68 (*b*) and SCC13 (*c*) cells were pre-incubated with antibodies against JunB, c-Jun, JunD and Fos as indicated above each lane and then incubated with FPIII probe containing an AP1-1 site. In the control sample, antibody against SP1 was pre-incubated with extracts. NS represents non-specific binding. Multiple bands were observed in SCC13 cells and only the bottom one was identified as an AP1-specific band (data not shown).

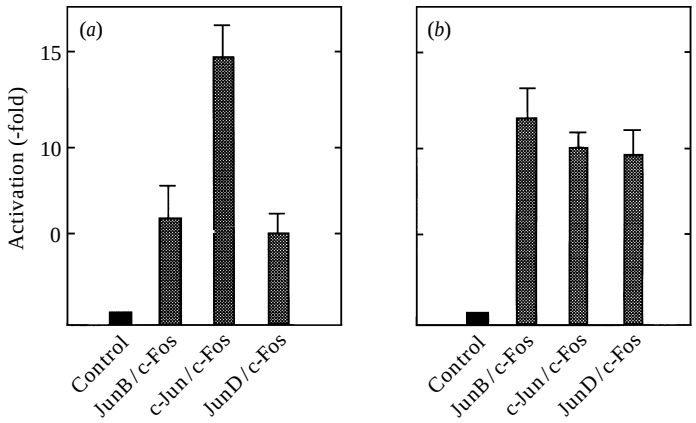

Fig. 5. Induction of AP1 in the cells in which HPV enhancers are inactive. Expression plasmids for JunB, c-Jun and JunD were each co-transfected with c-Fos expression plasmids as well as a luciferase reporter plasmid containing the wild-type LCR of HPV-31b into C33A (*a*) and HepG2 (*b*) cells and transient expression assays were performed. In control samples, SV2-Neo plasmid was co-transfected with a reporter plasmid . At 48 h after transfection, cells were collected and luciferase assays were performed.

which was diminished by addition of the AP1 consensus motif but not by addition of unrelated sequences or oligonucleotides containing substitution mutations in their AP1-like consensus motifs, suggesting that AP1 binds to these sites. Additional interactions were observed with the AP1-3 and AP1-4 sites, which were competed by addition of Oct1 and TEF-1 consensus motifs, respectively, suggesting that Oct1 and TEF-1 factors also bind these sites. We next sought to examine the functional significance of the AP1 sites. Luciferase reporter plasmids containing substitution mutations in each AP1 site were transfected into HeLa or CIN612 cells and luciferase assays were performed (Fig. 2). Mutations introduced are shown in Fig. 2 and Table 1. They were confirmed to abrogate AP1 binding in our previous study (Kyo *et al.*, 1995) and by EMSA in the present study. In HeLa cells, each mutation resulted in an 80 to 90% reduction in luciferase activity, showing the drastic effect of AP1 factors on HPV-31 transcription. Similarly, in CIN612 cells, mutation of AP1-1

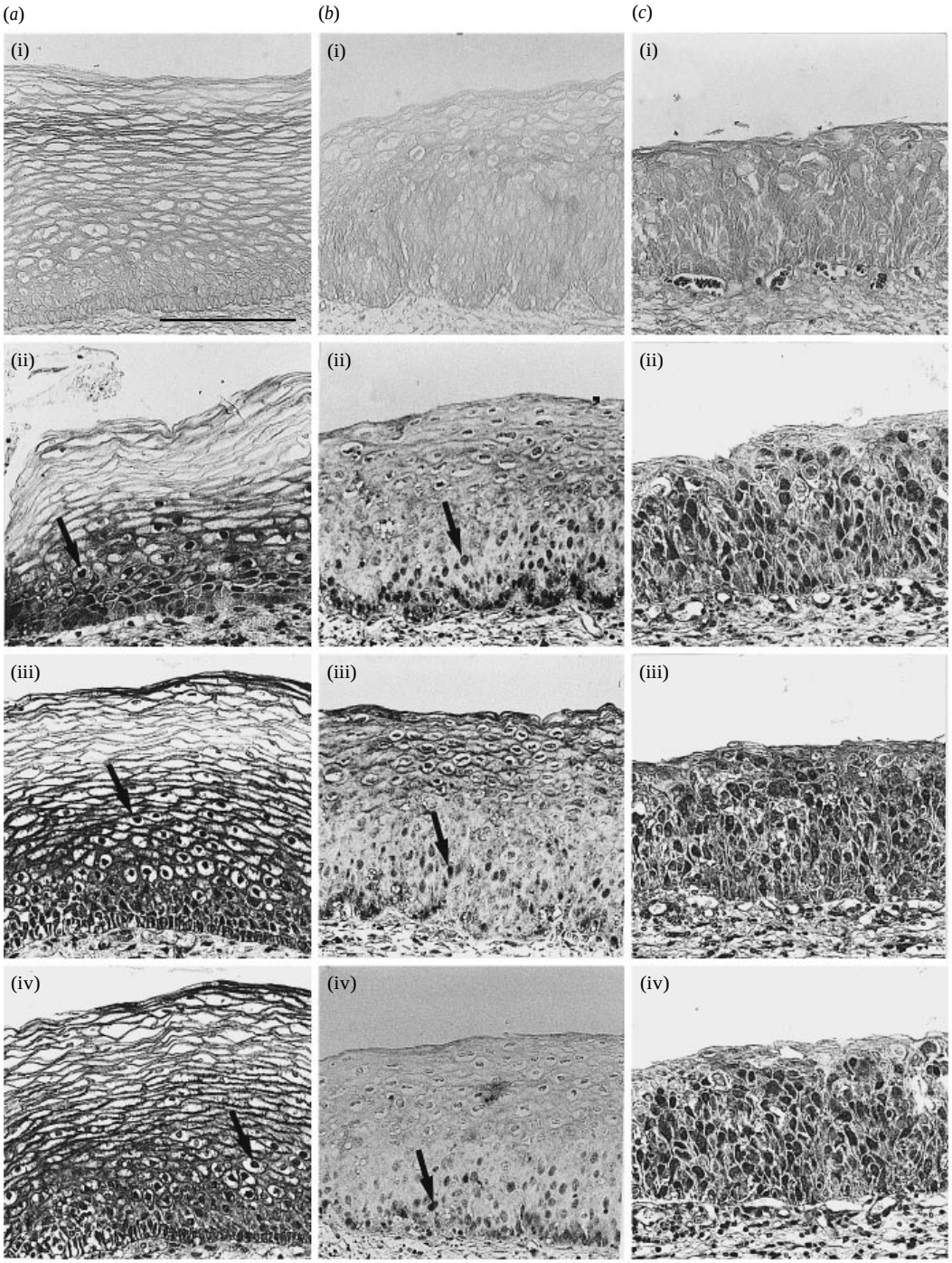

Fig. 6. Immunohistochemical analysis to identify AP1 expression in the stratified epithelia of normal cervix (*a*), CIN II (*b*) and CIN III (*c*). Bar marker represents 200 μm. Sections were incubated with normal goat serum for controls (i) or with primary antibodies against JunB (ii), c-Jun (iii) or JunD (iv). Arrows indicate significant AP1 signals.

had a severe effect while disruption of AP1-2, AP1-3 and AP1-4 had a less severe effect. These data suggest that AP1 sites outside the minimum enhancer also contribute to LCR transcription. Since each mutation was effective independent of the other sites, it appears that these sites function synergistically for full activation of enhancer.

Differential effect of each Jun type on the HPV enhancer in different cell types. To examine the distribution of Jun family members in a variety of cell types and to identify the predominant Jun type binding to the HPV-31 LCR, an EMSA was performed using an AP1 site (AP1-1) as a probe with nuclear extracts from different cell types (Fig. 4). In CIN612

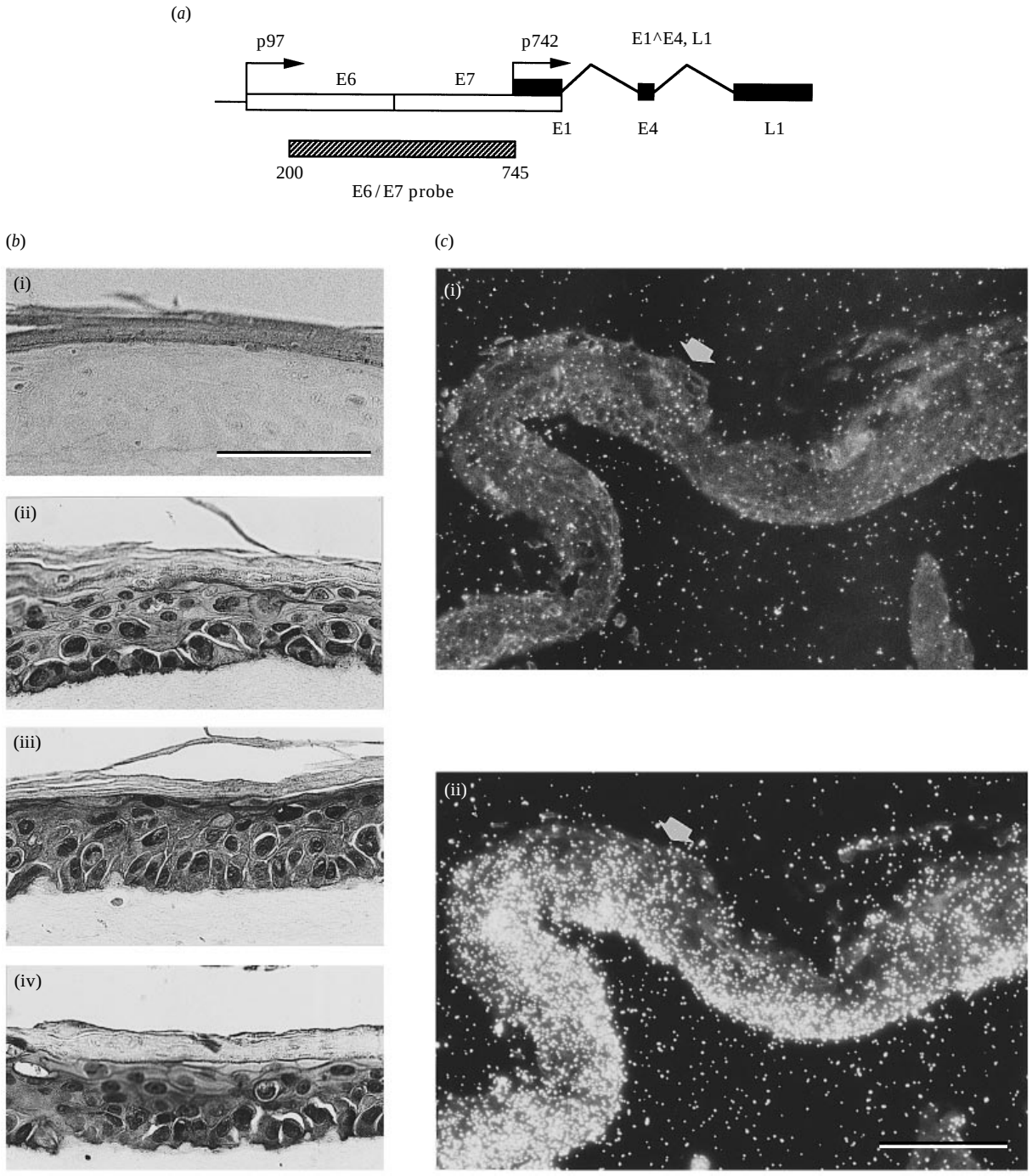

Fig 7. (*a*) Probe sequences used in *in situ* mRNA hybridization. The p97 early promoter of HPV-31b is shown. The p742 E7 promoter which directs E1 ∧ E4 differentiation-dependent transcripts is also indicated. (*b*) Immunohistochemical analysis to identify AP1 expression in the differentiating epithelia of organotypic raft cultures using CIN612 cells. Sections were incubated with normal goat serum for controls (i) or with primary antibodies against JunB (ii), c-Jun (iii) and JunD (iv). Bar marker represents 200 μm. (*c*) *In situ* mRNA hybridization to detect HPV-31b E6/E7 signals in raft tissues. Sections were hybridized with sense probes (i) or anti-sense probe (ii). Bar marker represents 200 μm. Arrows indicate terminally differentiated layers.

cells, an AP1-specific shifted band was observed and it was significantly reduced following incubation with a JunD antibody, while it was only moderately inhibited by addition of JunB and c-Jun antibodies. A different spectrum of inhibition was observed in Hs68 cells, where the shifted band was completely inhibited by addition of c-Jun antibody but moderately competed by addition of JunD antibody. No change was observed on addition of JunB antibody. In the

Table 1. Oligonucleotides used for Electrophoresis Mobility Shift Assay

Name	Position (nt)	Sequence*
AP1-1	7636–7660	5'-ATTATACTATGAATCATGTT TGT TT-3' 3'-TAATATGATACTTAGTACAAACAAA-5'
AP1-3	7796–7822	5'-CTGCCAAGGTTGTGTCATGCAT TATAA-3' 3'-GACGGTTCCAACACAGTACCTAATATT-5'
AP1-3MT	7796–7822	5'-CTGCCAAGGT CA TGT A ATGCATTATAA-3' 3'-GACGGTTCCA GT ACA T TACCTAATATT-5'
AP1-4	7825–7851	5'-AAGTTGTATGTTACTCATATAATTAAT-3' 3'-TTCAACATACAATGAGTATATTAATTA-5'
AP1-4MT	7825–7851	5'-AAGTTGTATG CTTCACATATAATTAAT-3' 3'-TTCAACATAC GAAGTGTATATTAATTA-5'

* Mutations introduced are underlined.

squamous cancer cell line SCC13, which lacks HPV sequences, the AP1 shift was equally inhibited by addition of each antibody. These results suggest that each of the Jun members can bind the AP1 sites in the HPV-31 LCR but the predominant type appears to vary between different cell types.

We next sought to determine if there was any functional difference between the Jun types in transactivation of the HPV-31 LCR. Three types of Jun expression plasmids (*junB*, c-*jun* and *junD*) were each co-transfected with c-*fos* expression plasmids and a luciferase reporter plasmid containing the HPV-31b LCR into C33A cells or HepG2 cells. Transient expression assays were then performed. These cells exhibited low levels of HPV-31 expression in transient assays (Fig. 1*a*). As shown in Fig. 5, in C33A cells, co-transfection of c-*jun* and c-*fos* led to an activation of 15-fold above background, while cotransfection of *fos* with *junB* and *junD* increased expression six- and fivefold, respectively. In HepG2 cells, transfection of *junB* increased expression 12-fold, while c-*jun* and *junD* activated expression 10-fold. Expression of each Jun member without co-expression of Fos had only marginal effects (data not shown). These findings suggest that each Jun family member is capable of activating HPV expression in cooperation with Fos, though there was some preferred activity in different cell lines. These studies also demonstrate that HPV expression can be increased by higher levels of AP1 factors, indicating that the levels of these proteins can contribute to the transcriptional activity of HPV.

Expression of the Jun family in stratified epithelia of the uterine cervix

To characterize the distribution of Jun family members in stratified epithelial cells of uterine cervix, immunohistochemical analyses were performed using JunB, c-Jun and JunD antibodies with paraffin-embedded biopsy specimens from normal cervix, various degrees of CIN and cervical cancer. Representative results are shown in Fig. 6. In normal cervix and low-grade CIN II, which retain markers of terminal differentiation, JunB and JunD were mainly expressed in the basal cell and suprabasal cell layers, although faint signals were observed in more differentiated layers. No expression was observed in the most terminally differentiated layers. It thus appeared that JunB and JunD were preferentially expressed throughout lower layers of the epithelia. c-Jun expression was mainly observed in the lower layers but weak signals were also observed in more differentiated layers. In contrast, in high-grade CIN and invasive cancer, which are composed of undifferentiated cells, AP1 expression was observed throughout the layers and a similar distribution was observed for all three Jun family members

E6/E7 and AP1 expression in an *in vitro* differentiation system

We next sought to investigate how HPV early gene expression was regulated during the virus life cycle in differentiating epithelia, using an *in vitro* raft system for epithelial differentiation with CIN612 cell lines containing HPV-31b genomes as episomes. Organotypic raft cultures using CIN612 cells stratify in a manner similar to that seen in low-grade CIN lesions and are able to synthesize HPV-31 virions (Myer *et al.*, 1992). The probe used in this study was designed based on our previous analyses of the late promoter that directs synthesis of the E1–E4 transcript of HPV-31b (Hummel *et al.*, 1992). The probe includes sequences from E6 and stops at nucleotide 745 in the E7 ORF, and does not contain sequences from the E1–E4 differentiation-dependent transcript (Fig. 7*a*).

In situ mRNA hybridization using this probe revealed that E6/E7 transcripts were distributed in both the basal and suprabasal regions and at a lower level in the more differentiated cells (Fig. 7*c*). Only faint signals were observed in the terminally differentiated superficial layers. These signals were specific for HPV early transcripts because control specimens hybridized with sense probe gave no significant signal. When immunohistochemical analysis of *jun* expression was performed using rafts of CIN612 cells, we observed a similar distribution of expression to that seen in biopsy materials and a strong correlation with HPV-31 early gene expression (Fig. 7*b*). These findings demonstrated that E6/E7 expression was closely associated with AP1 expression and further implicate AP1 factors in regulating E6/E7 expression in differentiating epithelia.

Discussion

In the present study, we found that AP1 binding to the HPV-31b LCR was reduced in C33A cells in which the HPV enhancer was inactive and that enhancer activity was restored when AP1 expression vectors were transfected. These findings suggest that quantitative differences between AP1 levels in cell types is a critical factor in determining enhancer tropism. Previous studies have demonstrated an elevated level of AP1 in keratinocytes as compared to fibroblasts, possibly owing to a lack of degrading enzyme resulting in increasing stability of AP1 factors (Offord *et al*., 1993). The present EMSA analyses also revealed that the predominant types of Jun that bind to sites in the LCR varied in different cell types. Co-transfection of the individual Jun proteins together with c-Fos into C33A and HepG2 cells indicated the differential effect of each Jun type on the HPV enhancer in different cell types. Thus, differences between the Jun family members present in different cell types may also contribute to enhancer tropism of HPV. It has been suggested that AP1 interacts with some cellular factors such as the glucocorticoid or retinoic acid receptors to regulate gene transcription (de Groot *et al*., 1990; Jonat *et al*., 1990; Schule *et al*., 1990). Such interactions could affect or alter AP1 function in each cell type, also contributing to specificity of HPV expression.

Previous studies on the expression of AP1 in differentiating epithelia suggested that JunB expression was restricted to differentiating cells (Wilkinson *et al*., 1989). In our study, JunB as well as JunD were detected in the lower layers of stratified epithelia in biopsies from normal cervix as well as low grade CIN. c-Jun seemed to be more highly expressed in differentiated layers. In contrast, in CIN III or invasive cervical cancer, they were expressed throughout all layers. Studies by Welter & Eckert (1996) using biopsies of normal foreskin keratinocytes demonstrated JunB and JunD expression in most layers, while c-Jun was found in the stratum granulosum. These observations are similar but not identical to our studies; this may be due to the differences in tissue types.

Despite several studies examining E6/E7 expression in uterine cervical lesions using *in situ* mRNA hybridization, some confusion still exists concerning the distribution of E6/E7 transcripts in the differentiating epithelia. One of the major reasons for this may be due to selection of probe sequences. As previously described, E1–E4 differentiation-dependent transcripts, which are expressed in the superficial cell layers of differentiating epithelia, initiate at a promoter located within the E7 ORF at nucleotide 742 in HPV-31 (Hummel *et al*., 1992) and between 498 and 767 in HPV-16 (Higgins *et al*., 1992). These late transcripts contain E7 sequences, although they do not encode functional E7 proteins. Therefore, the use of probes containing the entire E7 ORF would identify both early and late transcripts. Another factor affecting the expression of early genes in differentiating epithelia is the physical state of HPV DNA, which can be episomal or integrated. The state of virus DNA may vary among biopsy specimens and is sometimes heterogeneous even in a sample, making correlations between E6/E7 expression and cell differentiation difficult to investigate. To examine the expression of HPV genes in a homologous population of cells, we utilized an *in vitro* differentiation system with CIN612 cells derived from a CIN I lesion, and found that E6/E7 transcripts were distributed throughout the majority of the lower layers of the stratified epithelia. This is consistent with our Northern blot analysis of cells grown in monolayers and rafts, which indicated similar levels of E6/E7 expression following stratification (Hummel *et al*., 1992). Another study reported differentiation-independent constitutive expression of HPV-16 E6/E7 using a monolayer system in which CaSki cells were treated with calcium for promoting cell differentiation (Choo *et al*., 1994). These investigators observed that E6/E7 expression was not altered by cell differentiation, further supporting the present results. Our previous *in situ* hybridization studies indicated that HPV genomes are amplified and late proteins are expressed in differentiated cell layers (Bedell *et al*., 1991). These findings together with the present results suggest that increasing the copy number of virus genomes does not lead to elevated levels of E6/E7 expression and that there may in fact be a reduction in early transcription in cells that express late functions.

In contrast to E6/E7 expression, previous studies have identified that L1, L2 and E1–E4 differentiation-dependent transcripts are found in the suprabasal cell layers (Bedell *et al*., 1991; Higgins *et al*., 1992; Hummel *et al*., 1992; Pray & Laimins, 1995). Thus, E6/E7 and late gene expression appear to be regulated by different mechanisms, which is not surprising since late expression requires episomal templates while early expression does not (Frattini *et al*., 1996). Although the factors regulating the p742 promoter have not been identified, it is possible that differentiation-dependent cellular factors are involved in its regulation.

HPVs preferentially infect differentiating epithelia in genital mucosa. Initial infection is thought to occur in the basal cell layer where AP1 expression is significant. This pattern of AP1

expression possibly promotes early gene expression, contributing to the maintenance of initial infection and subsequently leading to replication in the upper layers. HPV infection induces cell proliferation, which is probably owing to specific interactions of the E6 and E7 products with p53 and Rb proteins, respectively. It is also likely that elevated AP1 activity, which activates promoters of the genes involved in cell proliferation, also contributes to the increased proliferation ability of the cells. It thus appears that the coordinated action of AP1 in inducing E6/E7 expression is important for maintaining HPV-infected cells in a proliferation-competent state during epithelial differentiation.

In conclusion, we have demonstrated that AP1 is a significant regulator of HPV expression and that it contributes to the specificity of virus expression in epithelial cells. Expression of HPV-31b E6/E7 was observed throughout lower layers of stratified epithelia and correlates with AP1 expression. This suggests a role for AP1 in regulating E6/E7 expression in differentiating epithelia.

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