Epidermal Growth Factor (EGF) Elicits Down-Regulation of Human Papillomavirus Type 16 (HPV-16) E6/E7 mRNA at the Transcriptional Level in an EGF-Stimulated Human Keratinocyte Cell Line: Functional Role of EGF-Responsive Silencer in the HPV-16 Long Control Region

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We have found that epidermal growth factor (EGF) elicits negative regulation of human papillomavirus type 16 (HPV-16) E6/E7 at the mRNA level in the HPV-16-immortalized human keratinocyte cell line (PHK160b). This down-regulation of HPV-16 E6/E7 expression was achieved when the cells were stimulated to proliferate with the concomitantly enhanced *c-myc* expression by EGF in a dose-dependent manner. By using partly synchronized PHK160b cells, negative and positive regulations of the HPV-16 E6/E7 expression was correlated to EGF-linked cell cycle events in this particular human keratinocyte cell line. In order to study transcriptional control mechanisms of the HPV-16 E6/E7, transient expression assays were performed with CAT expression plasmids that the transcription could be directed by the 5'-deleted HPV-16 long control region (LCR) including the virus P_{97} promoter. We demonstrated that the HPV-16 LCR contained EGF-responsive elements and that a predominant silencer activity was mapped in the proximal 124-bp region (EGFRE) of the LCR. This restricted LCR region had significant influence on HPV-16-homologous promoters in lowering the CAT expression in the presence and absence of EGF. EGFRE was thus considered to be a conditional transcription-controlling element on HPV-16 E6/E7 expression in this EGF-responsive human keratinocyte cell line. This suggests that specific sequences in the LCR play a critical part in the EGF-induced down-regulation of E6/E7 expression at the transcriptional level. Since the results obtained from the transient expression assay agreed with the mode of expression of the endogenous HPV-16 E6/E7, the present study strongly suggests that the transcriptional regulation of the HPV-16 E6/E7 oncogene is mediated by growth-related specific cellular factors interacting with HPV-16 LCR elements.

Human papillomaviruses (HPVs) are useful in control mechanism studies of gene expression, since HPV gene expression appears to be closely associated with events specific for the control of growth and differentiation of squamous epithelium (8). Recently, it has been demonstrated that HPVs, particularly types 16 and 18 (HPV-16 and -18), immortalize human squamous epithelial cells including human foreskin keratinocytes (18, 43, 49, 61) as well as cervical keratinocytes (60). Establishment of these cell lines enables the analysis of a virus-cell interaction approaching the *in vivo* situation of these pathogenic viruses. Transcriptional control mechanisms of HPVs have been extensively studied by using mostly types 11, 16, and 18 (10–14, 16, 17, 21–24, 28, 29, 31, 46, 54). Putatively functional regulatory elements mapped in the viral long control region (LCR) bind proteins of both viral and cellular origins (11, 13, 21–24). Although specific interactions of cellular factors with certain nucleotide sequences in the LCR upstream of E6/E7 genes have been documented (16, 21), only a few of these factors have been correlated to transcriptional HPV gene regulation (10, 13, 16, 17, 22, 24). The highly conserved viral E2 gene products bind to the specific E2 motif, ACCN₆GGT, within the LCR (1). This contributes to negative or positive regulation of the viral early genes (12, 28, 29, 31, 35, 39, 57), though the effect of E2 is not considered to be cell type

specific (29, 35, 39). Recently, a DNA segment which functions as an E2-independent, cell-specific regulator has also been mapped to the proximal region of the HPV LCR (16, 17, 23). It has been postulated that some cellular factors that bind to the LCR, such as glucocorticoid receptors (11, 13, 22–24), nuclear factor I (13, 16, 22, 23), and the AP1/*jun* protein family (10, 13, 16), stimulate viral gene expression. These factors are somewhat ubiquitous in a variety of cell types, and therefore epitheliotropic gene regulation of HPVs correlating with these factors remains to be elucidated.

Since most epithelial cells require growth factors such as epidermal growth factor (EGF) or fibroblast growth factor for their proliferation, and in some cases, differentiation, we attempted to characterize the mode of HPV-16 gene regulation in an appropriate natural host by using a human foreskin keratinocyte cell line which responds to EGF. EGF modulates cell proliferation and differentiation in various cells and cell lines and is necessary for the multiplication of cultured human epidermal keratinocytes (2, 45). EGF induces a variety of early and late responses in cells that have EGF receptors (EGF-Rs). Relatively earlier responses are EGF-R kinase activity, increased ion flux, activation of cellular metabolism, and induction of specific mRNA followed by cellular DNA synthesis (9). Signal transduction may be an ongoing process after EGF binding to EGF-Rs (50), subsequently resulting in positive and negative regulations of specific genes leading to cell proliferation and differentiation.

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However, specific target genes and nucleotide sequences are still poorly documented.

In the present study, we have identified a functional DNA portion within the HPV-16 LCR which confers negative transcriptional regulation on the virus promoter in the EGF-stimulated human keratinocyte cell line. The EGF-responsive silencer activity is overlapping in the region previously mapped to the keratinocyte-dependent enhancer (16, 17, 23, 24). This regulation may be due to the specific interaction of EGF-inducible cellular factors with nucleotide sequences in the restricted LCR region. EGF regulation of the HPV-16 gene expression is dissociated from EGF-induced cell proliferation and the enhanced *c-myc* expression. The biological significance of the HPV-16 gene regulation is discussed.

MATERIALS AND METHODS

Cells and cell culture. Stably growing human epidermal keratinocyte lines (PHK16 series) were obtained after transfecting HPV-16 DNA by the modified Ca^{2+} -phosphate coprecipitation method (43) or electroporation by using a Baekon model 2000 (Advanced Gene Transfer System, Baekon/CA) (27). The PHK160b line used in most of this study was maintained in complete DM152 medium supplemented with a hormone mix including 5 ng of EGF per ml and 0.5% dialyzed fetal calf serum as described previously (43). (More details on the cell line, PHK160b, are described in the text as well as in a previous report [27]).

Construction of plasmid DNAs. Chloramphenicol acetyltransferase (CAT) plasmids containing the 5' deletion of the HPV-16 LCR were constructed so that CAT expression occurred from the HPV-16 transcriptional promoter or the minimum unit of the simian virus 40 promoter (SVE; provided by M. Satake and Y. Ito) (55a). Briefly, each CAT plasmid contains LCR portions cleaved by various restriction enzymes which create 5' ends as follows: *Pst*I (7,003 nucleotides [nt]), *Eco*RI (7,454 nt), *Hinf*I (7,632 nt), *Rsa*I (7,643 nt), and *Hae*III (7,767 nt) for CAT plasmids pLCRCAT, pLCRECAT, pLCRHfCAT, pLCRRCAT, and pLCRHeCAT, respectively. The 3' end of each 5'-deleted segment is common at an *Ava*II site (113 nt). The plasmid DNAs were purified by cesium chloride (CsCl) density gradient centrifugation.

CAT assay. Transfection of CAT plasmid DNAs (10 to 15 μg) into 10^6 cells in 100 μl of the reaction buffer was performed by electroporation with a Baekon model 2000 as recommended by the supplier. The system settings for the electroporation with the Baekon model 2000 were as follows: amplitude, 7.5 kV; number of pulses, 2^8 U; burst time, 6.4 s; cycles, 20. Plating efficiencies of the electroporated cells was reproducibly 70% or more, and the copy number of the transfected plasmid DNA was consistent under the constant electroporation conditions. The CAT expression assay was carried out by the method of Gorman et al. (25). Radioactively labeled acetyl chloramphenicol was quantified by densitometric analysis of the autoradiogram and by the measurement of ^{14}C -labeled acetyl chloramphenicol with a scintillation counter.

Flow cytometry for population analysis in cell cycle and EGF-R dynamics. EGF-starved and -stimulated human keratinocyte cell lines were detached by EDTA treatment (2 mM in phosphate buffer) on ice for 20 min. Cellular DNA was stained with propidium iodide by using the Cycle TEST DNA reagent system and was analyzed by flow cytometry with the FACScan/CellFIT DNA system (Becton Dickinson). For the analysis of EGF-R dynamics, the cells were

incubated with mouse anti-EGF-R monoclonal antibody (Ab-1) reacting to the EGF-R outer membrane domain (Cosmo Bio, Tokyo, Japan) and stained with goat anti-mouse immunoglobulin G fluorescein isothiocyanate. The numbers of EGF-Rs on the cell surface membrane were shown by an arbitrary unit consisting of fluorescence intensity at 525 nm.

Extraction of RNA and dot hybridization. Total cellular RNA was extracted by 4 M guanidine isothiocyanate (pH 7.6). High-molecular-weight cellular DNA was fragmented by being passed through a 19-gauge needle several times. Viral and cellular RNAs were pelleted through a CsCl (5.7 g/ml) cushion as described previously (63). Purified RNA was dissolved in distilled water and was dot or Northern (RNA) blot hybridized, which was followed by autoradiography by using the standard methods described by Maniatis et al. (40). The quantity of mRNA was measured by densitometric analysis and was normalized by the expression levels of actin mRNA used as an internal standard.

RESULTS

EGF optimizes the proliferation of HPV-16-immortalized human epidermal keratinocytes. The PHK160b keratinocyte line used here has been established in our laboratory and harbors full-length HPV-16 genomes. The predominant viral mRNA expressed in the keratinocytes was the polycistronic 1.8-kb mRNA containing the E6/E7 early viral genes found in other HPV-16 transformants (27, 56, 63). The major viral transcriptional promoter, P_{97} , resides immediately upstream of the E6 initiation codon (52). This HPV-16-immortalized human keratinocyte cell line stably proliferated in the absence of high concentrations of Ca^{2+} and serum, and apparent differentiated keratinocyte phenotypes, such as the formation of cornified envelopes, were not evident. EGF was required for optimum cell growth but was unnecessary for maintaining cell proliferation (Fig. 1A and B). The growth rate and saturation density of PHK160b cells was reduced by 30% in medium lacking EGF. When the cells were treated with high concentrations (1.0 mM) of Ca^{2+} in the presence and absence of EGF, the cell-to-cell adhesion was induced, resulting in the epithelial island formation (Fig. 1C) typically observed in cultured normal epidermal keratinocytes (5, 30). Growth inhibition by added Ca^{2+} was more severe in the absence of EGF. EGF did not interfere with the cell-to-cell adhesion induced by Ca^{2+} . A cornified envelope subsequently appeared at a low frequency under this condition, suggesting that EGF starvation with high concentrations of Ca^{2+} potentiates the PHK160b cells to differentiate, but unlike nontransformed primary human keratinocytes, they were poorly committed to terminally differentiate. More than 90% of these cells were viable for a longer culture period in the presence of high concentrations of Ca^{2+} , as judged by vital staining with 0.4% trypan blue. The growth of PHK160b cells was thus optimized by the addition of EGF (5 to 10 ng/ml) and a low concentration (0.03 mM) of Ca^{2+} .

EGF suppresses HPV-16 E6/E7 expression with concomitant *c-myc* expression at the mRNA level in PHK160b cells. To investigate whether HPV-16 E6/E7 expression was conditionally regulated in association with cell proliferation, we examined the effects of EGF on viral gene expression at the mRNA level in PHK160b cells. Prior to EGF stimulation, the cells were starved in media lacking EGF for 2 to 3 days. The PHK160b cells were incubated with EGF in the range of 0.1 to 50 ng/ml and with low concentrations of dialyzed serum (0.5%) and Ca^{2+} (0.03 mM). Total cellular mRNA was

FIG. 1. Effects of EGF and Ca^{2+} on growth control and differentiation of HPV-16-immortalized human epidermal keratinocytes (PHK160b). (A) Combinations and treatments of the EGF and Ca^{2+} . EGF (10 ng/ml) was added to the cells after EGF starvation for 2 days. Concentrations of Ca^{2+} were adjusted in media with and without EGF starvation. (B) Each point represents the mean value of cell numbers in triplicate cultures. Standard errors were less than 1.2% of the mean values. (C) Morphologic changes in the presence and absence of high concentrations of Ca^{2+} . Magnification, $\times 13.4$.

isolated from cells 2 to 3 days after the EGF stimulation. The expression levels of E6/E7, *c-myc*, and actin genes were quantified by Northern blot and dot hybridization (Fig. 2A, inset), and the mRNAs of the E6/E7 genes and *c-myc* were normalized by the expression levels of actin mRNA. We found that EGF dramatically lowered the steady-state levels of HPV-16 early gene mRNA as probed by the E6/E7 genes, while the expression of the *c-myc* gene was stimulated by EGF in a dose-dependent manner (Fig. 2A). The numbers of EGF-Rs reactive to anti-EGF-R monoclonal antibody dynamically changed after the EGF stimulation, suggesting that PHK160b cells respond to EGF through internalization of the receptors (Fig. 2B).

Since the EGF effect on cell proliferation counteracts

Ca^{2+} signaling in the differentiation of cultured keratinocytes (19), we next examined whether the EGF effect on the expression level of E6/E7 and *c-myc* mRNAs could be influenced by the high Ca^{2+} concentrations. Total mRNA was similarly extracted from cells 48 h after the EGF stimulation, and RNAs (10 μg) were dot hybridized by using E6/E7, *v-myc*, and actin genes as probes. The steady-state level of the E6/E7 mRNA was elevated by the added Ca^{2+} (1.0 mM) (Fig. 3). In contrast, the mRNA level of *c-myc* declined. Maximum E6/E7 expression was achieved by high concentrations of Ca^{2+} and no EGF. The reverse was true for maximum *c-myc* expression. These results indicate that EGF is a predominant modulator in the regulation of HPV-16 gene expression in this particular cell line.

FIG. 2. (A) Effects of EGF on steady-state mRNA levels of *c-myc* and HPV-16 E6/E7 in PHK160b. The cells were stimulated with various concentrations of EGF following EGF starvation for 48 h. Total cellular RNA was extracted from cells 48 h after EGF stimulation. (A) Cellular RNA (10 μ g) was spotted onto nitrocellulose filters and probed by *v-myc* and β -actin gene (inset). Northern blot RNA (20 μ g/lane) was probed by an HPV-16 DNA segment covering the E6/E7 reading frame (1.75-kb *Pst*I fragment) (inset). Autoradiogram spots were quantified by densitometric analysis. The relative quantity of E6/E7 or *c-myc* mRNA was normalized by the amount of expressed actin mRNA. (B) Responses of PHK160b cells to added EGF (10 ng/ml) regarding dynamic changes of EGF-R that was reactive to mouse anti-EGF-R monoclonal antibody. Changes in EGF-R numbers were represented by an arbitrary unit of the 525-nm fluorescence of fluorescein isothiocyanate. Down-regulated EGF-R recovered with time and stably remained at the levels indicated by an arrowhead (48 h after EGF stimulation).

FIG. 3. Effects of EGF and Ca²⁺ on the mRNA levels of E6/E7 and *c-myc*. Dot hybridization was performed as described in the legend to Fig. 2. The relative mRNA levels 48 h after EGF stimulation were the comparison with the level of mRNAs from cells incubated in low concentrations of Ca²⁺ and in the absence of EGF (indicated by underlines). - and +, 0.03 and 1.0 mM of Ca²⁺, respectively. Treatments with Ca²⁺ and EGF were the same as shown in Fig. 1.

Flow cytometry of the cell population further demonstrated that 60 to 70% of the EGF-starved cells prior to EGF addition were in G1 phase and that approximately 25% of the cells moved into S phase within 24 h following EGF stimulation (Table 1). The population ratios in G1 and S phases were maximal at time zero and at 48 h after EGF stimulation. This indicates that a fraction of EGF-starved PHK160b cells was synchronously stimulated to enter S phase by the addition of EGF, further suggesting that the regulation of HPV-16 E6/E7 expression is associated with EGF-linked cell cycle events.

EGF suppression was correlated to transcriptional regulation of the HPV-16 LCR. To determine first whether the

TABLE 1. Population analysis in cell cycle of PHK160b^a by flow cytometry

Phase	% Cells in phase ^b (h):						
	-48	-24	0	10	24	48	120
G1	64	64	72	65	46	64	52
	53	54	63	ND	43	72	ND
S	27	26	19	26	44	25	33
	30	31	27	ND	41	20	ND
G2/M	9	10	9	9	10	11	15
	17	15	10	ND	16	8	ND

^a PHK160b is a near-diploid human keratinocyte cell line and requires EGF for optimum cell growth. This cell line was routinely maintained in the medium supplemented with 5 ng of EGF per ml.

^b To test EGF effects, cells were starved in media without EGF for 2 to 3 days before the addition of 10 ng of EGF per ml. The data include two independent cell population kinetics before and after EGF stimulation. ND, Not determined.

FIG. 4. (A) Effects of EGF on the transcriptional activity of the HPV-16 LCR as determined by the CAT assay. The LCR was ligated to the CAT gene so that transcription would start either from HPV-16 promoters (plasmid a, pLCRCAT) or from the SVE promoter (plasmid b, pLCRSVECAT). Relative CAT expressions were quantified by either densitometric analysis or direct measurement of ^{14}C -acetylated chloramphenicol (AC). Parts of the solid lines in the CAT constructs indicate polylinker sequences. (B) Dot hybridization of 10 μg of the extracted RNA. Relative intensities shown at the right side of each autoradiogram indicate up- and down-regulation of mRNA levels. RNAs were extracted from cells which were prepared in parallel with those for CAT assays.

effect of EGF on the transcriptional activity of the HPV-16 promoter is linked to the LCR function, two CAT plasmids containing different transcriptional promoters were constructed for the transient expression assay in the presence and absence of EGF (Fig. 4A). The pLCRCAT plasmid was expected to express CAT from the HPV-16 transcriptional promoter. The other plasmid contained an additional SVE promoter immediately downstream of the 3' LCR. We found that approximately 80% of the CAT expression was suppressed by added EGF in plasmid pLCRCAT-transfected keratinocytes, whereas EGF had little effect on CAT expression with the other plasmid, pLCRSVECAT. Since dot hybridization of the isolated mRNA in parallel experiments showed that E6/E7 suppression was approximately 50% (Fig. 4B), the transient expression assay appeared to be more sensitive in detecting the transcriptional level than the direct measurement of mRNAs by Northern blot or dot hybridization. The significance of these results could be correlated with functions of EGF-induced *trans*-suppressing factors which elicit transcriptional regulation from the HPV-16 promoters, suggesting strongly that specific sequences within the LCR were involved in this negative regulation.

An EGF-responsive silencer exists within the LCR. We then attempted to identify EGF-responsive HPV-16 LCR elements. To do so, we made CAT constructs carrying the 5'-deleted LCR (Fig. 5A). These CAT plasmids were transfected into the immortalized human keratinocyte cell line (PHK160b) to assess EGF-responsive elements. The results demonstrated that EGF negatively regulated CAT expression in all series of the CAT plasmid-transfected PHK160b line, possibly through effects on the LCR. Careful examina-

tions in repeated experiments on CAT expression indicated that deletion mutants from the 5' side of the LCR differentially responded to the added EGF, suggesting that a specific portion of the LCR plays a part in controlling transcriptional activity of the LCR (Fig. 5B). The highest CAT expression occurred in pLCRECAT-transfected keratinocytes in the absence of EGF. This plasmid carried an epithelial cell-specific enhancer (CTRE) in the presence and absence of EGF (55a). Plasmids c (pLCRHfCAT), d (pLCRRCAT), and e (pLCRHeCAT) achieved poor CAT expression in EGF-treated keratinocytes. Among these three plasmids, pLCRRCAT responded significantly in lowering CAT expression by the EGF effect. Since pLCRHeCAT represented the poorest response to EGF and gave rise to the lowest CAT expression among the plasmid series, the adjacent DNA segment at a position between *Rsa*I (7643) and *Hae*III (7767) appeared to function as an enhancer only in the absence of EGF.

We next tried to characterize these regulatory elements as to whether CTRE or EGFRE alone was biologically active regarding EGF responses. These two segments were ligated to pSVECAT plasmid to evaluate transcriptional activity from the SVE promoter in the presence or absence of EGF (Fig. 6A). CTRE enhanced CAT expression severalfold from the SVE promoter in the presence and absence of EGF (Fig. 5B, left panel). This element appeared to be a constitutive epithelial cell-dependent enhancer, since no enhancer activity was detected in fibroblasts (55a). Also, EGFRE did not have a significant enhancer activity on the SVE promoter. The effect of EGF on these two elements is represented in a slightly different manner on the SVE promoter activity. The CTRE element positively responded to EGF in a range of

FIG. 5. Identification of the EGFRE by using various CAT plasmids containing the 5'-deleted LCR. Plasmid d contains a unit which responded negatively to the added EGF. Functional units indicated on the map are as follows: E2, E2-responsive element; GRE, glucocorticoid-responsive element; KD, keratinocyte-dependent enhancer; HPV18, HPV-18-homologous enhancer. Nuclear protein binding consensus sequences (NFI and AP1) are indicated by arrowheads. EGF (10 ng/ml) was added after electroporation and incubated for 2 days as described in the legend to Fig. 4.

about 10%, and EGFRE responded negatively, resulting in about 15% suppression. Although these small differences in the transient expression assay hampered precise evaluation of the transcriptional activity, the effects of EGF on these two regulatory elements can be distinguished in a limited range when we reflected the basal EGF effect on the SVE promoter. The positive EGF effect on the SVE promoter alone averaged 22% in our assays, which were repeated at least five times. In contrast, the EGF suppression was more clearly detectable at significant levels in independently performed experiments using the same pLCRCAT plasmid series containing the HPV-16 homologous promoters (Fig. 6B), though the relative value of their CAT expressions could not be precisely determined in a range of certain variations. Again, the plasmid pLCRHeCAT showed the lowest CAT expression and the poorest response to EGF. The level of CAT expression from this shortest CAT plasmid that we made was comparable with that from the SVE

promoter in the absence of EGF and was significantly higher than that from the promoterless pCAT plasmid (data not shown). We therefore tentatively mapped a minimal transcription unit whose transcriptional activity is critically influenced by EGFRE (Fig. 6A) to this region. The EGF effect on CAT expression by using various CAT plasmids was summarized in Table 2.

On the basis of these results, we concluded that the HPV-16 LCR contained EGFRE capable of responding to EGF, suggesting that EGFRE is important in forming specific transcription complexes cooperating with the other control elements. The repressive function of EGFRE appeared to be predominant over other putative control elements, such as CTRE or the keratin-dependent enhancer, E2-responsive elements, and distal enhancers in the presence of EGF, though further analyses are necessary for elucidating functional roles of this conditional transcription-controlling element in relation to other complex elements.

FIG. 6. EGF-responsive silencer activity of EGFRE detected with HPV-16 natural promoter (MTU) but not with the SVE promoter. (A) pSVE-X-CAT plasmids containing EGFRE and CTRE, designated pSVE-EGFRE-CAT and pSVE-CTRE-CAT, respectively. (B) The CAT expression of the plasmids shown in the left panel was comparable with differential CAT expression by using pLCR-E-CAT, pLCR-R-CAT, and pLCR-He-CAT, shown in the right panel.

DISCUSSION

We have shown that the HPV-16 E6/E7 expression is down-regulated by EGF in a HPV-16-immortalized human keratinocyte cell line. Both transient CAT expression assay and direct measurement of E6/E7 mRNA expression strongly suggest that EGF-induced *trans*-suppressing factors

are involved in the down-regulation of HPV-16 E6/E7 at the transcriptional level. Functional dissection of EGF-responsive elements demonstrated that a proximal 124-bp LCR region played a critical part in the transcriptional regulation of HPV-16 E6/E7. This restricted LCR region, designated EGFRE, was mapped overlapping the keratinocyte-dependent enhancer that has been previously identified by Cripe et al. (17).

Correlations of the down-regulation in HPV-16 early genes with the growth and differentiation of epidermal keratinocytes. The PHK160b line used here was established after transfection of HPV-16 DNA (27). The cell line is considered immortal (more than 800 population doublings to date), but it does not have a tumorigenic phenotype in *nu/nu* mice. The chromosome number persisted at near diploid during serial passages over 800 population doublings. PHK160b cells retained cellular phenotypes regarding the morphologic and growth responses to Ca^{2+} and EGF, which is characteristic for normal epidermal keratinocytes (2, 5, 19, 30, 45). However, morphologic abnormalities were observed in the collagen-gel floating culture of the cell line used here (26a); these abnormalities have been shown in the other HPV-16-transformed human keratinocytes (38). Although HPV-16 alters epithelial differentiation, it seems that the regulation of HPV gene expression can be influenced by factors which normally affect the growth and differentiation of epidermal keratinocytes. By using the bovine papillomavirus type 1 system, the bovine papillomavirus type 1 E5 oncogene was shown to be a modulator of EGF-Rs in a rodent cell system (41). We do not know if HPV-16 E6/E7 oncogenes can similarly modulate EGF-Rs of keratinocytes. Reduced EGF-R distribution or an altered EGF response has been shown in differentiating epidermal cells (26, 32). Since HPV gene expression and replication appear to be associated with processes of keratinocyte differentiation in vivo (8, 14), the counteractive effects of EGF and Ca^{2+} may somehow reflect the in vivo situation in the viral and cellular events. Most recently, it was shown that tumor growth factor β (TGF- β) suppresses the HPV-16 early gene expression with the concomitant suppression of *c-myc* by using HPV-16-immortalized human foreskin and cervical keratinocytes (6, 62). We did not achieve consistent results concerning the TGF- β effect on HPV-16 gene regulation by using our PHK160b line (55a). Our partly inconsistent unpublished observations may be due to the usage of different cell lines which in fact represent a wide spectrum of cellular phenotypes including differential TGF- β sensitivities in the immortalized human keratinocytes (6, 62a). These differential cellular phenotypes can be observed in many independently established keratinocyte cell lines, possibly because of progressive changes associated with the HPV-16-induced transformation (62a). These differential cellular responses to factors could be reflected in the differential expression of HPV-16 genes in various transformed cell lines and progressive cervical tumors (33, 51, 63). Taken together with our results, differential TGF- β effects on the regulatory HPV-16 gene expression may further suggest that various complex factors and its combinations may be required for the specific regulation of HPV-16 gene expression.

Negative or positive factors may be closely associated with the physiological status of the cells, such as the differential inducibility of proto-oncogenes by EGF (7). Differential responsiveness of proto-oncogenes to growth factors may result in different growth advantages (36, 37, 53, 55). It is possible that malignantly transformed tumor cell lines such as SiHa or HeLa have lost their negative factors

TABLE 2. EGF effects on CAT expression in various CAT plasmids

Plasmid	CAT expression	
	Suppression or stimulation (mean $\pm$ SD) by EGF ^a	Relative activity ^b without EGF
pLCR-CAT	– (65 $\pm$ 17)	12.0
pLCR-E-CAT	– (51 $\pm$ 11)	14.0
pLCR-Hf-CAT	– (66 $\pm$ 5)	3.0
pLCR-R-CAT	– (68 $\pm$ 7)	7.0
pLCR-He-CAT	– (17 $\pm$ 7)	1.0
pSVE-CAT	+ (22 $\pm$ 11)	1.2
pSVE-LCR-CAT	– (10 $\pm$ 5)	11.0
pSVE-CTRE-CAT	+ (10 $\pm$ 4)	10.0
pSVE-EGFRE-CAT	– (15 $\pm$ 5)	2.0

^a –, Suppression; +, stimulation. Values shown are percentages of suppression or stimulation by 10 ng of EGF per ml and are calculated from independent CAT expression assays repeated three to five times.

^b Relative CAT expressions among CAT plasmids were deduced from two independent CAT expression assays by using PHK160b cells.

related to the growth regulation, as has been suggested by cytogenetic growth regulation of the tumor cell lines (34, 48). Some of these putative factors may be involved in transcriptional suppression of HPV-16 oncogenes (47). These previous reports also argue that the expression of HPV oncogenes is differentially controlled by cellular factors.

The relationship between cell cycle and E6/E7 transcriptional suppression. The differential CAT expressions were reproducibly detectable by using various CAT plasmids containing the 5'-deleted LCR segments with the natural promoters under our assay conditions, though the results obtained by the transient CAT expression assay have some limitations in the precise evaluation of the extent of regulation at a quantitative level. In some cases, expression levels in the CAT assay and in endogenous HPV-16 mRNA analysis were not perfectly consistent, possibly because of different assay conditions as well as different physical states of DNA in both assays. Alternatively, some variations of heterogeneous cell populations in cell cycle may have existed when the assays were performed. In fact, the preparation of a constant homogeneity of cell populations in cell cycle, such as ratios of cell populations in G1 to those in S, was critical to obtaining reproducible results in both CAT assay and mRNA analysis (unpublished data). In this study, we performed most CAT assays and mRNA analyses using cells between 36 and 48 h after EGF stimulation. Flow cytometry demonstrated that EGF starvation facilitated the accumulation of cells in G1 phase in up to 70% of the population. Approximately 25% of the G1 population was promptly stimulated to enter S phase during the following 24 h. We do not know why the entire G1 population was not induced to enter S phase. A likely explanation is given by the irreversible growth arrest at G1 phase in the EGF-starved keratinocytes, even in low concentrations of Ca^{2+} (59). Alternatively, heterogeneous G1 populations represented differential EGF stimulation to enter S phase. Since more than 90% of cells were viable as judged by the vital staining, the latter is more likely. Approximately 25% of the population appeared to be independent of EGF for cell proliferation. These results imply that a fraction of the HPV-16-immortalized PHK160b cells can proliferate independently from EGF. This proliferation may be because of the effects of E6/E7-associated growth promoting activity in the absence of EGF or acquired autocrine growth by factors such as TGF- α often induced in cultured human keratinocytes (2, 15). This hampers growth arrest with EGF starvation to synchronize population of human epidermal keratinocytes at the G1/S boundary (59). Although we could not precisely correlate specific cell cycle regulations to the HPV-16 gene regulation because of some limitations in these assays, the present results suggest that the transcriptional regulation of HPV-16 E6/E7 oncogenes may be implicated in some specific EGF-linked cell cycle events in this particular human keratinocyte cell line.

Prompt *c-myc* expression at the mRNA level responding to added EGF occurred prior to the stimulation of DNA synthesis, and relatively high steady-state levels of mRNA were maintained during S phase (unpublished data). This EGF-stimulated *c-myc* expression at the mRNA level as well as the cellular DNA synthesis has been demonstrated in a variety of cells that have EGF-Rs (7, 36, 37, 44, 54, 55). The Ca^{2+} effect lowered *c-myc* expression in PHK160b cells (about 10 to 20% suppression) (Fig. 3), and reduced cell growth was concomitant. In contrast, Ca^{2+} stimulation elevated the expression levels of E6/E7 genes in the absence of EGF in which *c-myc* expression and cell growth declined.

Although synchronization was not perfectly achieved, the present results suggest that the E6/E7 expression can be dissociated from these cellular events by specifically responding to added EGF. It is still unclear, however, whether the effect on E6/E7 is simply cell cycle dependent or is specifically linked to the EGF effect.

Functional elements within the LCR involved in the down-regulation of HPV-16 E6/E7 expression. It has been shown that various transcription control elements of HPV-16 may interact with cellular factors, resulting in the modulation of viral gene expression (13, 23). Glucocorticoids enhance the transforming ability of HPV-16 in certain cells, possibly because of the enhanced expression of viral E6/E7 genes through functions of the glucocorticoid response elements (11, 13, 42). The other functional elements have been correlated with the cell-type-specific control elements (13, 16, 55a). These specific sequences function mostly as transcriptional enhancers for the expression of E6/E7. To our knowledge, the viral E2 gene products have been the only identified repressors in the expression of papillomavirus early genes (12, 35, 39), although some negative cellular factors that control HPV gene expression have been presumed in particular cells treated by 5-azacytidine (47). More recently, some putatively negative regulatory elements have been dissociated from E2-binding sites, suggesting the presence of cellular repressors that bind to these restricted regions (13, 16, 17, 23). Although physical and functional dissections of regulatory elements are still incomplete, we have tentatively mapped the 124-bp EGFRE to the region within the 400-bp cell-specific enhancer (CRE) (13, 22, 23). The CRE region is a cluster of multiple functional elements such as the glucocorticoid response element (11, 13, 22), the keratin-dependent enhancer (16), and HPV-18-homologous enhancer sequences (54). The EGFRE found in this study was positioned at the 3' region of CRE where the keratinocyte-dependent enhancer and HPV-18-homologous enhancer sequences resided.

The EGFRE DNA segment contains three nuclear factor I motifs as well as a cytokeratin-specific promoter element (4), one AP1-binding site, and two functionally unidentified footprints (PVF and NFA) (13). It has been shown that the AP1 is responsible for the EGF-induced transcriptional activation of certain cellular genes (20). The AP1 site is also known as a phorbol ester-responsive element (10). There are three AP1 sites at positions 7621/7667, 7643/7659, and 7806/7822 in the HPV-16 enhancer region. Most recently, Cripe et al. have shown that Jun/Fos complexes bind to these AP1 sites, suggesting that HPV-16 P_{97} transcription may respond to growth factors and oncogene products via the AP1 pathway (16). The present study may support their prediction. Our preliminary results, however, suggest that the EGF-induced transcriptional suppression of E6/E7 may not be due to the AP1 site at position 7643/7659 in the proximal core of the HPV-16 enhancer, since pLCR HfCAT plasmid lacking this AP1 site still responded to EGF and since the plasmid pLCRRCAT containing the AP1 site poorly responded to phorbol ester (unpublished data). The other AP1 site at position 7806/7822 in the minimal transcription unit may also be dissociated from the EGF response (Fig. 6, plasmids pSVE-EGFRE-CAT and pLCR-He-CAT), though possibilities of the cooperation of these multiple protein binding sites to form biologically active transcription complexes cannot be excluded. E2 motifs specific for the promoter region of HPVs may also be involved in this process (41a, 46). It has been speculated that EGFRE-protein complexes interacting with other functional elements facilitate a

topological hindrance around unique TATA boxes containing E2 motifs immediately upstream of the P₉₇ promoter, resulting in the apparent promoter selectivity for transcription (3, 46, 58).

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