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Regulation of the Human Papillomavirus Type 11 E6 Promoter by Viral and Host Transcription Factors in Primary Human Keratinocytes

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Human papillomavirus (HPV) type 11 is strictly trophic for epithelial cells and induces benign condylomata of the external genitalia and also causes laryngeal papillomas. Primary keratinocytes are the appropriate hosts for studies of HPV gene regulation, but they are not frequently used, owing to difficulties in culturing and low transfection efficiencies. By modifying a Polybrene transfection procedure, we achieved consistently high transfection efficiencies in primary human foreskin keratinocytes and characterized the HPV type 11 enhancer in the context of the homologous E6 promoter. Contrary to previous studies with immortalized human cervical carcinoma C-33A cells, constitutive enhancer element II in the upstream regulatory region conferred no enhancer activity and did not abrogate repression by the homologous E2 protein. Rather, repression was strong, ranging from 5.6- to 20-fold for the various enhancer deletion mutations. By deletion analysis, a strong enhancer that included three nuclear factor 1 sites and one nuclear factor 1-associated factor-binding site was localized to a 45-bp region within constitutive enhancer element I, and it showed some degree of tissue specificity.

Human papillomaviruses (HPVs) are small DNA viruses that induce benign or malignant hyperproliferations in cutaneous or mucosal epithelium. The regulation of the upstream regulatory region (URR) containing the enhancer or the enhancer-E6 promoter of mucotrophic HPVs has been studied intensively via transient transfections of reporter genes, most commonly into immortalized human cervical carcinoma cells. Established cell lines usually have mutations in cellular genes associated with disregulation of transcription and proliferation and, in the case of cervical carcinoma cell lines, often contain integrated HPV DNA, the expression of which could complicate interpretation of data. Primary human foreskin keratinocytes (PHK) are an appropriate choice for transcriptional studies because they are a natural target for HPV infection. Such cells, however, are more difficult to culture and maintain and typically have very low transfection efficiencies (27). Thus, relatively few investigations of gene regulation have been performed with primary keratinocytes. Studies with both types of cells collectively have demonstrated that host transcription factors, as well as the family of virus-encoded E2 proteins, are modulators of the viral enhancer and the E6 promoter. The E2 proteins function by binding to a consensus sequence, $ACCN_6GGT$ (for reviews, see references 30 and 35). E2-C proteins devoid of the amino-terminal *trans*-activating domain of the full-length E2 protein act as competitive repressors of transcription (6, 8, 12, 40). The full-length E2 protein encoded by bovine papillomavirus type 1 and, to a much lesser extent, the homologous HPV E2 proteins repress transcription from the E6 promoter of HPV-16 and HPV-18 (1, 33, 40). For HPV-11, the intact URR-E6 promoter was repressed by the bovine papillomavirus type E2 protein but not by the homologous HPV-11 E2 protein (but see below) (8).

The URRs of all sequenced papillomaviruses trophic for the human genital tract contain many of the same binding motifs for ubiquitous transcription factors. For instance, the HPV-16 core enhancer, localized to a 232-bp segment, is epithelial cell specific and contains binding sites for AP-1, nuclear factor 1 (NF-1), and transcriptional enhancer factors TEF-1 and TEF-2 (9–11, 19, 24). All of these elements contribute to enhancer activity. The HPV-18 core enhancer has also been localized to a 230-bp fragment which exhibits a degree of tissue specificity (18, 39). Binding of the *junB* factor to the AP-1 sites in the HPV-18 URR and participation of other cellular factors are essential for enhancer function (29, 41).

The HPV-11 enhancer has two distinct domains, designated constitutive enhancer element I (CEI) (nucleotides 7769 to 7831) and CEII (nt 7677 to 7747) (see Fig. 2A). CEI functions as an enhancer when linked to the simian virus 40 minimal early promoter or to the E6 promoter in monkey CV-1 kidney cells and in human C-33A carcinoma cells (7, 23). CEI contains several overlapping motifs, including C/EBP, NF-1/CTF, and the NF-1-associated factor (NFA), some of which have been shown to bind cognate proteins in HeLa nuclear extracts (9, 10, 20) but none of which has been tested functionally. CEII contains novel sequences and an AP-1 site and conferred a high level of activity to the E6 promoter uniquely in human cervical carcinoma-derived C-33A cells only when in the presence of CEI. When CEII was deleted, E2 and E2-C proteins repressed the residual E6 promoter activity (7). CEII does not contribute to enhancer activity in monkey kidney CV-1 cells (23) and thus was postulated to function in a human keratinocyte-specific manner (7). In this study, we defined conditions for efficient

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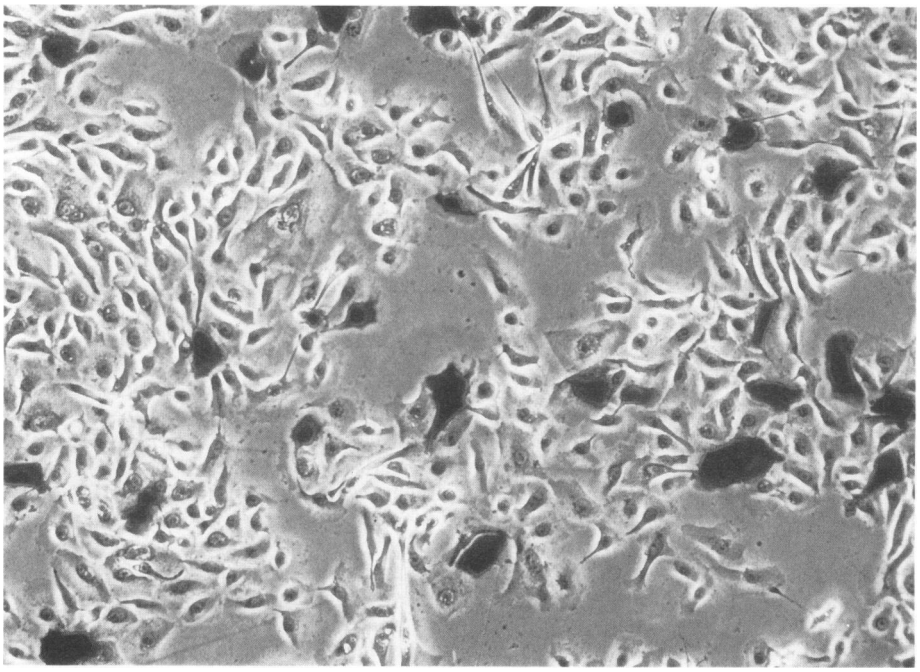

FIG. 1. Efficient transfection of PHK. A typical transfection obtained with the modified Polybrene protocol is shown. The darkly stained cells were positive for the reporter gene product, bacterial β-gal. The efficiency was approximately 9%. PHK were cultured from neonatal foreskins as previously described (45), with minor modifications. The recovered cells were grown in keratinocyte growth medium (KGM; Clonetics) or serum-free medium (SFM; GIBCO) in the presence of lethally irradiated NIH 3T3 feeder cells. To minimize the variations due to tissue source, several cultures derived from different foreskins were pooled for frozen stocks. For transfection, PHK grown to 80 to 90% confluence were passaged 1:4 after all fibroblasts were removed by treatment with 30 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–4 mM glucose–3 mM KCl–130 mM NaCl–1 mM Na_2HPO_4, pH 7.4 (43), with 0.005% trypsin and 0.05 mM EDTA. Transfection was performed 16 to 20 h later, when the plates were 20 to 30% confluent, as follows. A 1.5-ml solution of SFM or KGM with 30 μg of Polybrene per ml and 5 μg of cytomegalovirus–β-gal DNA was added to each 60-mm plate, which was then returned to the 37°C incubator. After 5 h, the cells were rinsed with Ham's F12 medium and treated with 15% glycerol in Ham's F12 medium for 2 min, rinsed, and refed with SFM or KGM containing 2.5 mM sodium butyrate. Forty to 48 h later, the cells were stained for β-gal activity, for 2 h at 37°C, as previously described (34).

transfection of PHK and performed extensive analyses of HPV-11 E6 enhancer-promoter function in these cells.

Efficient transfection of PHK. We tested several methods for transient transfection into PHK, including strontium phosphate (4), DEAE-dextran (44), calcium phosphate (22), and Polybrene (26) by the chloramphenicol acelyltransferase (CAT) assay. The calcium phosphate method initially gave the highest activity but is undesirable because the high levels of calcium induce irreversible differentiation of primary keratinocytes. Electroporation and lipofection yielded either poor cell viability or poor efficiency, as determined by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining for expressed bacterial β-galactosidase (β-gal) (see the legend to Fig. 1) (36a). The Polybrene protocol resulted in the next highest CAT activity while having no obvious deleterious effect, as judged by cell viability and morphology. We modified the protocol to use glycerol shock and optimized other conditions of transfection by monitoring pSV2CAT activities. A consistent 5 to 15% transfection efficiency was observed by counting cells positive for β-gal expressed from a cytomegalovirus immediate-early promoter in three experiments (Fig. 1). This transfection efficiency in PHK is a 10- to 50-fold improvement over that achieved in other studies. In one such study, the best efficiency obtained in PHK was 1,400 β-gal-positive cells per 60-mm-diameter plate (25). In other studies, in which only CAT activities were reported, we approximated the efficiencies on the basis of percent substrate converted per hour per milligram of protein in lysates of cells transfected with pSV2CAT or pRSVCAT (1, 13, 33).

Localization of the constitutive enhancer and efficient repression by the homologous E2 protein. To determine HPV-11 core enhancer activity and the effect of the homologous E2 protein in PHK, clones of a CAT reporter gene driven by the intact URR-E6 promoter or 5′ deletion mutations (Fig. 2) were transfected into keratinocytes in the presence or absence of an HPV-11 E2 expression vector. The results are summarized in Fig. 2B. In the absence of the E2 expression vector, the full-length HPV-11 URR-E6 promoter was highly active in these cells. The percentage of the chloramphenicol substrate converted by the most active URR construction was typically 70 to 80% of that of the SV40 full enhancer-CAT recombinant (Fig. 2B). Deletions to nt 7730 and to nt 7761 (clones 14-0 and 8-N), which removed a portion of or the entire CEII region, had no effect on intrinsic transcriptional activity of the E6 promoter (Fig. 2B). In contrast, deletion to nt 7792 in clone 16-3, which removed a portion of the CEI region, reduced the constitutive activity dramatically. These data are clearly different from the results obtained with transformed C-33A cells (7), in which deletion of CEII led to an 80 to 90% reduction of promoter activity. This suggests that CEI, but not CEII, functions in primary,

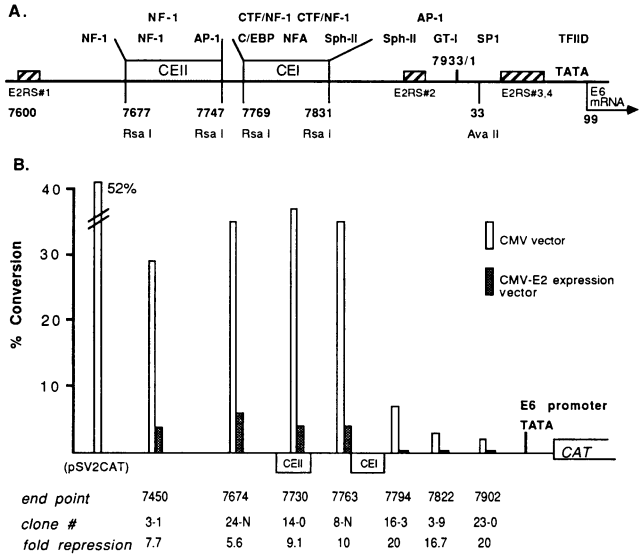

FIG. 2. 5′ deletional analysis of the HPV-11 enhancer-E6 promoter in PHK in the presence or in the absence of the homologous E2 protein. (A) The 3′ portion of the HPV-11 URR, encompassing previously defined enhancer regions CEI and CEII and the E6 promoter spanning nucleotides 7600-7933/1-99 are diagrammed with potential or proven binding sites for host transcription factors CTF/NF-1, AP-1, C/EBP, NFA, Sph-II, GT-1, Sp1, and TFIID and the four viral E2 transcription factor-responsive sequences (E2-RS 1 through E2-RS 4). (B) CAT activities of 5′ deletion mutations of the URR. The 5′ deletion mutations have already been described (7, 23) or prepared similarly. The name of the clones, the 5′ endpoint of the deletion, and the fold repression imposed by protein E2 are noted. Enhancer-CAT plasmids were cotransfected with either the HPV-11 E2 expression vector (hatched bar) or the cytomegalovirus (CMV) vector alone (open bar). Percent conversion is expressed relative to total conversion of the substrate by the purified CAT enzyme. The averages of three experiments, each performed in duplicate with different DNA preparations, are presented; the results of all of the experiments were very similar. The HPV-11 E2 protein is expressed from the cytomegalovirus immediate-early promoter (36). pSV2 CAT expresses the CAT gene from the simian virus 40 early promoter (21). Two micrograms of the CAT plasmid and 0.65 μg of the E2 expression vector were used per 60-mm dish. CAT assays were incubated for 1.5 h at 37°C by using the fluor diffusion method (31). Each reaction mixture was composed of 30 μg of lysate protein (3), 25 μl of 1 M Tris-Cl (pH 7.8), 50 μl of 5 mM chloramphenicol, and 0.1 μCi of [^{14}C]acetyl coenzyme A in a total volume of 200 μl.

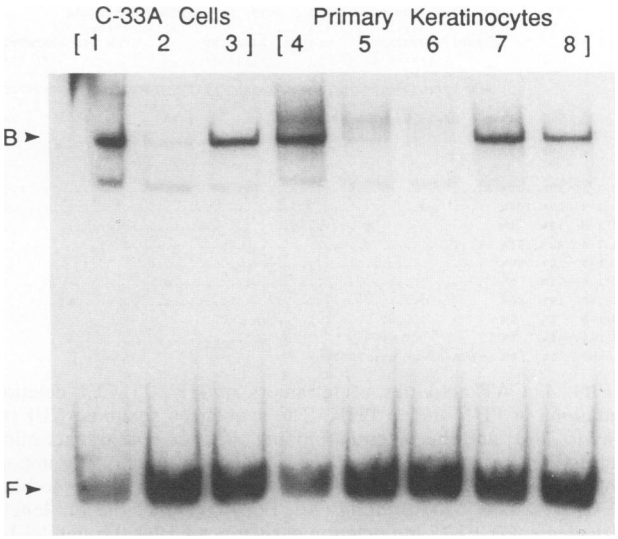

FIG. 3. Protein-DNA-binding assay by gel mobility shift. Assays were performed with nuclear extracts prepared from either C-33A cells (lanes 1 to 3) or PHK (lanes 4 to 8) (28). The probe was a 103-bp fragment containing the 71-bp CEII sequence (nt 7677 to 7747) and flanking polylinker sequences, which was end labeled as previously described (7). Five to ten micrograms of nuclear protein was mixed with 10,000 cpm (4 fmol) of DNA, and the gel shift assay was performed by using previously published protocols (14, 17) with modifications (7). The homologous competitor was CEII present in a plasmid. The mutant competitor was a plasmid containing a synthetic oligonucleotide spanning positions 7677 to 7747 with a deletion of an A at position 7732 which fails to bind to CEII cognate proteins (7). Lanes: 1 and 4, no competitor; 2 and 6, 1.0 μg of wild-type competitor; 3 and 8, 1 μg of mutant competitor; 5 and 7, 0.25 μg of wild-type or mutant competitor, respectively. B, bound probe; F, free probe.

undifferentiated keratinocytes. When cotransfected with a vector expressing the homologous E2 protein, the CAT activity from the intact URR-E6 promoter and each of the 5′ deletion mutations was strongly repressed, contrary to previous data obtained with immortalized C-33A cells (7). Repression ranged from 5.6- to 20-fold (Fig. 2B). These results are in general agreement with those obtained with HPV-16 and HPV-18 in primary keratinocytes (33, 40), except that repression in our studies was more dramatic.

Presence of CEII-binding proteins in PHK. Gel band shift experiments demonstrated that host proteins in nuclear extracts of C-33A or HeLa cells form a specific complex with the CEII DNA fragment (7). To determine whether the lack of CEII function in PHK was due to the absence of these host factors, we performed gel shift experiments on a DNA restriction fragment containing CEII by using nuclear extracts prepared from C33A cells and from PHK. The specific DNA-protein complex was identified by competition with the unlabeled wild-type CEII DNA fragment but not by a synthetic mutated oligonucleotide (nt 7700 to 7737). This oligonucleotide has a single base deletion at position 7732 that eliminates CEII function and is unable to compete for CEII factor binding (7). With either nuclear extract, a complex with the same mobility and patterns of DNA competition was observed (Fig. 3). These results suggest that PHK contain CEII-binding proteins similar, if not identical to those in C-33A cells, although they appear to be inactive by functional criteria.

This result is consistent with the observations of other investigators, who demonstrated that nuclear extracts from cells in which the viral enhancers have either a high level of activity or very little activity gave the same footprints (11, 16, 20). Thus, the cell type specificity of these viral enhancers may result from interactions among epithelium-specific factors which do not bind DNA directly, i.e., adaptors, and ubiquitous proteins that bind to the URR. Alternatively, tissue specificity could stem from the particular number and spacing of the various *cis* elements together with the relative abundances of the factors in a given tissue.

Further dissection of core enhancer CEI. Inspection of the CEI region revealed several known enhancer motifs (Fig. 2A). To define these motifs functionally, a series of 5′ and 3′ deletion mutations of the CEI region (nt 7769 to 7831) were generated. One copy of CEI conferred little activity, probably owing to loss of interactions with downstream elements or, possibly, the change of spacing between the enhancer

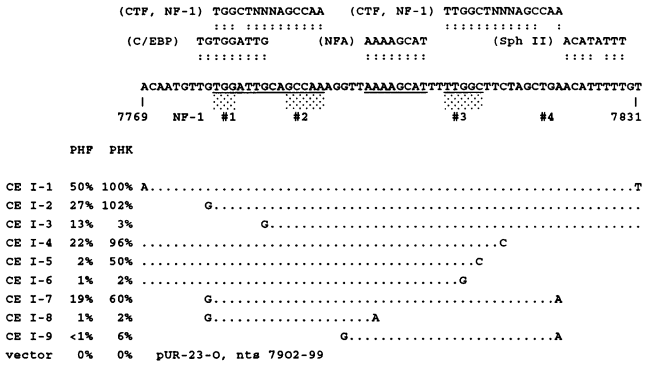

FIG. 4. CAT activities of tetramers of HPV-11 CEI deletion mutations in PHF and in PHK. The sequences spanning CEI (nt 7769 to 7831) and the potential binding sites for host transcription factors are presented; sequences implicated as functionally important are underlined. Shaded sequences therein were shown to bind NF-1 by DNase I footprinting (20). Four copies of the full-length CEI segment (CEI-1) and deletion derivatives CEI-2 through CEI-6 were each cloned into vector 23-0 upstream of the HPV-11 minimal E6 promoter (nt 7902-7933/1-99) and the CAT reporter. The orientation of each copy of CEI within each tetramer was determined by Sanger DNA sequencing analysis. To generate clones CEI-7, CEI-8, and CEI-9, four oligonucleotides representing the 5′ and 3′ halves of the minimally active CEI sequence (nt 7777 to 7821) were synthesized. The two halves were then cloned as tetramers, either separately or together, to reconstitute the minimal CEI sequence. All activities were normalized to that of CEI-1 in PHK. PHF were recovered from neonatal foreskins in the same manner as PHK, except that the cells were grown in Dulbecco modified Eagle medium with 10% fetal calf serum instead of serum-free medium. Transient transfections were done with 5 μg of DNA per 60-mm dish. PHF transfections were performed in the presence of a low CO_2 concentration (5) and followed by incubation in medium containing 2.5 mM sodium butyrate, as described previously (6). Additional details of CAT assays are in the legend to Fig. 2.

and promoter. Therefore, CEI was multimerized into tetramers and placed upstream of the enhancerless E6 promoter in clone 23-0. To ensure that regions of the URR that did not appear to be transcriptionally important do not acquire enhancer activity when cloned in multiple copies, two additional regions of the URR were tetramerized, cloned upstream of 23-0, and assayed in PHK. One region is CEII (nt 7677 to 7747), which includes an AP-1 site; the other spans nt 7831-7933/1-33 and contains sequences with some homology to Sph-II-, AP-1, and GT-I motifs (Fig. 2A). Only the CEI tetramer conferred a high level of activity on the minimal E6 promoter (data not shown). These results suggest that motifs within CEI may be categorized as class A enhancer elements which function when multimerized and that sequences downstream of CEI are either promoter elements or class B enhancer elements that contribute transcriptional enhancement by interacting with other enhancer elements but do not function by themselves, even when multimerized (15).

To delimit CEI, we prepared 5′ and 3′ deletion mutations of CEI by exonuclease III-mung bean nuclease digestions. Tetramers of each were cloned into vector 23-0 (CEI-2 through CEI-6) and transfected into PHK. The CAT activities were normalized to that of the full-length CEI fragment (CE I-1) tetramer and are summarized in Fig. 4. Clone CE I-2, deleted from the 5′ end into C/EBP, had the same activity as intact CEI. The activity decreased abruptly to only 3% in clone 3 when the 5′-end deletion extended into the CCAAT transcription factor (CTF) motif, which consists of two NF-1 sites (1 and 2), indicating that this motif was essential. CEI-4 had full activity. This deletion removed half of a CTF site (NF-1 site 4) and a sequence with 50% homology to a papillomavirus enhancer factor (PVF) site in HPV-16 shown to bind TEF-2 (9, 10). CEI-4 also deleted an Sph-II-like sequence which has been shown in HPV-16 to bind TEF-1 and to be functionally important (24). These results indicate that the putative PVF and TEF-1 sites do not play major roles in HPV-11 enhancer-E6 promoter activity in keratinocytes. Clone 5 removed part of the CTF but retained NF-1 site 3; it lost half of its activity. Removal of an additional two bases into NF-1 site 3 (clone 6) essentially eliminated all CAT activity, demonstrating that NF-1 site 3 at the 3′ border of the minimal CEI fragment is also necessary for activity. To confirm the interpretations based on 5′ and 3′ deletion mutations, oligonucleotides spanning the active region were synthesized. Either half of this region alone and the joined halves reconstituting the minimal CEI sequence were multimerized. The tetramer of the reconstituted CEI core (clone 7) had 60% of the activity of the full region, whereas tetramers of either half (clones 8 and 9) had little or no activity. Together, these results suggest that the 45-bp region containing NF-1 sites 1, 2, and 3 and an NFA site constitute the core CEI enhancer in PHK. All three sites have previously been shown to bind NF-1 by DNase I footprinting with HeLa cell extract (20) (Fig. 4). The less than 100% activities of clones 5 and 7 may be attributed to spacing constraints imposed on the NF-1 sites at the border. Whether the internal sequence (AAAAGCAT) with homology to NFA (10) is essential for activity cannot be deduced from our study. However, since the two CTF sites at the 5′ and 3′ ends of CEI are not equivalent, perhaps interaction with NFA, which is known to bind to this sequence in HPV-11 (9), compensated for the loss of an NF-1 site.

Transcriptional activity of CEI in PHF. To determine whether the entire URR and, especially, CEI confer epithelial cell specificity, several of the clones in this study were transfected into primary dermal fibroblasts (PHF) derived from neonatal foreskin (Fig. 4). The efficiency of transfection was similar to that in PHK, as judged by the percentage of cells positive for β-gal in two experiments. Differences in cell type preference were evident. CEI-1, CEI-2, CEI-4, CEI-5, and CEI-7 had much lower activities in PHF than in PHK (Fig. 4). In contrast, clone 3 retained more activity in PHF than in PHK. These results suggest that the 5′ and 3′ sequences flanking the minimal CEI region appear to be important for activity in PHF but not in PHK. Thus, the CEI region conferred a degree of specificity.

Even moderate activity of CEI in PHF was surprising, however, in view of the more stringent tissue tropism observed with the core enhancers of HPV-16 and HPV-18 (12, 18, 19, 39, 42). One possible explanation might be the different transfection efficiencies achieved in the various studies. A second explanation is that *cis* elements that restrict the activity in fibroblasts may exist. For instance, the full-length enhancer clone, nt 7072 to 99, had activity in PHF that was only 20% of that in PHK, whereas 5′ deletion clones 24-N, 14-0, and 8-N (Fig. 2) were all approximately 50% as active in PHF as in PHK. A third possibility is that the region downstream of CEI, which is important for E6 promoter activity in PHK, also contributes to cell type specificity. Deletion of CEI (16-3) virtually eliminated activity in PHF (data not shown), whereas in PHK, 15 to 20% of the activity remained (Fig. 2B). It is conceivable that relatively subtle differences in enhancer-promoter activity in

these experiments translate into significant differences in virus-host cell interactions in vivo.

Conclusion. We achieved consistently high efficiency in PHK transfection (Fig. 1) and showed by deletion analysis that the HPV-11 URR core enhancer in PHK is localized to a 45-bp region (nt 7777 to 7821) within the previously identified CEI region (23) (Fig. 2 and 4). The minimal CEI region contains three NF-1 sites and an NFA site and is, to a degree, tissue specific. It is worth noting that the two putative AP-1 sites, one within the CEII region and the other downstream of the CEI region, are not part of the HPV-11 core enhancer in PHK. Deletion of CEII did not affect activity (Fig. 2B). Conversely, multimerization of either fragment containing the AP-1 sites did not confer enhancer activity. This is in contrast to their essential role for HPV-16 and HPV-18 enhancer activity in cervical carcinoma cells and in PHK (9, 10, 29, 41). These results may reflect the importance of interactions among different host factors or proper spacing in effecting a final transcriptional activity. Such dissimilarities among the papillomaviruses that infect mucosa may help explain why HPV-11 is usually found in the vulva or larynx, whereas HPV-16 and HPV-18 tend to infect the cervix.

A weak repression of the E6 promoter of HPV-16 and HPV-18 by the homologous viral E2 protein in cervical carcinoma cells and in PHK has been reported (1, 33, 40). We demonstrated that in PHK, HPV-11 E2 protein is an exceptionally strong repressor for the homologous viral E6 promoter (Fig. 2B). Furthermore, the CEII enhancer element critical for overcoming E2 repression in human C-33A cells did not exhibit such a function in PHK.

The current model for the role of the E2 protein in a natural infection is that of autoregulated repression of transcription of the E6 and E7 genes transcribed from the E6 promoter. For the high-risk viruses HPV-16 and HPV-18, viral integration leading to loss of E2 expression and consequent derepression of the E6 promoter is thought to be a critical event in the progression to neoplasias (32, 37). Our results suggest that autoregulation also exists for the benign virus HPV-11, in keeping with the low levels of viral transcription and DNA replication in undifferentiated basal and parabasal cells (2, 38). We propose that during the course of differentiation or neoplastic conversion (as in C-33A cells), CEII cognate or related proteins may be activated or their cofactors may become available. E2 repression is then overridden, and transcription becomes highly activated, a necessary step for the productive phase of the infection.

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