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Transcription Activities of Human Papillomavirus Type 11 E6 Promoter-Proximal Elements in Raft and Submerged Cultures of Foreskin Keratinocytes

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Human papillomaviruses (HPVs) replicate only in differentiated squamous epithelia in warts and in epithelial raft cultures grown at the medium-air interface. Virus-encoded and host transcription factors are thought to be responsible for repressing the viral enhancer and promoter located within the upstream regulatory region (URR) in the undifferentiated basal and parabasal cells while up-regulating their activities in the differentiated spinous cells. Using recombinant retroviruses, we acutely transduced neonatal foreskin keratinocytes (PHKs) with a *lacZ* reporter gene driven by the wild-type URR of the low-risk HPV type 11 or by a URR with individual mutations in seven promoter-proximal elements, some of which have not been characterized previously. β-Galactosidase activities were detected in the submerged, proliferating PHKs and also in the differentiated spinous cells, but not in the steady-state proliferating basal cells, of stratified raft cultures. In particular, mutation of an Oct1, an Sp1, or a previously unknown promoter-proximal AP1 site severely reduced the reporter activity, whereas mutation of either of two NF1 sites flanking the Oct1 site had no effect. These results demonstrate changes in cellular transcription factor profiles under different culture conditions and begin to characterize the naturally differentiation-dependent activation of the URR. They provide one molecular explanation for the patterns of HPV expression in warts and help validate epithelial raft cultures as an important experimental system for genetic dissection of HPV regulatory elements.

Human papillomaviruses (HPVs) are a large group of human pathogens that contain a circular, double-stranded DNA genome of 7.9 kbp. They infect epithelial tissues at preferred body sites and reproduce only in squamous cells undergoing terminal differentiation (13). Among the subset of virus genotypes which infect mucosal epithelia, HPV type 6 (HPV-6) and HPV-11 cause benign anogenital condylomata and recurrent respiratory papillomatosis while HPV-16 and HPV-18 can cause cervical and penile intraepithelial dysplasias and carcinomas (71). All HPVs have essentially the same organization of protein coding regions and a noncoding, upstream regulatory region (URR) (also known as the long control region) which contains a DNA replication origin, transcription regulatory sequences, and one or more promoters which control the expression of the viral oncoproteins E6 and E7. It is assumed that the URR dictates host, tissue, and differentiation specificities of the various members of the papillomavirus family.

Using submerged cultures of epithelial cell lines and neonatal foreskin keratinocytes (primary human keratinocytes [PHKs]), various investigators have performed extensive deletion, reconstitution, and site-directed mutagenesis analyses of HPV-11, -16, and -18 URRs linked to the native promoter (E6 or P1 for HPV-11, P105 for HPV-18, and P97 for HPV-16) or to a surrogate promoter to express a reporter gene such as the chloramphenicol acetyltransferase or luciferase gene. The results show that the URR is regulated both by virus-encoded E2 proteins and by host transcription factors, many of which are ubiquitous rather than tissue specific. For instance, multiple copies of NF1 sites, a glucocorticoid responsive element, and a novel Sp1 site invariably bind positive factors (1, 6, 9–11, 14, 20, 21, 27–29, 32, 33, 35, 38, 40, 56, 57, 62). An Oct1 site (previously termed NFA) is highly conserved among these viruses, but the results of mutagenic analyses vary (11, 16, 32, 47, 48, 58). One or two AP1 sites that bind to Jun-Fos heterodimeric proteins function as positive elements in the HPV-16 and HPV-18 URRs (10, 11, 15, 50, 64). In the HPV-11 URR, we note that there are two AP1-like sequences, but their functions have not been investigated. In addition, transcription factor binding sites specific for individual HPV types have also been identified. For instance, the 5′ region of the HPV-11 URR, including the sequence CCNGTNAC, binds unknown proteins and functions to enhance the simian virus 40 (SV40) promoter in laryngeal and papilloma cells (2). TEF-I has been shown to be a positive factor for the HPV-16 URR (35), whereas KRF (42) and Epoc1 (70) enhance HPV-18 URR activity. It appears that the overall balance of the multiple factors confers the species and cell type specificity of the viral promoters (for reviews, see references 4, 5, and 34).

In productively infected benign lesions in patients and in experimental animal xenograft systems, HPV transcription, including that of the E6 and E7 genes, is very low in the proliferating basal and parabasal cells and is up-regulated in the more differentiated spinous cells (7, 12, 23, 61). It has been suggested that the family of virus-encoded E2 proteins may be responsible for this low activity in the basal strata. Evidence in support of this hypothesis comes from the analysis of carcinomas which express high levels of the E6 and E7 genes in the undifferentiated basal cell-like keratinocytes. In these lesions, the viral DNA is often integrated, disrupting the expression of the E1 and E2 genes (reviewed in reference 13). In transiently transfected cells, the E2 proteins expressed from a strong surrogate promoter invariably function as a repressor of the E6 promoter, as they displace TBP or Sp1 from their respective binding sites. The full-length E2 protein can, however, also

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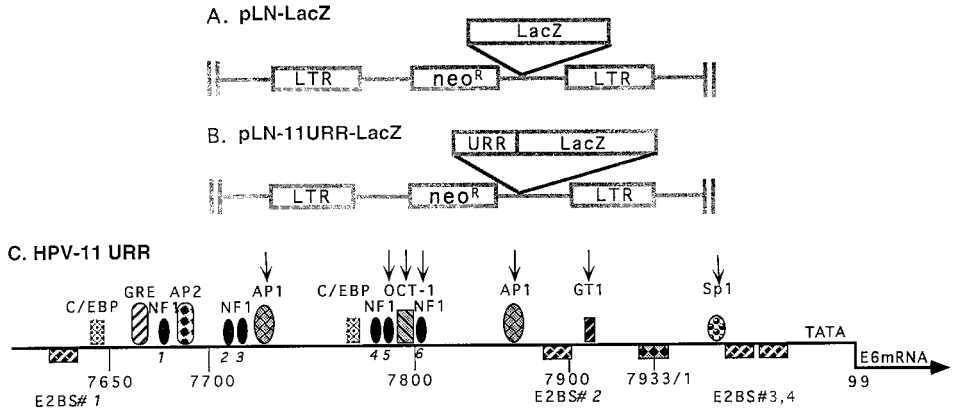

FIG. 1. Structures of retroviral vectors. (A) Schematic diagram of the retroviral vector pLN-*lacZ* in which the *lacZ* reporter gene is cloned downstream of the neomycin resistance gene (*neo*) in the absence of a dedicated promoter. (B) pLN-11URR-*lacZ*, in which the wild-type HPV-11 URR (nt 7072 to 7933 and nt 1 to 99) or mutated URR was cloned downstream of *neo* in the orientation to promote *lacZ* expression as described in Materials and Methods. (C) Schematic diagram of the viral E1 and E2 sites (below the horizontal line) and cellular factor binding sites (above the line) within the promoter-proximal region of the HPV-11 URR. The locations of the motifs are not to scale. Arrows indicate the motifs mutated in this study.

stimulate the E6 promoter when it is expressed from a relatively weak promoter (8, 9, 14, 15, 18, 21, 22, 26, 52, 59, 63). Nonetheless, there has not been direct proof as to whether the viral E2 protein is mainly responsible for the low viral activity in warts or the viral promoter is naturally up-regulated in response to differentiation and is further modulated by the E2 proteins. The recent development and characterization of organotypic epithelial raft cultures that faithfully recapitulate squamous differentiation capable of supporting virus production in cells that harbor episomal HPV DNA now make it possible to begin such experimental investigations in vitro (19, 25, 39, 45, 68). In particular, the differentiation-dependent up-regulation of the HPV-18 and the HPV-11 E6-E7 genes under the control of their respective native enhancer-promoter elements has been demonstrated when transduced into PHKs via recombinant retroviruses. The expression of the HPV-18 E7 gene alone is necessary and sufficient to reactivate the entire host DNA replication machinery in otherwise differentiated cells so that the viral genome can also replicate (7).

Using the highly efficient method of retrovirus-mediated gene transfer, we have recently developed an HPV-18 and HPV-11 URR-driven *lacZ* reporter system (pLN-18URR-*lacZ* and pLN-11URR-*lacZ*) (Fig. 1B) and initiated a mutagenic analysis of the HPV-18 URR to identify *cis* elements that are important for E6 or P105 activities in differentiated raft cultures of PHKs (51). We showed that the expression of the β-galactosidase (β-Gal) from either clone is primarily restricted to the differentiated cell layers. In contrast, the vector pLN-*lacZ*, in which the reporter gene is located immediately downstream of the neomycin resistance gene (*neo*) (Fig. 1A) in the absence of a dedicated promoter, expressed no β-Gal activity due to an inability to reinitiate translation from transcripts generated from the retroviral long terminal repeat (LTR) promoter. The β-Gal activities of two HPV-18 URR mutants examined correlated with the amounts of *lacZ* transcripts relative to the wild-type URR clone. We concluded from these results that the β-Gal protein was indeed translated from RNA initiated from the HPV URR-E6 promoter.

We have now performed a mutagenic study of the promoter-proximal enhancer elements in the HPV-11 URR-*lacZ* in PHKs acutely transduced with amphotropic retroviruses. We show that the reporter activities of wild-type and mutated elements in the proliferating, submerged cultures were qualitatively comparable to those in the differentiated cells in stratified rafts but were distinct from the inactivity in the proliferating basal keratinocytes of the mature raft cultures. We also show that a previously uncharacterized E6 promoter-proximal AP1 site indeed binds JunB–c-Fos proteins and is critical for the promoter activity. The Oct1 site is also important, but its reported synergy with a flanking NF1 site was not observed. Additional differences from and similarities to previous relevant studies are also discussed.

MATERIALS AND METHODS

Site-directed mutagenesis. Site-directed mutations in all transcription factor cognate sites in the HPV-11 URR were performed by the PCR amplification method, with the exception of mutations in Sp1 and in GT1, both of which were described previously (21). All mutations targeted critical consensus nucleotides for factor binding as reported in the literature. Briefly, the pGEM-H11URR plasmid containing the entire HPV-11 URR (nucleotides [nt] 7072 to 7933 and nt 1 to 99) (31) was used to generate overlapping PCR fragments with mutagenic primers and the flanking T7 or SP6 universal primers. Gel-purified fragments were then mixed at an equimolar ratio to serve as second PCR templates with T7 and SP6 primers. The products were digested with *Bam*HI and *Hin*dIII, which flank the URR, and recloned into pGEM-1. After confirmation by the dideoxynucleotide sequencing method, the *Bam*HI and *Hin*dIII fragments containing each mutation were made blunt ended with the Klenow fragment of the *Escherichia coli* DNA polymerase I and cloned into the *Nru*I site of the pLN-*lacZ* vector described below.

Preparation of HPV-11-driven *lacZ* in the retroviral vector. Plasmid pβG, which contains the bacterial *lacZ* gene with an optimized Kozak protein translation initiation sequence, was a gift of J. Anthony Thompson. To construct pLN clones, the SV40 promoter in pLNSX (46) was removed by restriction digestion with *Nru*I and *Hin*dIII. pLN-*lac* contains the reporter gene immediately downstream of the bacterial neomycin resistance gene (*neo*) in the absence of a dedicated promoter (Fig. 1A) (51). The pLN-11URR plasmids were prepared by inserting the wild-type HPV-11 URR or mutations therein after blunt-end ligation in the correct orientation at the *Nru*I site located 5′ to the *lacZ* reporter gene (Fig. 1B). The orientation of the URR was established by restriction digestions.

Production of retroviruses and acute infection of PHKs. Amphotropic recombinant retroviruses were produced from the helper cell line pG+enAM12 (43) as previously described (51). Titers of serial dilutions of retroviruses were determined by a reverse transcriptase assay. Equivalent titers of viruses were then used to infect the same batch of PHKs in several independent experiments, each with PHKs from a different donor. PHKs were recovered from neonatal foreskin as described previously (67). The same number of PHKs in the first or second passage (at 30% confluence in 60-mm-diameter plates) was infected with recombinant retroviruses and then selected for 2 days with G418 (Geneticin; 400 μg/ml) in serum-free medium (SFM) (both products from GIBCO/BRL) as described previously (51). All uninfected cells died after such a selection. Usually, 70% or more of the cells were infected and survived, and they were used immediately for subsequent experiments without further expansion.

β-Gal activities in PHKs cultured as raft cultures or as submerged proliferating cultures. G418-selected PHKs or uninfected PHKs (2×10^5) were seeded on dermal equivalents consisting of type 1 rat tail collagen (Collaborative Research) with Swiss 3T3 J2 fibroblasts (a gift of Elaine Fuchs) and cultured at the medium-air interface for 9 days as described previously (7, 51). One set of raft cultures was fixed with 2% formaldehyde plus 0.2% glutaraldehyde in phosphate-buffered saline (pH 8.0) at room temperature for 45 min, incubated with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) solution (55) at 37°C overnight, and then embedded in paraffin. Sections (5 μm thick) were cut for histologic analysis and for detection of β-Gal-positive cells. The slides were photographed after a light counterstaining with hematoxylin and eosin (H&E) to correlate β-Gal activity with tissue morphology. Slides that were not counterstained were also examined and photographed to reveal the even weakly positive cells. Each mutation was tested along with the wild-type URR and with uninfected PHKs in several independent experiments.

For submerged cultures, aliquots of uninfected or G418-selected PHKs after acute infections with retroviruses as described above were also plated on 60-mm-diameter plates in the absence of any feeder cells. After 2 to 3 days, one set of plates was used to determine the percentage of cells positive for β-Gal activity after incubation with X-Gal solution. Positive cells from five random fields were counted with a Nikon Diaphot inverted phase microscope with a 20× objective lens and were averaged. Cells from the second set were trypsinized, lysed, and used for in vitro β-Gal enzymatic assays (54). These experiments were performed twice.

Histology and immunofluorescence analyses. Sections were deparaffinized and stained with H&E for histologic assessment. Immunofluorescence analyses were performed on uninfected PHKs grown for 36 h on coverslips in SFM, on PHKs that were acutely infected with retroviruses and selected in SFM with G418 (400 μg/ml) for 2 days, and on uninfected PHKs that were stimulated to differentiate for 36 h in SFM to which 1 mM Ca^{2+} was added (17). The cells were fixed with cold methanol-acetone and probed with a mouse monoclonal antibody against involucrin as described by the supplier (Novocastra Laboratories, Ltd.). Reactivity was detected with fluorescein-conjugated anti-mouse antibody (Pharmagen). Hoechst 33258 stain (20 μg/ml in 1× phosphate-buffered saline) was used to stain the nuclei to reveal all cells.

Electrophoretic mobility shift assay (EMSA). One microgram each of T7-b-cfos and T3-Blue-junB supercoiled plasmid DNA (kind gifts from Françoise Thierry) was used for in vitro translation using the TNT-coupled reticulocyte lysate system (Promega). The double-stranded oligonucleotide containing the putative promoter-proximal AP1 binding site 5′-CAAAATGAGTAACCTAAG GTCA-3′ was end labeled by using [γ-^{32}P]ATP (10 mCi/ml; Amersham) with the T4 DNA kinase following standard procedures. Unlabeled wild-type double-stranded oligonucleotides and mutated oligonucleotides 5′-CAAAAT**ac**GTAA CCTAAGGTCA-3′ (where lowercase letters in boldface type are positions differing from the wild-type sequence) were used as competitors. Briefly, 20 μl of reaction mixture contained binding buffer (15% glycerol, 75 mM KCl, 0.375 mM phenylmethylsulfonyl fluoride, and 12.5 mM NaCl), 0.5 μg of poly(dI-dC), 0.5 ng of probe, and 1 μl each of in vitro translation products in the presence or absence of competitors at a 71-fold or 142-fold molar excess. The mixture was incubated at room temperature for 20 min and then separated in a 4% nondenaturing acrylamide gel by electrophoresis at 4°C in 0.25× Tris-borate-EDTA (TBE) buffer at 100 V. For the supershift assay, 1 or 2 μl of monoclonal antibody against JunB (Promega) was preincubated with in vitro-translated JunB–c-Fos proteins on ice for 10 min and then added to the DNA in binding buffer for an additional 20 min. The mixture was then electrophoresed as described above. The gel was dried, and the autoradiogram was exposed overnight.

RESULTS

Uninfected PHKs and PHKs infected with recombinant retroviruses were grown as raft cultures. All cultures expressed the differentiation marker proteins, keratin 1 (K1) and profilaggrin (data not shown), and had four well-organized, morphologically distinct layers: the proliferating basal cells, the differentiating spinous cells, the terminally differentiated granular cells, and the superficial squames (Fig. 2). To correlate tissue morphology with β-Gal activities, sections were reviewed after counterstaining with H&E (Fig. 2A to F and H). To reveal even very weak signals that may have been masked by the counterstains, the sections were also examined and photographed without counterstains (Fig. 2G and data not shown). Many more weakly positive cells were detected in the latter slides; however, the conclusions with regard to the relative activities and topographical distribution of the positive cells remained the same. In agreement with our previous studies (51), uninfected PHKs did not demonstrate β-Gal activity (data not shown), nor did cultures infected with pLN-*lacZ* virus (Fig. 1A), which does not contain the HPV-11 URR (Fig. 2H). In contrast, the wild-type pLN-11URR-*lacZ* (Fig. 1B) expressed β-Gal activity primarily in suprabasal cells, while only a few basal cells were positive (see Fig. 2A). Wild-type or mutated URR-*lacZ* viruses (indicated by arrows in Fig. 1C) were then used to infect PHKs, selected with G418 to eliminate all uninfected cells, and developed into raft cultures. Each of the mutations disrupted the binding of cognate transcription factors to consensus sites according to previous studies and this investigation (Table 1; see Fig. 3). The sections were examined side by side with the negative control cultures in several independent experiments, and examples are shown in Fig. 2. The criteria for evaluation include the number and topographical locations of cells positive for β-Gal and the intensity of the blue color of the positive cells. The results are described below.

Mutations that significantly down-regulate reporter gene expression. The Oct1 protein is considered ubiquitous (37). The presence of an Oct1 binding site (5′-AAAAGCAT-3′) flanked by NF-1 sites 5 and 6 is highly conserved in the HPV-11, HPV-16, and HPV-18 URRs. In HPV-11 and HPV-16, Oct1 protein binding to this site functions synergistically with NF1 bound to site 6 to confer high E6 promoter activity in HeLa cells, as mutations of both NF1 and Oct1 binding sites reduce the promoter activity no more severely than individual mutations (48). However, mutations of the Oct1 site in the HPV-18 URR either increased or had no effect on promoter activity in HeLa cells (5, 32). In the raft cultures, mutation of the Oct1 site in the HPV-11 URR dramatically reduced the number of β-Gal-positive cells in the suprabasal layers, and the positive cells had much weaker activities relative to the wild-type-virus-infected culture (Fig. 2F).

There are two AP1-like sequences in the HPV-11 URR. The promoter-distal site (designated AP1d) at nt 7738 to 7744 (5′-TGACTAA-3′) is identical in sequence to an AP1 site in the HPV-18 URR known to bind JunB (64). The promoter-proximal site (designated AP1p) at nt 7865 to 7871 (5′-TGAGT AA-3′) has a 1-bp mismatch with the consensus sequence (TG ACTAA). It had not been shown to bind AP1. We mutated both putative binding sites individually. The AP1d mutations only moderately reduced β-Gal activity in the suprabasal cells (data not shown), to a level between those of the GT1 and Sp1 mutations (described below). In contrast, mutations in AP1p dramatically reduced both the number of β-Gal-positive cells and their activities (Fig. 2E and G).

AP1 enhancer factors consist of homodimers and heterodimers of c-Jun, JunB, JunD, and c-Fos family proteins, and they regulate a large number of genes, including K1, involucrin, and profilaggrin, that are specifically expressed in differentiated keratinocytes (24, 36, 41, 53, 65, 66), and junB preferentially binds to AP1 sequences in the HPV-18 URR (64). We performed EMSAs to examine the ability of the putative AP1p site to bind the in vitro-translated JunB–c-Fos heterodimeric proteins (Fig. 3). A specific complex was detected with the wild-type oligonucleotides (Fig. 3, compare lanes 1 and 2). The complex was competed by unlabeled wild-type oligonucleotides but not by the same mutated oligonucleotides used in the reporter clones (Fig. 3, lanes 3 to 6). Furthermore, the complex was supershifted by antibody to JunB (Fig. 3 lanes 8 and 9). These results demonstrate that the promoter-proximal motif is indeed an AP1 site and that its integrity is extremely important for the E6 promoter activity in the differentiated cells. Since our raft cultures expressed involucrin, K1, and profilaggrin (68) (data not shown), we believe that members of the AP1 protein family are indeed present in the differentiated strata as shown by others.

The Sp1 binding site is ubiquitous in the promoters of

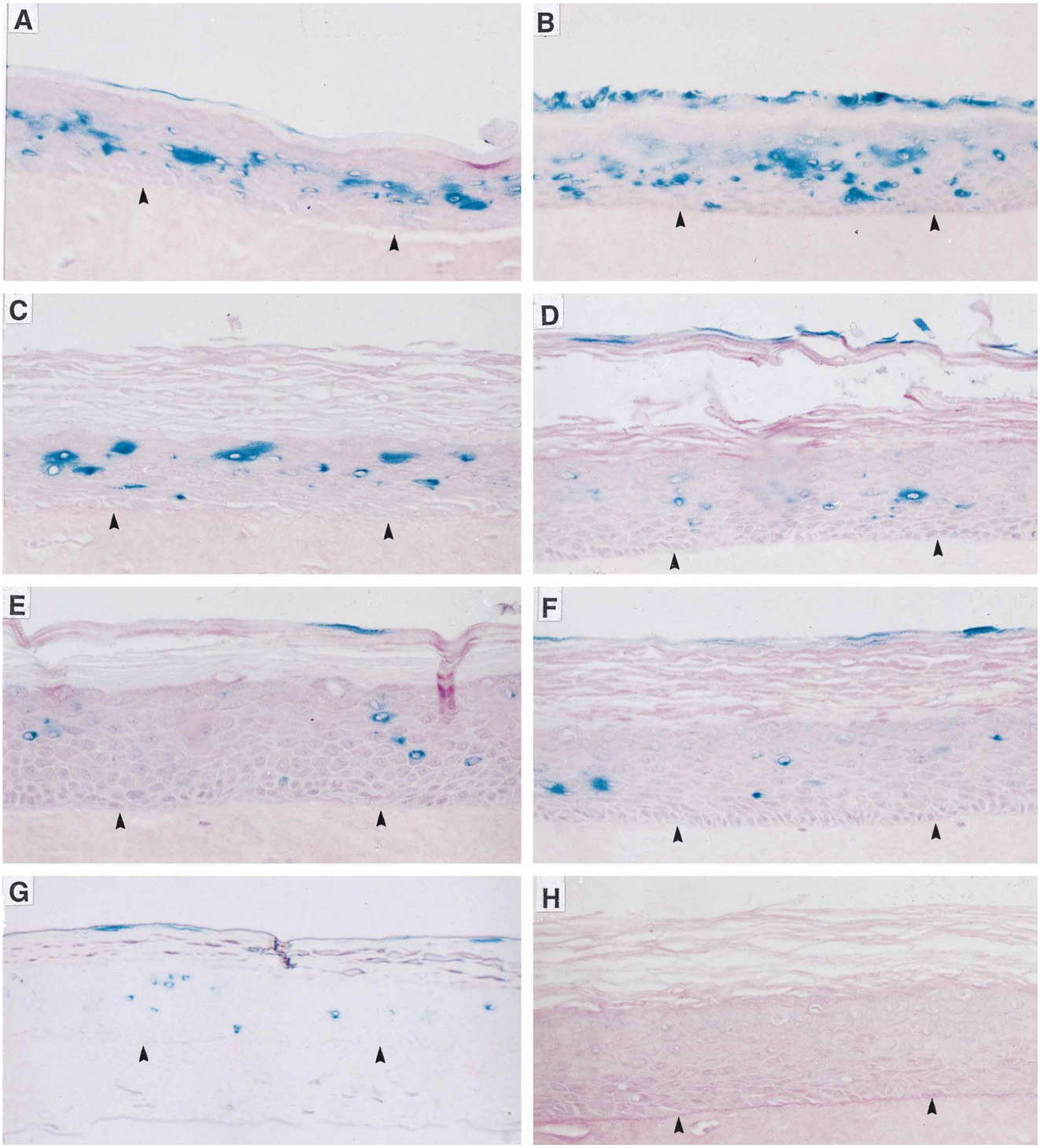

FIG. 2. β-Gal activities in epithelial raft cultures of PHKs infected with recombinant retroviruses. The β-Gal activities in paraffin-embedded sections were revealed after reacting with an X-Gal solution as described in Materials and Methods. The sections were counterstained (A to F and H) to reveal the topography of β-Gal-positive cells in the epithelium. One set of sections was not counterstained, revealing even cells with very weak β-Gal activities that were masked by the counterstains (G). The blue area near the top of panel B is associated with desquamated tissues, whereas the bulk of the squames in panel A was lost during processing (as shown in panel A). There was little or no difference in β-Gal activities in the living tissues between the sections shown in panels A and B. Arrowheads point to basal cells. The great majority of the positive cells were located in suprabasal layers in cultures infected with wild-type or mutated URR retroviruses (A to G), whereas pLN-*lacZ* control retrovirus generated no signal (H). (A) pLN-11URR-*lacZ*; (B) pLN-11URR-NF1(#6)M-*lacZ*; (C) pLN-11URR-GT1-M-*lacZ*; (D) pLN-11URR-Sp1-M-*lacZ*; (E and G) pLN-11URR-AP1pM-*lacZ*; (F) pLN-11URR-Oct1M-*lacZ*; (H) pLN-*lacZ*.

TABLE 1. Mutations in the HPV-11 URR[a]

Binding site	Type	Initial nt	Sequence
Sp1	WT	33	5′-AGGAGGG-3′
	MT		5′-AG**ctcaa-3′**
GT1	WT	7909	5′-ACCCACACCCT-3′
	MT		5′-**tCCCtCt**CCCT-3′
AP1p	WT	7865	5′-TGAGTAA-3′
	MT		5′-T**ac**GTAA-3′
AP1d	WT	7738	5′-TGACTAA-3′
	MT		5′-T**acCg**AA-3′
NF1 (site 5)	WT	7808	5′-TTGGC-3′
	MT		5′-T**ca**GC-3′
NF1 (site 6)	WT	7787	5′-GCCAA-3′
	MT		5′-GC**tg**A-3′
Oct1	WT	7797	5′-AAAAGCAT-3′
	MT		5′-AAAA**at**AT-3′

[a] The 5′-nucleotide positions and sequences of wild-type (WT) transcription factor binding sites and mutations (MT) are shown. Lowercase letters in boldface type are mutated positions.

housekeeping genes. It can be inferred that proteins of the Sp1 family are present in most if not all cells. One novel Sp1 binding site, which varies in sequence among the different HPV types, is located adjacent to the viral E2 protein binding site 3 in the E6 promoter-proximal region of all genital HPVs (Fig. 1C). Mutations of this site in HPV-11 eliminate Sp1 binding in vitro and also consistently lead to a reduction of the E6 promoter activity in transfected epithelial cell lines (21). In the raft cultures, point mutations (Table 1) resulted in a significant reduction in β-Gal activity in the suprabasal cells (Fig. 2D). However, in several independent experiments, the Sp1 mutant always had a higher number of very weakly positive cells than did cultures containing the Oct1 or the AP1p mutations when viewed in sections without counterstains (data not shown).

Mutations that have little or no effect on *lacZ* reporter gene expression. The GT1 sequence in the SV40 enhancer (5′-ACTTTCCACACCT-3′) binds the Sp1 protein (69), but the GT1-like sequence in the HPV-11 URR (5′-CCACACCCT-3′) does not (21). An unknown protein in C33A nuclear extracts binds to this motif, and mutations that eliminated protein binding slightly reduced the promoter activity in C33A cells (21). One of these mutations was tested (Table 1). It also slightly reduced the number of positive cells in the suprabasal cells (Fig. 2C).

The consensus NF1 transcription factor binding sites (5′-TTGGC-3′) are clustered in two regions in the HPV-11 URR, and three of them (sites 4 to 6 in Fig. 1C) are situated in the promoter-proximal region. As stated, in HeLa cells, NF1 bound to site 6 (nt 7807 to 7811) synergizes with the Oct1 bound to a juxtaposed site. We mutated NF-1 sites 5 and 6 individually (Table 1). Neither mutation exhibited any discernible effect on the β-Gal activity in raft cultures compared to the wild-type clone (Fig. 2B and data not shown). Thus, we observed no synergy between Oct1 and NF1.

Different transcriptional environments in proliferating PHK monolayer cultures and in proliferating basal cells in raft cultures. The HPV URR-E6 promoter, including that of HPV-11, is active in epithelial cell lines and in submerged PHKs cultured as monolayers under conditions in which these cells proliferate. However, in benign lesions and in the stratified raft cultures presented in this study, very few proliferating basal cells exhibited viral or reporter activities (7, 19, 61). There are several possible explanations. First, the submerged PHKs and established cell lines are not equivalent to the proliferating basal cells in the raft culture in their transcriptional environment, and the URR is down-regulated when the cells are placed on the dermal equivalent. Only upon leaving the basal layer and committing to differentiation does the promoter become reactivated. Second, PHKs positive for β-Gal activity in previous studies are not proliferating cells but are cells that have already differentiated. Finally, there are some differences in the URR constructions used in the various studies and thus they may not be directly comparable.

To distinguish among these possibilities, we examined the β-Gal activities in proliferating PHKs in submerged cultures after acute infection with the wildtype and a 2-day treatment with G418. Uninfected PHKs were used as negative controls. As before, all uninfected cells died after drug selection. Uninfected PHKs that were not selected with G418 were uniformly negative for β-Gal. About 70% of PHKs infected by the wild-type URR-*lacZ* virus were strongly positive for β-Gal. This high percentage of positive cells immediately suggested that many of the positive cells were not differentiated. Had all positive cells differentiated, there would not have been sufficient proliferating cells to support the development of mature raft cultures.

To rule out the possibility that β-Gal-positive cells in the submerged cultures were already differentiated, we used indirect immunofluorescence to detect the expression of involucrin, an early marker for epithelial differentiation; for total cell count, the nuclei in the field were revealed by Hoechst 33258 stain (Fig. 4). Only occasional involucrin-positive cells were detected from either uninfected or retrovirus-infected PHKs

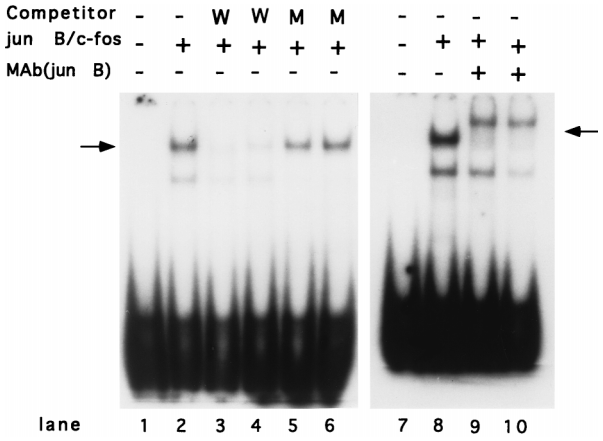

FIG. 3. EMSA of the putative Ap1p binding site within the HPV-11 URR. Double-stranded oligonucleotides containing the wild-type HPV-11 AP1p binding site were end labeled with [γ-^{32}P]ATP. In vitro-translated JunB and c-Fos proteins were used for the binding assay. The unlabeled wild-type (W) or mutated (M) oligonucleotides were used as the competitors as indicated. The specific DNA-protein complex (lower arrow, left) was supershifted (upper arrow, right) when monoclonal antibody against JunB [MAb(jun B)] was preincubated with the in vitro-translated proteins prior to addition of the probe. Lanes 1 and 7, probe only; lanes 2 and 8, probe plus 2 μl of in vitro translation reaction mixture containing JunB–c-Fos proteins; lanes 3 and 4, addition of unlabeled wild-type competitor DNA at 71- and 142-fold molar excess, respectively; lanes 5 and 6, addition of mutated competitor at 71- and 142-fold molar excess, respectively; lanes 9 and 10, 0.1 and 0.2 μg of anti-JunB polyclonal antibody (Santa Cruz) were preincubated with 2 μl each of in vitro translation mixture for an additional 10 min on ice, and this mixture was then added to the full reaction cocktail.

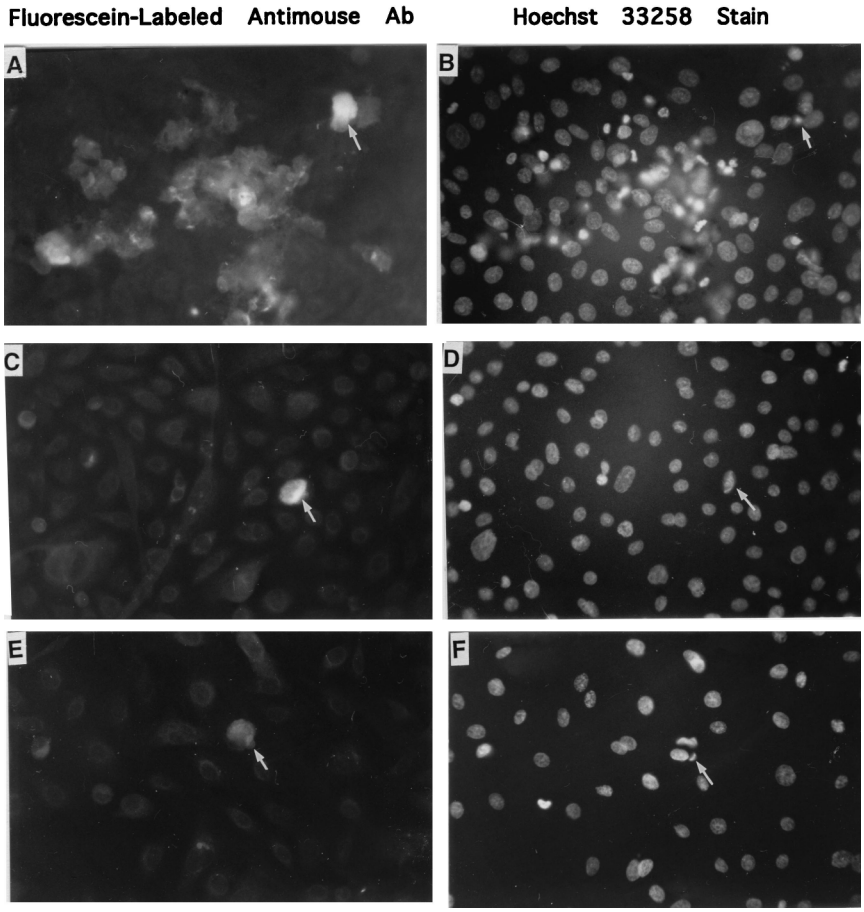

FIG. 4. Involucrin expression in submerged PHK cultures. PHKs were grown on coverslips in SFM in the absence of fibroblast feeders. The expression of the differentiation marker gene involucrin was detected by indirect immunofluorescence using a fluorescein-conjugated secondary antibody (Ab) (A, C, and E), while the nuclei of all the cells present on the coverslips were revealed by Hoechst 33258 stain (B, D, and F). (A and B) Uninfected PHKs cultured for 36 h in SFM to which 1 mM Ca^{2+} was added to induce differentiation; (C and D) the same number of uninfected PHKs as shown in panels A and B cultured for 36 h in the absence of Ca^{2+}; (E and F) PHKs infected with pLN-11URR-*lacZ* and selected for 2 days in SFM with G418 in the absence of Ca^{2+} and cultured for an additional 36 h. Arrows point to corresponding cells viewed under different filters. Few positive cells were detected in cultures to which Ca^{2+} was not added. In contrast, in the presence of Ca^{2+}, the cells piled up and were positive for involucrin, both indications of differentiation.

(Fig. 4C to F). As a positive control for the assay, PHKs in a submerged culture were stimulated to differentiate by the addition of 1 mM Ca^{2+} in SFM for 36 h. The cells piled up, and a large fraction of the cells were positive for involucrin (Fig. 4A and B), both signs of epithelial differentiation. Thus, we conclude that the β-Gal-positive PHKs in submerged cultures in SFM in the absence of differentiation stimuli are not differentiated and that the transcriptional environment in proliferating PHKs in monolayers is distinct from that in the proliferating basal layer in stratified and differentiated raft cultures.

Effects of URR mutations in submerged proliferating PHK cultures. The wild type and selected mutants were used to infect PHKs in submerged cultures grown in SFM, treated with G418 for 2 days, and assayed for β-Gal activity. The results are presented in Table 2. The relative percentage of cells infected with mutated viruses that are β-Gal positive qualitatively reflected the β-Gal activities in the raft cultures. In general, mutations that more dramatically reduced β-Gal activities in raft cultures also had lower percentages of positive cells in the submerged cultures, whereas mutations that had no effect in raft cultures had a similar number of positive cells relative to cultures infected by the wild-type virus. The Sp1 mutant, which had more abundant but weakly positive cells in raft cultures

TABLE 2. Mutational analyses of HPV-11 URR-lacZ in submerged PHK cultures[a]

HPV-11 URR-*lacZ*	% Positive cells	% Relative β-Gal activity
WT	100.0, 100.0	100.0, 100.0
AP1p	40.0, 41.7	22.1, 23.9
Oct1	42.8, 48.1	17.1, 23.6
Sp1	71.1, 68.0	45.0, 39.0
GT1	85.7, ND	ND
NF1#5	102.6, 92.3	98.9, 100.9
NF1#6	103.5, 105.1	102.9, 104.5

[a] PHKs were infected with equal titers of wild-type (WT) virus or viruses with mutations in individual transcription factor binding sites. After infection with recombinant retroviruses, cells were selected with G418 to eliminate uninfected cells and were cultured in SFM for 2 or 3 days. The initial percentages of positive cells from five random fields were counted with an inverted phase microscope and averaged. The calibrated percentages of positive cells for each mutation were then obtained by comparison with the percentage of positive cells after infection with the WT clone, which was about 70%. Duplicate plates were harvested for β-Gal activity assays, with the activities of the WT-infected PHKs and uninfected cells set as 100.0 and 0.0%, respectively. Data from two independent experiments are presented. ND, not determined.

than the AP1p and Oct1 mutants, also had a higher percentage of positive cells in the submerged cultures. β-Gal activities from cell lysates were also quantified (Table 2). Neither NF-1 mutation affected the activity significantly. However, the relative activities of the Oct1, Ap1p, and Sp1 mutants all were more severely reduced than was the percentage of positive cells. These results suggest a reduced β-Gal activity in positive cells, consistent with a decrease in promoter activities caused by these mutations.

DISCUSSION

In this investigation of a low-risk HPV type and in a recent study of the high-risk HPV-18 URR (51), we have demonstrated the feasibility of performing mutagenic analyses of the HPV enhancer-promoter to identify motifs and, by inference, the transcription factors or related family members that bind to these same sequences, which are important in the regulation of differentiation-dependent expression in the context of stratified epithelia. We chose retrovirus-mediated gene transfer because it can efficiently transduce the reporter into PHKs that are refractory to most transfection procedures. The design of the retroviral vector is such that β-Gal activity was not detected when the *lacZ* reporter gene (Fig. 1A) did not have a dedicated promoter located immediately upstream (Fig. 2H). Since a large number of acutely infected PHKs were examined simultaneously after a minimal expansion of the primary keratinocytes in culture, the pattern of reporter expression represents the typical behavior of the URR rather than a particular proviral integration event which may have different growth properties or impose specific local chromatin effects on the URR activity. By using these approaches, we show that the β-Gal activity is indeed detected primarily in the differentiated cells, as are viral activities in vivo, despite the facts that viral DNA exists as integrated proviral DNA and that there are no virally encoded E2 proteins which can additionally modulate the promoter activity (51; this study). A naturally differentiation-dependent HPV-18 and HPV-11 URR activity from integrated provirus independent of the viral E2 protein has also been demonstrated in our previous study in which the viral oncogenes in their normal enhancer-promoter context serve as reporters (7).

Point mutations in individual transcription factor binding sites in the promoter-proximal region showed that each contributed differently to the differentiation-dependent expression but none caused an up-regulation in the basal cells. The order of positive contributions (from greatest to least) is AP1p, Oct1, Sp1, AP1d, GT1. NF1 sites 5 and 6 have no discernible function. That mutations in individual transcription factor binding sites in the URR significantly affected the β-Gal activities in the URR-*lacZ* clones (Fig. 2) strongly supports our interpretation that URR-derived transcripts were responsible for the β-Gal activities observed. That the reduction in β-Gal activities in monolayer cell lysates from the AP1p, Oct1, and Sp1 mutants was more severe than the reduction in percentage of positive cells also supports the conclusion that the promoter activities were negatively impacted by these mutations (Table 2).

Although it is difficult to rule out a positive influence of the LTR enhancer on the URR promoter activity, we believe that it cannot be very significant on the basis of the present data. First, β-Gal was expressed constitutively and strongly throughout the epithelial layer from pLJ-*lacZ* in which the reporter was driven by the LTR directly (51), whereas it was expressed primarily in the differentiated spinous cells from pLN-URR-*lacZ* and few basal cells were positive. These results indicate that the LTR cannot overcome the down-regulation of the URR in the basal cells. Second, point mutations in the AP-1, Oct-1, or Sp1 site severely reduced the β-Gal activities in submerged and differentiated cells. The extent of down-regulation is not dissimilar to those found in previous studies with monolayer cell cultures in which the reporter clones did not have an LTR. Thus, the strong constitutive LTR enhancer appears unable to rescue the impaired URR in these cells. Third, the fact that β-Gal-positive cells were not detected in the basal cells (51; this study [Fig. 2]) harboring these debilitating mutations also demonstrated that the LTR enhancer had little influence on even the crippled URR in the basal cells.

Some of the above-mentioned results concerning the functions of various transcription factor binding sites are consistent with those from a recent study of HPV-18 (51). Both showed that the promoter-proximal AP1 sites were more important than the promoter-distal AP1 site and that the Oct1 and Sp1 sites were also critical for high viral promoter activity in the differentiated cells. However, the reduction in the β-Gal activity in raft cultures caused by the Sp1 mutation in the HPV-11 URR appeared less severe than that caused by mutation in the similarly located Sp1 site in the HPV-18 URR. With the HPV-18 URR, the Sp1 mutation nearly abolished the β-Gal activity, which was detectable only when the slides were not counterstained. Similarly, the Sp1 mutation had a less debilitating effect in HPV-11 than in HPV-18 when examined in submerged PHK cultures (51). These results suggest that the interactions among the transcription factors bound to the URR, rather than the mere presence of individual factors, play important roles in regulating the overall promoter activity.

Interestingly, the reporter activities of mutations in monolayer PHKs relative to those of the wild-type clones were qualitatively similar to those in the differentiated cells for both HPV-11 (Table 2) and HPV-18 (51). We have presented evidence that PHKs in the submerged cultures in SFM were not differentiated (Fig. 4), yielding the important conclusion that basal proliferating cells in stratified raft cultures are not equivalent to proliferating PHKs in submerged cultures or established epithelial cell lines in their transcriptional milieu. Rather, they mimic the basal and parabasal cells in benign lesions that also have very low viral activities (30, 60, 61). These results may provide an explanation for the reactivation of HPV present in subclinically infected keratinocytes at the margins of surgically treated lesions during reepithelialization of the wound bed as well as in the outgrowth apron of HPV-infected cells when warts are explanted onto collagen rafts (19). Once stratification is complete, HPV expression is down-regulated in the basal layers both in vivo and in raft cultures.

We surmise that the basal cells are low in the abundance of certain positive transcription factors or, alternatively, they may express transcription repressors that are absent, reduced, or counteracted by new positive factors in monolayer PHKs, in transformed epithelial cell lines, and in differentiated PHKs. The host factor YY1 has been shown to inhibit the HPV-16 and HPV-18 URRs (3, 44, 49). A YY1 site has not been reported for the HPV-11 URR. However, a C/EBPβ binding site functions as a negative element for the HPV-11 URR-E6 promoter (65). Work is in progress to determine whether it also serves as a repressor binding site in the epithelial raft cultures.

In comparing our results to those of previous studies of the HPV-11 URR conducted with cervical cell lines or with PHKs, we also note some differences. In the C33A cell line, the integrity of the Sp1 site is very important, and mutations of this site lead to a 70% reduction in promoter activity (21). However, in submerged cultures of proliferating PHKs, the same

mutations reduced the activity by a more moderate amount. A side-by-side comparison of the Sp1 mutation in C33A cells and in submerged PHKs confirmed that the number of β-Gal-positive cells was more reduced in the C33A cells than in PHKs (data not shown). This disparity suggests differences in the type or in the abundance of the Sp1 family of proteins or in their interactions with other transcription factors that vary between these two types of cells. Furthermore, in neither the submerged proliferating PHKs nor the raft cultures did we observe synergy between Oct1 and NF1 (Table 2, Fig. 2), as had been previously reported for HeLa cells (48). Our results are also different from those of a previous reconstitution experiment in which multimerization of a promoter-proximal restriction fragment containing NF1 sites 4 to 6 conferred high E6 promoter activity in submerged PHKs, whereas deletion of either site 4 or 6 reduced the activity substantially (20). We are currently testing the hypothesis that the redundancy of NF1 sites may have masked the effects of mutations in individual sites. Nevertheless, our results suggest that it is important to use the entire URR in the normal host PHKs for such studies.

In summary, we have established an experimental system to examine HPV promoter regulation in differentiating PHKs and have begun to evaluate contributions of sequence elements to the URR-E6 promoter under different culture conditions. Using this organotypic system, we are continuing to investigate additional *cis* elements to identify those responsible for the regulation of HPV promoters during epithelial differentiation.

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