A Distal Element in the HPV-11 Upstream Regulatory Region Contributes to Promoter Repression in Basal Keratinocytes in Squamous Epithelium

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In benign squamous lesions and in organotypic epithelial cultures, the human papillomavirus (HPV) E6 and E7 genes are transcriptionally up-regulated in differentiated, spinous keratinocytes. We previously identified sequence elements in the enhancer-promoter regions of HPV types 18 and 11 important for this promoter regulation by using the bacterial LacZ reporter gene in stratified raft cultures of primary human keratinocytes (PHKs) or in submerged, proliferating cultures acutely transduced with recombinant retroviruses. Notably, mutations in the promoter-proximal Sp1, Oct1, and AP1 sites each significantly reduce reporter activity in differentiated cells, indicating that the bound factors are transcription transactivators. In the present study, we performed further mutagenesis on distal motifs in the HPV-11 regulatory region in PHKs in submerged and raft cultures. Mutations in an AP2-like site, three individual NF-1 sites, or five NF-1 sites collectively reduced promoter activity slightly in differentiated cells. A mutation in a putative glucocorticoid response element had no discernable effect in the presence or the absence of dexamethasone. However, mutations in a C/EBP binding site, especially the distal site, strikingly up-regulated reporter gene expression, particularly in basal and lower spinous cells, implicating bound protein as a transcription repressor. Collectively, these results demonstrate that the overall differentiation-dependent papillomaviral gene expression observed in vivo and in vitro involves promoter repression in the lower strata and activation in the upper, differentiated strata.

INTRODUCTION

Human papillomaviruses (HPVs) are widespread human pathogens. Infections of genital and oral epithelia by the mucosotropic HPVs can lead to benign papillomas and condylomata. Certain high-risk HPV types can also cause dysplasias that progress to carcinomas in a small fraction of patients (zur Hausen and de Villiers, 1994). Productive infections by HPVs are observed only in benign squamous lesions. Viral activities increase in the more differentiated, upper spinous strata, culminating in the production of progeny virions in the superficial, terminally differentiated cells (Chow and Broker, 1997a). Species and tissue tropism and differentiation dependence are largely attributed to transcription regulatory elements in the noncoding region termed the upstream regulatory region (URR) or the long control region (LCR), which is contiguous with the promoter located immediately upstream of the viral E6 and E7 genes (the E6 or P1 promoter for HPV-11, P97 for HPV-16, or P105 for HPV-18). These viral gene products, respectively, inactivate the host tumor suppressor protein p53 and pRB family. The function of E7 is to reactivate host DNA replication machinery to support viral DNA replication in postmitotic, differentiated cells (Kuo et al., 1994; Cheng et al., 1995; Jian et al., 1998; reviewed by Chow and Broker, 1997a).

Inappropriate expression of these genes in stem cells is a critical event in carcinogenesis initiated by high-risk HPV types. Therefore, it is important to understand how the URR is regulated in primary human keratinocytes (PHKs), the natural host cells, both in proliferating and in differentiated states.

Both virus-encoded and host transcription factors regulate the URR-E6 promoter. In brief, the URR contains consensus binding sites for the family of viral E2 proteins that either positively or negatively regulate the E6 promoter (Chin et al., 1989; Dong et al., 1994a; Tan et al., 1994; reviewed by Ham et al., 1991; McBride et al., 1991). Host transcription factors such as Sp1, AP1, Oct1, NF1, KRF, TEF-I, TEF-II, Epoc-1/Skn-1, and YY1 as well as glucocorticoid receptor function as transcription activators (Bernard and Apt, 1994; Hoppe-Seyler and Butz, 1994; Khare et al., 1996; O’Connor and Bernard, 1995; Yukawa et al., 1996; Kanaya et al., 1997; Parker et al., 1997; Zhao et al., 1997). Cis elements that bind host transcription repressors have also been identified, including YY1 in the URR of HPV-16 and -18 (Bauknecht et al., 1992; Dong et al., 1994b; May et al., 1994; O’Connor and Bernard, 1995) and C/EBP in HPV-11 (Wang et al., 1996), HPV-16 (Sibbet and Campo, 1990), and BPV-4 (McCaffery and Jackson, 1994).

The majority of these studies were conducted in established epithelial cell lines. Only a few investigations
were performed in proliferating PHKs because of the very low efficiency of DNA transfection. We have developed a model system to examine URR-E6 promoter regulation in PHKs. Highly efficient retrovirus-mediated gene transfer was used to introduce a bacterial LacZ reporter gene under the control of either a wildtype or a sited-mutated HPV-11 or HPV-18URR-E6 promoter (pLN-11URR-LacZ or -18URR-LacZ) (Fig. 1A). Acutely infected PHKs were selected for 2 days with Geneticin under conditions where all uninfected cells died. β-Galactosidase (β-gal) assays were then conducted either with lysates of submerged cultures or by an in situ method in organotypic raft cultures that were generated on a dermal equivalent after 9 days of growth at the air–medium interface (Parker et al., 1997; Zhao et al., 1997). In this system, the LacZ reporter gene without a dedicated promoter (pLN-LacZ) (Fig. 1A) does not exhibit any β-gal activity (see Fig. 2F), whereas the LacZ reporter gene directly controlled by the retrovirus LTR in pLJ-LacZ is expressed in both basal and differentiated strata. pLN-11URR-LacZ or pLN-18URR-LacZ expresses the reporter gene predominantly in spinous cells. Very few positive cells are observed in the basal stratum, recapitulating the differentiation-dependent viral gene expression observed in vivo. In particular, for both HPV types, a promoter-proximal AP1 site, an Oct1 site, and a novel Sp1 site are each critical for promoter activities, as mutations in any one of them reduce reporter activity dramatically, suggestive of synergistic interactions.

Interestingly, β-gal is also expressed from both the HPV-11 and HPV-18URR in a substantial fraction of PHKs in proliferating, submerged cultures, demonstrating a different transcription milieu from the proliferating basal cells in the stratified epithelium. Differences from observations made in established cell lines or with deleted or reconstituted URRs were noted. For instance, an Oct1 binding site in the HPV-18URR was thought to function as a negative element in cell lines (Hoppe-Seyler et al., 1991), whereas another report suggests that a similarly located Oct1 site synergises with a promoter-proximal NF1 site to confer high activity in HPV-11 and HPV-16URR (O’Connor and Bernard, 1995). However, we observed no discernable effect in raft or submerged cultures in mutations of either of the two NF1 sites flanking the Oct1 site in the context of the entire HPV-11URR (Zhao et al., 1997).

These studies demonstrate that binding of transcription activators to the cognate binding sites is responsible for the reporter activity in the differentiated keratinocytes. But what is the reason for promoter shutoff in the basal cells of stratified and differentiated raft cultures? It could be due to the absence of certain key activators, an induction of repressors in the basal cells, or both. In this report, we present mutagenic analyses of a number of E6 promoter-distal motifs in the HPV-11URR in PHKs. No motifs were found that bind a strong transcription activator. However, we discovered that two C/EBP binding sites bind transcription repressors. Mutations in either site up-regulated the reporter activity, particularly in the basal and lower spinous cells, but also in submerged,
FIG. 2 β-Gal activities in epithelial raft cultures of PHKs infected with recombinant retroviruses. β-Gal activities were revealed in paraffin-embedded sections after reacting with an X-gal solution. The sections were counterstained lightly to reveal the distribution of β-gal-positive cells in the stratified epithelium. β-Gal activities in raft cultures of PHKs infected with (A) pLN-11URR-LacZ; (B) pLN-11URR-AP2M-LacZ; (C) pLN-11URR-C/EBP(p)-LacZ; (D) pLN-11URR-C/EBP(d)-LacZ; (E) pLN-11URR-24N-LacZ; (F) pLN-LacZ. All raft cultures were prepared in parallel using the same batch of PHKs. Each mutation was tested three or more times and the results were consistent. Arrows in panel F point to basal stratum.
proliferating cultures. The up-regulation of promoter activity in response to the loss of the distal site was more substantial than that of the proximal site. Thus, we conclude that the combined effects of promoter repression in the lower strata and activation in the differentiated, upper strata contribute to an overall differentiation-dependent URR-E6 promoter activity during squamous differentiation.

**RESULTS**

PHKs were acutely infected with equal titers of retroviruses in which the LacZ reporter was under the control of the wildtype or a mutated HPV-11URR. In parallel, the negative controls include pLN-LacZ, which does not have a dedicated promoter or uninfected PHKs (Fig. 1, data not shown). The URR mutations affect the consensus nucleotides known to abolish cognate protein binding or to exert an effect on promoter activity.

<table>
<thead>
<tr>
<th>Mutations of Transcription Factor Binding Sites in the Distal Region of the HPV-11URR</th>
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<tr>
<td>Sequences</td>
</tr>
<tr>
<td>C/EBP(p)†</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>NF1(3)</td>
</tr>
<tr>
<td>NF1(2)</td>
</tr>
<tr>
<td>GRE</td>
</tr>
<tr>
<td>NF1(1)</td>
</tr>
<tr>
<td>AP2</td>
</tr>
<tr>
<td>C/EBP(d)†</td>
</tr>
<tr>
<td>24-N</td>
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</table>

*Note.* The DNA fragment containing the HPV-11URR spans nucleotides 7072–7933/1–99. The 5' nucleotide positions and sequences of wildtype (W, upper line) and mutated (M, lower line, with mutations in bold) binding sites are shown. All mutations were introduced into consensus nucleotides known to abolish cognate protein binding or to affect transcription activity.

†(p), the proximal site.

‡(d), the distal site.

Up-regulation of the reporter expression in both basal and differentiated keratinocytes by mutations in C/EBP binding sites

C/EBP proteins comprise a family of transcription factors with at least seven members and are involved in the regulation of a variety of promoters either as a repressor or as an activator (Wedel and Ziegler, 1995). There are two C/EBP binding sites in the HPV-11URR (Fig. 1B) (Wang et al., 1996; K. Auborn, personal communication). The promoter-distal site preferentially binds C/EBPβ and negatively regulates the HPV-11URR promoter, as determined from an experiment in which cotransfection of an HPV-11URR-driven reporter with a polynucleotide containing this binding site stimulated the reporter activity in proliferating, submerged PHK cultures (Wang et al., 1996). However, mutagenic analyses of either C/EBP binding site have not been reported.

We individually mutated the distal (7456–7466) and the proximal (7770–7784) C/EBP sites (denoted as C/EBP(d) or (p) in Table 1). In raft cultures of PHKs transduced with C/EBP(d)-LacZ, the reporter activity was significantly up-regulated relative to the wildtype URR. The signal intensity of many positive cells in the spinous strata appeared to be slightly increased. But the strongest induction was observed in the basal stratum and the lower spinous cells, as these cells were completely or largely negative for β-gal in cultures containing the wildtype URR-LacZ (compare Figs. 2D and 2A). The C/EBP(d) mutation also up-regulated the reporter activity, but to a lesser extent in the basal cells based on comparison of raft culture sections from end to end in several independent experiments (compare Figs. 2C and 2D). These results indicate that the two C/EBP sites function individually to bind a repressor protein in both basal and spinous cells in the raft cultures.

A slight down-regulation of reporter activity by a mutation in an AP2 binding site

A putative AP2 binding site (TACCCCCCCACT) located at nucleotide positions 7574 to 7587 has been proposed based on partial sequence homology and DNase I footprinting assays with HeLa cell nuclear extracts (Gloss et al., 1989). There was no previous AP2 binding assay nor functional analysis. We performed gel mobility shift assays by using oligonucleotides contain-
URR-E6 promoter in the stratified epithelium. Functions as a moderate enhancer element for the HPV-11URR can specifically bind the AP2 protein and observations indicate that the AP2-like binding site in the proximal AP1, Oct1, and Sp1 sites characterized was not as significant as those resulting from mutations (Zhao et al., 1997) (data not shown). These results indicate that this putative GRE in the HPV-11URR did not mediate dex regulation of the promoter activity.

A small reduction of reporter activity by individual and multiple mutations in the NF1 sites

We previously reported that mutations in either of two NF1 sites located proximal to the E6 promoter flanking the October site (sites 5 and 6 in Fig. 1B) did not lead to detectable effects on reporter expression in raft cultures or in submerged cultures of PHKs (Zhao et al., 1997). These mutations are known to abolish binding of NF1 in vitro (O’Connor and Bernard, 1995). To investigate whether this lack of effect might have been masked by the presence of redundant NF1 motifs, which appear three more times in the promoter-distal region (Fig. 1), we introduced the same mutations into these three additional motifs (sites 1, 2, and 3) individually and also prepared a mutation in which all five sites were simultaneously mutated (Table 1). The LacZ reporter genes driven by each of the single-site mutations, NF1M(1), (2), or (3), showed only a small reduction relative to the wildtype clone in the presence or absence of dex (Fig. 4, compare A with B; C with D). These results indicate that this putative GRE in the HPV-11URR did not mediate dex regulation of the promoter activity in our system.

Absence of a discernable effect by a mutation in GRE

Ligand-bound glucocorticoid receptor associated with the responsive element (GRE) alters chromatin structures for a short duration and facilitates RNA polymerase transcription (Slater et al., 1994). The URR of the mucosotropic HPVs each contains a sequence highly homologous to the GRE and is activated by progesterone and dexamethasone (dex) in epithelial cell lines (Jantzen et al., 1987; Chan et al., 1989). A single mutation in the highly conserved C residue to T or A in the GRE of HPV-18 and HPV-16URR abolishes glucocorticoid receptor binding or reduces transcription activation by these hormones in epithelial cell lines (Chan et al., 1989; Medina-Martinez et al., 1996). A 2-bp mutation in HPV-18 GRE including the same C residue increased the reporter gene activity but abolished the responsiveness to dex in one study (Butz and Hoppe-Seyler, 1993). However, the same mutation failed to exert any discernable effects in submerged, proliferating PHKs or PHK raft systems (Parker et al., 1997). We introduced this same C to T mutation and also changed the highly conserved flanking G and A residues in the putative GRE (Table 1). In raft cultures, the wildtype pLN-11URR-LacZ exhibited a small but discernable increase in reporter activity after a treatment with 10 nM of dex for 1, 2, or 3 days prior to harvest. The increase appeared the highest after a 1 day treatment (compare Figs. 4A and 4C, and data not shown). The 3-bp mutation had no effect on reporter activity relative to the wildtype clone in the presence or the absence of dex (Fig. 4, compare A with B; C with D). These results indicate that this putative GRE in the HPV-11URR did not mediate dex regulation of the promoter activity in our system.

![Graph showing electrophoretic mobility shift assay of purified AP2 protein binding to the putative cognate site within the HPV-11URR.](image)

**FIG. 3.** Electrophoretic mobility shift assay of purified AP2 protein binding to the putative cognate site within the HPV-11URR. Lane 1 contains 25 fmol 32P-labeled SV40 AP2 oligonucleotide probe and 150 ng purified AP2 protein. All other lanes contain 25 fmol of the wildtype (WT) HPV-11 AP2 probe, 600 ng AP2 protein (lanes 2, 6), and additional reagents (lanes 3–9) as described below. Lanes 3 and 8, binding reactions in the presence of a 120-fold excess of unlabeled HPV-11URR WT AP2 oligonucleotides. Lanes 4 and 9, reactions in the presence of a 120-fold excess of unlabeled, mutated AP2 oligonucleotides. Lane 5, a reaction in the presence of 100 ng of anti-AP2 polyclonal antibody. Lane 7, a reaction in the presence of a 120-fold excess of unlabeled SV40 AP2 oligonucleotides. Arrows point to the AP2:DNA complex and the supershift complex in the presence of anti-AP2 antibody.
TABLE 2

Relative β-Gal Activities of the HPV-11URR in Submerged PHK Cultures

<table>
<thead>
<tr>
<th>HPV-11 URR</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
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<tbody>
<tr>
<td>WT</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>WT + dex (1 day)</td>
<td>127.8</td>
<td>ND</td>
</tr>
<tr>
<td>C/EBPm(p)</td>
<td>112.0</td>
<td>124.9</td>
</tr>
<tr>
<td>NF1M(3)</td>
<td>80.9</td>
<td>77.1</td>
</tr>
<tr>
<td>NF1M(2)</td>
<td>75.3</td>
<td>ND</td>
</tr>
<tr>
<td>GREM</td>
<td>99.4</td>
<td>100.8</td>
</tr>
<tr>
<td>GREM + dex (1 day)</td>
<td>130.8</td>
<td>ND</td>
</tr>
<tr>
<td>NF1M(1)</td>
<td>85.8</td>
<td>79.9</td>
</tr>
<tr>
<td>AP2M</td>
<td>82.0</td>
<td>74.6</td>
</tr>
<tr>
<td>C/EBPm(d)</td>
<td>155.9</td>
<td>154.7</td>
</tr>
<tr>
<td>24-N</td>
<td>159.2</td>
<td>216.0</td>
</tr>
<tr>
<td>NF1M(12356)</td>
<td>67.2</td>
<td>70.5</td>
</tr>
<tr>
<td>Uninfected PHKs</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Note. Proliferating PHKs in submersed cultures were infected with equal titers of recombinant retroviruses containing wildtype or mutated HPV-11URR (Table 1), selected with Geneticin for 2 days. The surviving cells were cultured and then harvested at 24 h immediately prior to harvest. The activities of uninfected and unselected PHK lysates were taken as 0, whereas those from PHKs transduced by the wildtype (WT) clone were set as 100%. Treatment with dexamethasone (dex) was for 1 day at 10 nM for 24 h.

*NF1 site 4 consists of just 3 bp 5'-TGG-3' (Gloss et al., 1989) and was not mutated in this study.

both the wildtype and the GRE mutation and that the mutation had no effect on reporter activity in the absence of dex. Furthermore, the NF1M(12356) clone had reporter activity similar to that of the individual mutations. In contrast, an increase up to 150% of the wildtype clone in β-gal activities was observed in the C/EBP mutations and the distal site mutation had a more significant enhancement than did the proximal site mutation.

**DISCUSSION**

We and others have previously established that the PHK raft culture system is a faithful model system for investigating papillomavirus protein functions upon retrovirus-mediated gene transfer (reviewed by Chow and Broker, 1997b). By using this approach, we have developed a URR-LacZ reporter system to examine HPV promoter regulation during squamous cell differentiation and have initiated mutagenic analyses of a number of promoter-proximal transcription factor binding sites (Parker et al., 1997; Zhao et al., 1997). Mutations in the promoter-proximal AP1 site, the Oct1 site, and the Sp1 site each down-regulated LacZ reporter activity significantly. We have now extended the investigation to sequence elements in the promoter-distal region of the HPV-11URR.

As in our previous reports, the qualitative results in the differentiated cells in raft cultures and the quantitative β-gal activities in submerged cultures were concordant, even though the submerged cultures were not differentiated (Zhao et al., 1997) (Figs. 2 and 4 and Table 2). Furthermore, the submerged cultures had a similar percentage of β-gal-positive cells for five passages, further supporting our conclusion that vast majority of the cultures were proliferating cells (our unpublished results). After a 30-h exposure to BrdU, most the basal and parabasal cells in 9-day raft cultures are positive for BrdU incorporation, indicating that they are proliferating cells that actively replicated DNA (Jian et al., 1998). We suggest that submerged, proliferating cultures may be akin to the cellular state during wound healing and reepithelialization, whereas the basal cells in established raft cultures are in a state of maintenance and thus have different transcriptional environments.

We note that the reporter activity was often heterogeneous among adjacent cells in the differentiated as well as in submerged cultures (Figs. 2 and 4 and our unpublished results). We do not believe that a difference in proviral DNA copy number is the primary reason, as G418-selected PHKs infected with serially diluted recom-
binant retroviruses were also heterogeneous in reporter activity in submerged cultures when the activity was visualized in situ (our unpublished data). PHKs transduced with high dilutions of retroviruses when most of the culture died upon selection should have a similar and minimal copy number of provirus. The reasons for the heterogenous promoter activities are not yet understood. A heterogeneity in viral mRNA abundance in spinous cells is commonplace in patient specimens in which the viral DNA remains extrachromosomal. However, in those cases, the viral DNA copy number could also have played a role.

Most significantly, mutations in either of two C/EBP binding sites increased reporter activity in submerged PHK cultures and also in raft cultures, particularly in basal proliferating cells (Table 2, Fig. 2). Mutations of the distal site had a much more dramatic effect than those of the proximal site. Thus, the bound C/EBP proteins function as transcription repressors in all three types of transcriptional environments: the proliferating submerged PHKs, the proliferating basal keratinocytes in stratified epithelium, and the suprabasal, differentiated keratinocytes. These results suggest that lack of promoter activity in basal cells is not likely due to the absence of transcription activators. C/EBP proteins comprise a family of proteins that have a related DNA binding domain but different effector domains. The basal and differentiated strata in laryngeal epithelium express different members of the C/EBP family (Jin et al., 1998). In particular, much C/EBPα is cytoplasmic and nuclear localization was detected only in spinous cells, whereas C/EBPβ, which binds preferentially to the distal site (Wang et al., 1996), is predominately expressed in the nucleus in spinous cells. Interestingly, in laryngeal papillomas, most C/EBPβ was found in the nucleus of basal cells, whereas nuclear C/EBPα was found in all cell strata (Jin et al., 1998). Since mutations in either site up-regulated reporter gene expression moderately in upper spinous cells and strongly in basal and lower spinous cells, more than one C/EBP protein may down-regulate HPV-11URR. This result is different from that of a report on the HPV-18URR, in which C/EBPβ functioned as an activator in HeLa cells (Bauknecht et al., 1996). A C/EBP binding site has been suggested to confer promoter activity to the human involucrin gene, which is a differentiation-specific gene (Welter and Eckert, 1997). Thus, the specific interactions among URR-bound transcription factors influence the overall protein function.

The AP2 and related transcription factor KDF-1 are thought to confer epithelial-specific gene expression, as one or more binding sites have been identified in a number of genes expressed only in squamous epithelia (LaPres and Hudson, 1996; Magnaldo et al., 1993; Eckert et al., 1997). Limited mutational analyses of this element indicated that bound proteins function as transactivators in genes that are active in basal cells, such as keratins 5 and 14, and also in genes that are active in differentiated cells, such as keratin 1 and involucrin (Ohitsuki et al., 1993; Leask et al., 1991). Three AP2-like sequences located in the transglutaminase-1 promoter function as enhancers and bind an 87-kDa protein present in extracts of several epithelial cell lines (Mariniello et al., 1995). The present study demonstrates that the putative AP2 site located in the HPV-11URR can indeed bind the purified AP2 protein in vitro (Fig. 3). At present, the identity of the protein bound in vivo to this HPV-11URR element remains to be elucidated. But unlike keratinocyte-specific cellular genes, this HPV-11URR element does not appear to play a critical role in regulating viral promoter activity either in submerged raft cultures or in stratified raft cultures (Fig. 2 and Table 2), in agreement with the observation made in the HPV-18URR (Parker et al., 1997).

NF1 elements have been reported to function as positive regulators in the sequence context of truncated URR of HPV-11, -16 and -18 (Gloss et al., 1989; Chong et al., 1990; 1991; Dollard et al., 1993). The 2-bp mutation introduced into the HPV-11 NF1 sites in our study has previously been shown to abolish NF1 protein binding (O’Connor et al., 1995). These sites have also been suggested to play important roles in the cell type specificity of the URR-E6 promoter (Gloss et al., 1989). On the other hand, in the context of the entire HPV-18URR, individual mutations in three NF1 sites failed to decrease promoter activity, while combined mutations in all three sites only reduced reporter activity to 75 or 88% in HeLa cells or HaCat cells, respectively (Butz and Hoppe-Seyler, 1993). Our results in HPV-11 are in excellent agreement with the latter report in that mutations in any one of the five NF1 sites had either no effect (Zhao et al., 1997) or at most a very moderate effect on URR activity (Fig. 4, Table 2). Furthermore, mutations of all five sites do not reduce reporter activity more than individual mutations in site 1, 2 or 3 (Fig. 4, Table 2). Thus, NF1 sites are largely dispensable for high transcriptional activity of the HPV-11URR promoter in PHKs.

Mutational analyses of a putative glucocorticoid receptor response element in the URR of HPV-16 and HPV-18 abolished or diminished responses to dexamethasone in certain epithelial cell lines (Chan et al., 1989; Medina-Matinez et al., 1996). The wildtype HPV-11URR also responded to these hormones in epithelial cells, and a sequence highly homologous to the GRE of HPV-16 and HPV-18URR was hypothesized to mediate this induction (Chan et al., 1987). Our data show that although the HPV-11URR was indeed slightly stimulated by a brief treatment with dexamethasone, the GRE mutation had no effect on reporter activity in the presence or in the absence of dexamethasone relative to the wildtype in either raft or submerged cultures (Fig. 4, Table 2), in agreement with our mutational analysis of the GRE in HPV-18URR (Parker et al., 1997). Thus, it is likely that the stimulation by dex
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is an indirect consequence on the cells rather than a direct action on the HPV-11 and HPV-18URR-E6 promoter. This interpretation could account for the discrepancies in mutational analyses of HPV-18 GRE in different cells (Chan et al., 1989; von Knebel-Doeberitz et al., 1991; Butz and Hoppe-Seyler, 1993; Khare et al., 1996; Medina-Martinez et al., 1996; Parker et al., 1997). Perhaps these cells differ in other components comprising the GRE receptor-mediated pathway (Slater et al., 1994; Muchardt and Yaniv, 1993). However, it cannot be ruled out that there are additional GREs in the URRs that have not been identified.

The site-directed mutagenesis in this study suggested that several previously identified cis elements, including AP2, NF1, and GRE, did not contribute significantly to URR-E6 promoter activation in differentiated cells. This conclusion is supported by a 5’ URR deletion mutation 24-N in which the distal C/EBP site, the GRE, the AP2, and one NF1 site were all removed (Fig. 1). In submerged cultures, this deletion had reporter activity similar to or slightly higher than that of the C/EBPM(d) mutation alone (Table 2). In raft cultures, basal cells were also strongly positive for reporter activity (Fig. 2E), similar to the C/EBPM(d) clone.

Collectively, we infer from our previous (Zhao et al., 1997) and present studies that the most important transcription transactivator binding sites are those for AP1, Oct1, and Sp1 located in the promoter-proximal region, whereas the distal C/EBP site binds a strong repressor primarily in basal and lower spinous cells. These studies and those in numerous laboratories on host and viral promoters clearly demonstrate that the functions of these transcription factors depend on the promoter sequence context. Recently, we also found that trichostatin A, a specific inhibitor of histone deacetylases, can additionally up-regulate the promoter activity of wildtype HPV-11URR and C/EBPM(d), suggesting that histone remodeling also plays an important role in regulating HPV-11URR activity (W. Zhao, L. T. Chow, W. Y. Chen, T. M. Townes, and T. R. Broker, unpublished results).

In summary, the use of highly efficient retrovirus-mediated gene transfer has allowed us to reexamine transcription regulatory elements in the HPV-11URR-E6 promoter in the natural host cells of HPVs, primary human keratinocytes, grown either as organotypic cultures or as submerged proliferating cultures. These investigations have revealed two mechanisms by which the epithelial differentiation-dependent viral gene expression first observed in benign human lesions is achieved: the binding of dominant transcription repressors that inhibit the HPV-11URR-E6 promoter primarily in lower strata and the binding of strong transcription transactivators that activate the promoter in the differentiated upper strata. Without either methodology, neither mechanism would have been revealed. The combination of these two methods constitutes a powerful experimental system generally applicable to the investigation of promoter regulation or gene function in squamous epithelium.

MATERIALS AND METHODS

Site-directed mutagenesis and retroviral vectors

Site-directed mutations in all transcription factor binding sequences located in the HPV-11URR (7072–7933/1–99) between the BamHI and HindIII sites (Hirochika et al., 1988) were generated with polymerase chain reaction (PCR) amplification, as described previously (Zhao et al., 1997). All mutations targeted critical nucleotides (Table 1) that have been reported previously to eliminate binding of the cognate factors or to exert an effect on reporter gene expression. All mutations were confirmed by dideoxynucleotide sequencing. Briefly, the cloning vector pLN-LacZ is derived from pLNSX (Miller and Rosman, 1989) in which the SV40 promoter was removed and LacZ was placed downstream of the neomycin resistance gene (neo) without a dedicated promoter. In pLN-11URR-LacZ, the wildtype or mutated URR was inserted in the correct orientation into the pLN-LacZ upstream of the LacZ reporter (Fig. 1A). A 5’ deletion clone, 24-N, which retains HPV-11 nucleotides 7674–7933/1–99 (Chin et al., 1989), was constructed similarly.

Production of retroviruses and acute infection of PHKs

Ectotropic and amphotropic recombinant retroviruses were produced from helper cell lines 3cre and pG+envAM12 (Markowitz et al., 1988) as previously described (Parker et al., 1997; Zhao et al., 1997). Viral titers were estimated from the number of surviving PHKs after infection with serial dilutions of virus stocks and Geneticin selection (see below). PHKs were recovered from neonatal foreskins as described (Wilson et al., 1992). The same number of first passage PHKs at 30 to 50% confluence were infected with equal titers of recombinant retroviruses and then selected for 2 days with 400 μg/ml Geneticin in serum-free medium (SFM, GibCO/BRL). More than 70% of the cells usually survived the selection, while all uninfected cells died. The surviving cells were allowed to recover for 24 h and were then used immediately for experimentation without further passage. This procedure allowed us to examine mass cultures rather than descendants of selected clones.

β-Galactosidase assays

Epithelial raft cultures were prepared and harvested after 9 days at the air–medium interface and embedded in paraffin as described (Zhao et al., 1997). The β-gal activities were assayed according to established procedures (Sanes et al., 1986). Briefly, 5-μm sections were cut for histologic analysis and for detection of β-gal-positive cells. The slides were photographed after a light coun-
terstaining with hematoxylin and eosin to correlate β-gal activity with tissue morphology. Each mutation was tested in duplicate along with the wildtype URR-LacZ, with uninfected PHKs, and with PHKs infected with vector pLN-LacZ without a dedicated promoter for LacZ expression in several independent experiments using different batches of PHKs.

The same number of infected and Geneticin-selected PHKs or of uninfected and unselected PHKs were plated onto 60-mm plates and then assayed for β-gal enzymatic activities after additional culturing in SM for 24 to 36 h when the cells reached 90% confluence. Enzymatic assays of lysates of subcultured merged cultures were conducted using the same amount of total protein as described (Sambrook et al., 1989; Zhao et al., 1997). The results were averages of two experiments.

Electrophoretic mobility shift assay

Double-stranded oligonucleotides containing the putative AP2 binding site (underlined) 5′-CTATGTACCCTCCCACCAGCA-3′ in the HPV-11 URR and the AP2 site 5′-TGCTGGGACGCTGCGGACTTCCACACC-3′ derived from the SV40 enhancer were end-labeled in parallel with [γ-32P]ATP using phage T4 polynucleotide kinase and standard procedures. The protocol from the supplier of the purified AP2 protein (Promega, Madison, WI) was followed for binding reactions. Briefly, 10-μl reaction mixtures containing reaction buffer (10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 1 mM MgCl2, 4% glycerol), 150 ng of AP2 protein for the SV40 AP2 site, or 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 1 mM MgCl2, and 25 fmol of labeled oligonucleotides were incubated at room temperature for 20 min. The mixtures were then loaded onto a 5% native acrylamide gel and electrophoresed at 4°C in 0.25× Tris–borate–EDTA (TBE) buffer. The gel was dried and exposed overnight for autoradiography. For competition, a 120-fold excess of unlabeled HPV-11URR AP2 oligonucleotides, of the SV40 AP2 oligonucleotides, or of the mutated HV-11 URR AP2 oligonucleotides 5′-TACTATGTATTCAACCTCCACTGCA-3′ (with mutations in bold) was included in the binding reaction. One hundred nanograms of polyclonal anti-AP2 antibody (Promega) was used for the supershift under conditions where the free probes migrated out of the gel in order to visualize the supershifted DNA–protein band.

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