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The Enhancer of Human Papillomavirus Type 16: Binding Sites for the Ubiquitous Transcription Factors oct-1, NFA, TEF-2, NF1, and AP-1 Participate in Epithelial Cell-Specific Transcription

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The enhancer of human papillomavirus type 16 (HPV-16) is considered to be specific for epithelial cells, in particular for cervical carcinoma-derived cell lines. We reexamined this hypothesis with the complete enhancer as well as nonoverlapping subclones and found all clones to be active in epithelial cell lines derived from the epidermis and from carcinomas of the cervix, mammary gland, and colon, but inactive in fibroblast, lymphoma, and embryonal carcinoma cells. Although the virus infects only human mucosal epithelia, enhancer activity was independent of the exact type or of the species of origin of the transfected epithelial cell. In spite of epithelial cell specificity, we found that the activity of the HPV-16 enhancer varied strongly from a cytomegalovirus enhancer and the simian virus 40 enhancer in a cell line-dependent manner. This suggests varying quantitative contributions of enhancer elements rather than regulation by an all-or-none switch. Cell type specificity was maintained by a 91-bp subclone of the 400-bp enhancer. Most of the enhancer activity of this fragment was eliminated by alternative mutations in binding sites for the ubiquitous factors AP-1, nuclear factor 1 (NF1), or TEF-2. These three types of factors bind this 91-bp enhancer without cooperation, although activation appears to be synergistic. Outside the 91-bp fragment, a motif typical for papillomavirus enhancers, namely an octamerlike sequence flanked by an NF1-binding site, contributes to enhancer function, as the activity was strongly reduced upon its deletion. In HPV-16, this motif is bound by the oct-1 factor as well as by a probably novel factor, NFA, whereas a related motif of HPV-11 is recognized only by NFA. On examination, none of the five types of transcription factors involved in HPV enhancer activation was restricted to epithelial cells, but NF1, AP-1, and oct-1 were present in higher concentration in HeLa cells than in fibroblasts. Only NF1 showed some qualitative cell type-specific differences. We propose that the epithelial specificity of the HPV-16 enhancer is brought about via binding sites for supposed ubiquitous transcription factors. The mechanism of this activation apparently involves synergism between factors that vary in concentration and may include cell-specific functional differences residing outside the DNA-binding domain of these factors.

Human papillomavirus type 16 (HPV-16) is the most frequently encountered member of a group of papillomaviruses that infect the epithelia of the female and the male genital region. Epidemiological studies of genital cancer suggest that an infectious agent is involved in carcinogenesis of the cervix uteri (for reviews, see reference 57). Papillomaviruses are favored candidates, first because their genome is present in most if not all cervical cancers (57), and second since they encode proteins whose mechanism of transformation is moleculary understood: the products of the genes E5, E6, and E7 modify responses mediated by tyrosine kinase-type receptors and form complexes with the products of the tumor suppressor genes p53 and Rb, respectively (19, 36, 52).

HPVs are strictly epitheliotropic. This selectivity seems to be at least partially brought about by the viral transcriptional enhancer, which is active in epithelial cells but not functional in cell lines of other differentiation types (13, 25). We have previously found that the HPV-16 enhancer can be activated by numerous binding sites for apparently ubiquitous transcription factors, namely seven for nuclear factor 1 (NF1) (27), three for AP-1 (7), one for the progesterone and the glucocorticoid receptor (8), and one each for two factors that we provisionally designated PVF and NFA (11). These data have been confirmed and extended by the identification of two or three additional DNase I protections that identify the binding of undefined factors (12, 39, 56). Figure 1 gives a summary model based on these studies. The enhancers of HPV-11 and HPV-18 may be activated by a similar if not identical composition of factors (9, 23). Confusingly, neither our own work nor any other study could pinpoint a factor that binds to HPV DNA in epithelial cells but is absent in cells in which the enhancer is inactive. In particular, the conspicuous motif TTTGGCTT of the enhancers of all genital papillomaviruses and the promoters of cytokeratin genes is recognized by pure NF1 as well as by a factor in HeLa nuclear extracts with the same binding specificity observed for the adenovirus NF1-binding site (12, 26, 27).

The experiments reported here aimed at extending the identification and characterization of transcription factors that bind the HPV-16 enhancer and to assess their functional contribution. Our data do not support the possibility that activity of the HPV-16 enhancer depends critically on factors that bind in epithelial cells but are absent elsewhere. Instead, functional specificity is brought about by transcription factors that are ubiquitous and may stem from a combination of transcription factor synergism, concentration differences of qualitatively identical factors, and func-

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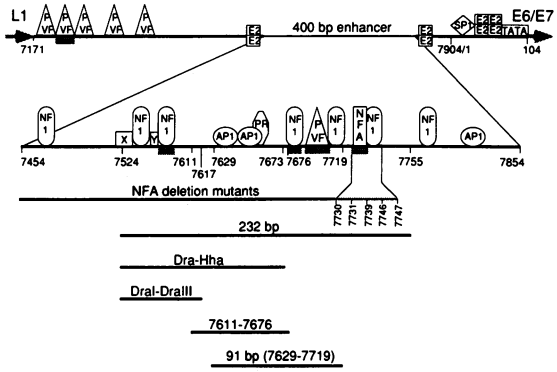

FIG. 1. The HPV-16 LCR. The upper portion and the blow-up of the enhancer region show the relative position of transcription factor-binding sites as previously published (11). The symbols X and Y refer to footprints derived from unidentified factors (12, 39). This paper shows that the PVF motifs are bound by the factor TEF-2 and that the NFA motif is bound by oct-1 and a possibly novel factor, NFA. The lower part of the figure represents deletions and subclones that were examined in this study.

tional variants of factors through means other than their sequence recognition specificity.

MATERIALS AND METHODS

Plasmids and oligonucleotides. The HPV-16 enhancer test plasmids (Fig. 1), as well as the 91-bp enhancer point mutants (see Fig. 4), were described previously (11, 25). The plasmids pORFEXCAT (3) and pSV2CAT (38), which had, respectively, the cytomegalovirus (CMV) enhancer-promoter and the simian virus 40 (SV40) enhancer-promoter driving the expression of the bacterial chloramphenicol acetyltransferase (CAT) gene (28), were used as positive controls in transfection experiments. The construct HPVe/pOVEC (24) was the internal control plasmid in RNase I protection assays. *Bal* 31 deletion generation of the enhancer NFA deletion mutants was done by following established protocols (49). The five deletion mutants were cloned in ptkCATdH/N (53) and sequence verified by the dideoxynucleotide termination reaction (50).

All oligonucleotides (Table 1) used for band shift experiments were synthesized on a Pharmacia gene synthesizer and obtained from B. Li, Institute of Molecular and Cell Biology, Singapore. The genomic positions of the oligonucleotides representing segments of HPV-16 are given in parentheses, and the sequence of the other oligonucleotides were based on published data: globin CAC (15); SV40 GT-1 (20); adenovirus NF1 (17), octamer/heptamer (37); collagenase AP1 (1).

Cell culture and functional assays. All cell lines used to test for enhancer function were grown in minimal essential medium supplemented with 10% fetal calf serum. A total of 14 cell lines were tested for their ability to support HPV-16 enhancer function. They include HeLa, SiHa, CaSki, MCF-7, and NIH 3T3 cells, all used previously in our laboratory (24, 26). HaCat and HD2 cells were a kind gift of N. E. Fusenig (5, 21), and the rest were commercially available: HT-3 (ATCC HTB 32), Colo320HSR (ATCC 220.1), C127 (ATCC 1616), Rat2 (ATCC CRL 1764), F9 (ATCC CRL 1720), Daudi (ATCC CCL 213), and MRHF (Whittaker M. A. Bioproducts). Transient transfections achieved by electroporation with the Bio-Rad Gene Pulser and CAT assays followed procedures standardized in our laboratory (8); 10 μg of DNA was used for CAT assay transfection. Each value of CAT activity represents the average of at least four separate transfections. For analysis of RNA transcripts, in addition to 10 μg of plasmid, 10 μg of HPVe(pOVEC) was cotransfected into HeLa cells by elec-

TABLE 1. Oligonucleotides used for band shift experiments[a]

Name (genomic position)	Oligonucleotide sequence[b]	Size (bp)
HPV-16 PVF 6e (7697–7710)	CTAGATTAGG**CACA**TATTT TAATCC**GTGT**ATAAAGATC	23
HPV-16 PVF 6l (7212–7199)	CTAGACAAG**CACA**TACAAT TGTTC**GTGT**ATGTTAGATC	23
PVF mutant (7697–7199)	CTAGATTAGGatCcTAaTc TAATCCtaGgATtAgGATC	23
GlobinCAC	CTAGAGGAGC**CACA**CCCTT TCCTC**GGTGTG**GGAAGATC	23
SV40 GT-1	CTAGACTTT**CCACAC**CCTT TGAAA**GGTGTG**GGAAGATC	23
HPV-16 NF1 fp6e (7708–7727)	TCGACTCTAGTTT**TTGGCTT**GTTTTAACTAGAG GAGATCAA**AAACCGAA**CAAAATTGATCTCCTAG	37
HPV-16 NF1 fp3e (7577–7596)	TCGACTCTAGTTGCACTGCTT**GCCAA**CCATTACTAGAG GAGATCAACGTGACGA**ACGGTT**GGTAATGATCTCCTAG	42
Adenovirus NF1	AATTCTTATT**TTGG**ATTGA**AGCCAA**TAATCG GAATAA**AACC**TAACTT**CGGTT**ATTAGCTTAA	35
HPV-16 NFA (7728–7741)	CTAGACCT**AATTGCAT**AT TGGA**TTAACGTA**TAGATC	22
HPV-11 NFA	CTAGAGT**TAAAAGCA**TTT TCAA**TTTTCGTA**AAGATC	22
Octamer-heptamer	CTAGATG**CTCATGA**AT**ATGCAAAT**CAATTGT TAC**GAGTACTTA**T**ACGTTTAG**TTAACAGATC	35
Collagenase AP1	CTAGATATAAAGCA**TGAGTCA**GACACCTCTT TATATTTCGT**ACTCAGT**CTGTGGAGAAGATC	35

[a] Published DNase I protection experiments (26, 27) have identified the following footprints: HPV-16 PVF 6e and PVF 6l, 3 bp 5′ and 3 bp 3′ of the CACA motif; HPV-16 NF1 fp6e, the TTTGGCTT motif plus 7 bp in the 3′ direction; HPV-16 NF1 fb3e, 7 bp 3′ and 3 bp 5′ of the TTGGC motif (lower strand).

[b] Conspicuous sequence motifs believed to be relevant for binding specificity are underlined.

troporation. The isolation of total RNA by the guanidium isothiocyanate method, with a subsequent CsCl centrifugation step, and the generation of SP6 polymerase-synthesized RNA probe followed by the analysis of the transcripts obtained were performed as detailed previously (24). A 10-μg portion of total RNA was used for each reaction in the RNase protection assay.

Footprint and band shift procedures. Nuclear extracts were prepared by following published protocols (18). Probes for footprint reactions (22) were generated by filling in a polylinker site of the ptkCATdH/N constructs that carried the prototype and mutant 91-bp HPV-16 enhancer fragments. The end-labeled DNA probe thus generated was gel purified and incubated with HeLa nuclear extracts for DNase I footprint titration performed as previously published by us (24). Annealed oligonucleotides for band shift assays were labeled with ^{32}P by filling in the protruding ends with Klenow polymerase and subsequently purified via gel electrophoresis. For the band shift assay the incubation mixture and incubation conditions were similar to those for footprint reactions. The band shifts were carried out by using a previously established procedure from our laboratory (7): 1 ng of labeled DNA (approximately 5,000 cpm Cerenkov) were band shifted, and competitions with homologous or heterologous oligonucleotides were done with a 100-fold excess of unlabeled DNA.

RESULTS

The HPV-16 enhancer and nonoverlapping enhancer subclones are active in epithelial cells but inactive in fibroblasts. It had been proposed that the HPV-16 enhancer is active in cell lines derived from cervical carcinomas, or even generally in keratinocytes, but that it is inactive in cell lines of other differentiation types (13, 25). We initiated a systematic study of several enhancer subclones in the hope of getting an indication of the specific elements in this enhancer which may be decisive for epithelial or keratinocyte specificity.

We use here the word "keratinocyte" synonymously with "epithelial cell," although we give preference to the latter term. The former term is often restricted to cells derived from squamous epithelia or, even more specifically, to those from the epidermis, but has been used in the papillomavirus literature to identify cells that express keratin, thus encompassing columnar and simple epithelia.

We selected a total of 14 cell lines as recipient cells. Of epithelial origin were SiHa, CaSki, and HeLa cells. These are derived from cervical carcinomas and contain transcriptionally active papillomavirus genomes recombined into their chromosomes (2, 54). They have the desirable property of being the most appropriate environment for papillomavirus transcription, but the undesirable property of expressing papillomavirus gene products with suspected transcriptional activities, which may alter enhancer properties, namely E6 and E7 (33, 43). To exclude this possibility, we included HT-3, which is derived from a cervical carcinoma and is free of endogenous papillomavirus genomes. MCF-7 cells, derived from a carcinoma of the mammary gland, were included, although they are not considered to be a natural target cell of papillomavirus infection, since they have a keratin gene expression pattern similar to that of cervical epithelia (42). HaCat cells represent a line derived from a cornifying human epidermis (5), and HD2 is a similar differentiation type from the mouse (21). The latter cells could reveal species specificity. A species-specific transcriptional element has been identified in bovine papillomavirus type 1 (58), although it does not seem to have a functional equivalent in the enhancer of genital HPVs. The cell line Colo320HSR was included to examine whether we would find HPV enhancer activity in this type of keratinocyte, which, just like mammary gland epithelia, has not been found to be naturally infected by HPV. As cell lines of nonepithelial origin, we used MRHF fibroblasts and Daudi Burkitt lymphoma cells from humans, mouse c127 and NIH 3T3 fibroblasts, Rat2 fibroblasts, and mouse F9 embryonal carcinoma cells.

This screen of cell lines for HPV-16 enhancer activity was done with expression vectors for the bacterial test gene coding for CAT (28). The HPV-16 enhancer constructs used were the full-sized 400-bp fragment (genomic positions 7454 to 7854), a 232-bp subclone (positions 7524 to 7755), and a 91-bp fragment, which was the smallest fragment retaining significant enhancer activity and containing a composition of transcription factor-binding sites that seems to be typical for HPV enhancers, namely two sites for NF1, two for AP-1, and one for PVF (11).

These fragments were cloned into the vector ptkCATdH/N. The CAT gene of this vector is expressed from a herpes simplex virus thymidine kinase promoter. By itself, this vector gives a very low and sometimes undetectable activity. Consequently, enhancer strength was not calculated as an induction ratio relative to the baseline of this vector, but as a fraction of the activity of a strong cytomegalovirus enhancer-promoter, which is considered to be ubiquitously active (6). As well as such a reference vector, termed pORFEXCAT (3), we transfected as an additional reference the plasmid pSV2CAT (38), which contains the supposedly ubiquitously active SV40 enhancer.

Figure 2 summarizes the outcome of these experiments. In all cell lines, CAT activity was highest under the influence of the CMV enhancer in pORFEXCAT, whose corresponding CAT values were set for each cell line as a 100% reference point. pSV2CAT shows considerable quantitative variability from 44% of pORFEXCAT in HeLa cells down to 1.3% in MRHF cells, but without qualitative preference for a differentiation type. It should be noted that the latter 1.3% activity translated to a more than 10-fold CAT stimulation when viewed independently of the activity of pORFEXCAT. In epithelial cells the 400- and 232-bp fragments of the HPV-16 enhancer showed enhancement of 1.1 to 19.5% relative to the CMV enhancer, but were practically inactive in fibroblasts. In this comparison some of these levels appear to be low, but in absolute numbers they still represent a 10- to 100-fold induction over the basal level (Fig. 2). Interestingly, the 91-bp fragment shows a similar activity range, although its activity is quantitatively reduced. The figure does not contain our findings for HeLa and CaSki cells, which were similar to those with SiHa cells, and for Colo320HSR cells, which resembled the HaCat data. The data obtained with Daudi and F9 cells resembled those for the four fibroblast lines, namely activity of the CMV and the SV40 enhancers with lack of activity of the three HPV-16 constructs. We conclude that the HPV-16 enhancer and its subclones are specific for epithelial cells irrespective of the species and the histologic origin. Neither deletion of nearly 80% of the 400-bp enhancer nor any one of numerous other deletions not shown here (10) results in a gain of ubiquitous activity. This excludes the possibility that cell type specificity is brought about by negative regulatory elements.

We next asked whether the 91-bp fragment may be the only subclone with elements relevant for cell type specificity or whether parts of the strong 232-bp enhancer clone that are

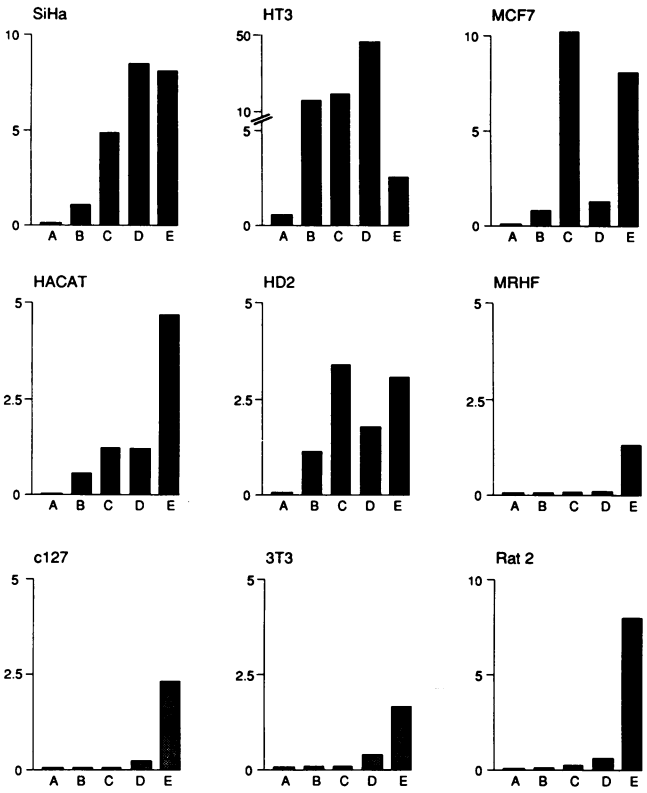

FIG. 2. Activity of three different HPV-16 enhancer clones in five epithelial cell lines (black bars) and four fibroblasts (gray bars). The diagram represents CAT expression relative to the one induced by a CMV promoter-enhancer vector and is expressed as a percentage of the activity obtained with this vector. The constructs tested were enhancer-free vector ptkCATdH/N (A), HPV-16 91-bp segment (B), HPV-16 232-bp segment (C), HPV-16 400-bp segment (D), and pSV2CAT (E). Although enhancer activities are generally lower than those obtained with the CMV vector, it is clear that the SV40 vector activates CAT expression in epithelial and fibroblast cells alike, whereas the HPV-16 clones give significant activity only in epithelial cells.

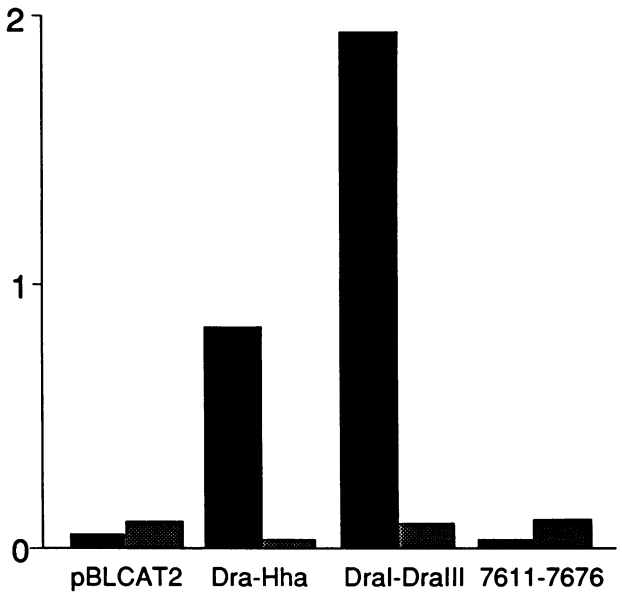

FIG. 3. Keratinocyte-specific enhancer activity of a 94-bp HPV-16 enhancer fragment (*Dra*I-*Dra*III) that is located 5′ of and without overlap with the 91-bp core enhancer examined in Fig. 2. The inactive fragment from 7611 to 7676 contains two of the three AP-1 sites of the HPV-16 enhancer; the keratinocyte-specific clone *Dra-Hha* represents a contiguous segment of the enhancer containing both the *Dra*I-*Dra*III and the 7611 to 7676 enhancer. The enhancer subfragments are graphically represented in Fig. 1. Symbols: ■, HeLa; ▨, MRHF.

not present in this 91-bp segment may also contain specific functions. We examined a 94-bp segment 5′ of the 91-bp enhancer between a *Dra*I site at position 7524 and a *Dra*III site at position 7621. This segment (positions 7524 to 7617) has two NF1 sites as common elements with the 91-bp fragment (26) (Fig. 1) and may also bind two additional yet undefined factors (12, 39) (Fig. 1). Figure 3 shows that this fragment, as well as a larger one (*Dra-Hha*, positions 7524 to 7673) that overlaps with the 91-mer and thus includes the two AP-1 sites, shows cell type-specific activity. However, a fragment from positions 7611 to 7676, which contains only the two AP-1 sites but no NF1 sites, does not function as an enhancer. Hence, at least two, if not several, modules of the enhancer must be responsible for its epithelial preference.

The 91-bp HPV-16 enhancer is synergistically activated by AP-1, NF1, and PVF in the absence of cooperativity in DNA binding. We have shown above that the 91-bp subfragment of the HPV-16 enhancer has reduced but still cell type-specific transcriptional activity. Numbers of important transcription factor-binding sites on this fragment in addition to a glucocorticoid-progesterone response element, are two for AP-1, two for NF1, and one for a factor binding the novel motif AGGCACATAT, which we previously termed PVF (11).

We have published CAT expression data that show that enhancer function of this 91-bp fragment is dependent on these three types of binding sites. To examine the role of these factors in enhancer activation, we decided to determine first the transcript level under the influence of these mutations and second the alterations that occurred in binding of these factors upon mutation. To determine the CAT mRNA levels, we performed the RNase protection experiments whose results are shown in Fig. 4. The nearly complete loss of transcriptional activity by each of these mutants

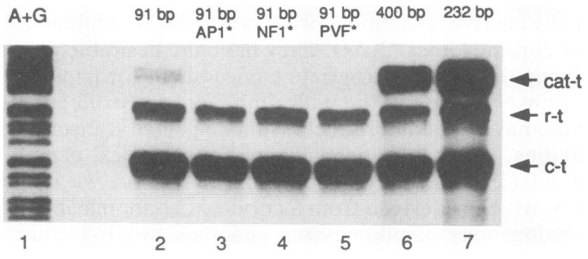

FIG. 4. Transcriptional analysis of HPV-16 enhancer mutants. Lanes 2, 6, and 7 document the relative activities of the 91-bp, the 400-bp and the 232-bp subclones, respectively; lanes 3, 4, and 5 document the loss of enhancer activity of the 91-bp fragment after alternative mutation of the AP-1, the NF1, or the PVF motif, respectively. Lane 1 represents is sequencing lane used as a size marker. The transcripts r-t and c-t were derived from the contransfected internal control plasmid HPVe(pOVEC) (24), while cat-t represents the CAT transcripts from the test constructs.

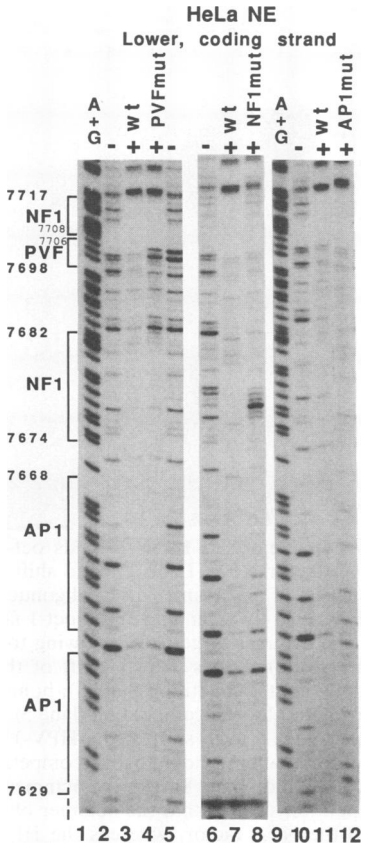

FIG. 5. DNase I protection analysis of the HPV-16 91-bp enhancer fragment with the wild-type sequence and alternative mutations in the PVF, NF1, or AP-1 motifs reveals lack of cooperativity in the binding of heterologous factors. Upon mutation, protections are reduced or disappear without influence on unrelated elements. A+G is the reference sequencing lane. The symbols − and + refer to DNase I cleavage in the absence and presence, respectively, of HeLa nuclear extract.

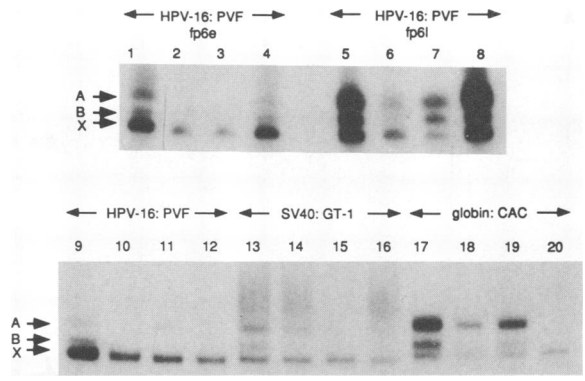

FIG. 6. Identity of the factor that binds the HPV-16 PVF motif of the enhancer, the HPV-16 PVF motifs in the 5′ LCR, the SV40 GT-1 motif, and the globin CAC box, and its identification as TEF-2. Bands A and B were specifically inhibited, whereas band X was partially resistant to competition by all oligonucleotides, indicative of an undefined specific factor or an unspecific activity. Upper panel: band shift of the HPV-16 PVF motif AGGCACATAT of the enhancer (fp6e) and AAGCACATAC at the 5′ segment of the LCR (fp61). Lanes 1 and 5, no competition; Slot 2 and 6, homologous competition; lanes 3 and 7, competition with the respective heterologous oligonucleotide; lanes 4 and 8, competition with a mutant oligonucleotide (Table 1). Lower panel: lanes 9, 13, and 17, band shift of the HPV-16 PVF oligonucleotide the SV40 GT-1, and the globin CAC box oligonucleotide, respectively; lanes 10, 14, and 18, competition with the PVF oligonucleotide; lanes 11, 15, and 19, competition with the SV40 GT-1 oligonucleotide; lanes 12, 16, and 20, competition with the globin CAC box oligonucleotide.

suggests synergistic activation by the combination of these three factors.

To examine whether functional cooperativity is brought about by cooperative binding, we examined the 91-bp fragment in a footprint study. Figure 5 shows that the three types of mutations exclusively eliminate binding of the corresponding factor, while binding of the other factors is retained. We conclude that synergism is brought about by functional cooperativity rather than cooperativity in the binding of the factor (34).

The PVF motif binds the factor TEF-2. The activity of the 91-bp clone occurred cell type specifically, although two of the factors that activate this enhancer, namely NF1 and AP-1, are not considered to be cell type-specific factors. To look into the properties of the third element, previously termed PVF, we examined the factor that binds this element.

The PVF motif has the sequence AGGCACATAT in HPV-16, HPV-18, and HPV-33, and related motifs occur in HPV-6, HPV-11, and HPV-31 (11). These elements have the sequence CACA in common, which is reminiscent of the CCACACCC promoter element of globin genes and of the GT-I-binding sites, which has the same sequence in the SV40 enhancer. It has been shown that both sequences bind the same factor, TEF-2 (14, 15, 20). To examine whether the PVF motif binds TEF-2, we performed band shifts with oligonucleotides representing these three sequences. We included in this study an oligonucleotide with the sequence AAGCACATAC (Table 1, HPV-16 PVF 61), which is repeated five times outside the enhancer at the 5′ end of the HPV-16 long control region (LCR) (26). Figure 6 shows that each of these four oligonucleotides gives bands at similar positions and that bands A and B are eliminated by homologous as well as heterologous competition with suspected or proven TEF-2-binding sites. We conclude that the HPV-PVF motifs bind factor TEF-2. An additional band, X, differed in relative intensity to A and B depending on the oligonucleotide used and may represent an additional undefined specific factor or a nonspecific DNA binding activity.

The cytokeratin-octamer TTTGGCTT of HPV-16 binds a factor with properties indistinguishable from the adenovirus NF1 site. We have previously shown that the two NF1-like binding sites that strongly activate the 91-bp fragment bind pure NF1 of heterologous origin and that binding of the corresponding NF1-like factor in HeLa extracts is inhibited by the bona fide NF1 site of adenovirus (see Table 1 for sequences) (27). The uniqueness of one of these two motifs, TTTGGCTT (positions 7710 to 7717), which occurs in all enhancers of genital papillomaviruses and in the promoter of cytokeratin genes (4), has repeatedly led to the hypothesis that this motif can bind a factor different from NF1. To elucidate whether this motif binds a factor different from other NF1-binding sites in HeLa cells, we analyzed this motif against another, less conspicuous NF1-binding site of HPV-16 and against the adenovirus NF1 site in a band shift experiment. Identical band shifts and complete heterologous competition (Fig. 7) exclude that this motif binds a factor differing from that bound by the other two NF1-binding sites, an observation that confirms the results of a similar

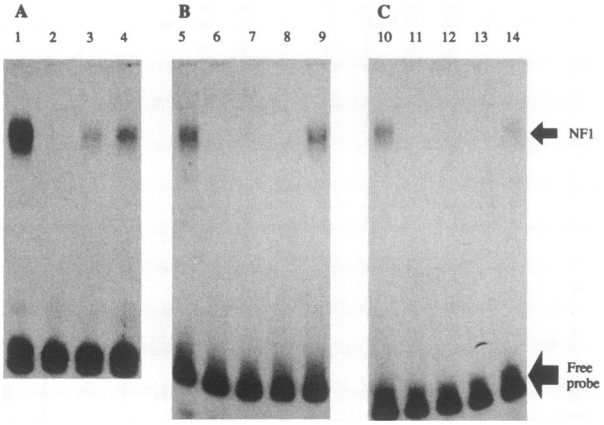

FIG. 7. The HPV-16 enhancer motif TTTGGCTT, a different HPV-16 NF1 motif, and the adenovirus NF1 site bind the same factor in HeLa nuclear extracts. (A) Adenovirus sequence. (B) HPV-16 fp3e motif (26). (C) HPV-16 fp6e TTTGGCTT motif. Lanes 1, 5, and 10 show the shift with HeLa nuclear extracts. The adenovirus shift can be completely inhibited by its homologous sequence (lane 2) and strongly (but incompletely, owing to the lower affinity [30]) by the two HPV-16 motifs (lane 3, fp3e; lane 4, TTTGGCTT motif). Binding to the two HPV-16 motifs can be completely inhibited by the adenovirus sequence (lanes 6 and 11) or the two HPV-16 motifs (lanes 7, 8, 12, and 13). Lanes 9 and 14 show lack of competition with a nonspecific PVF oligonucleotide.

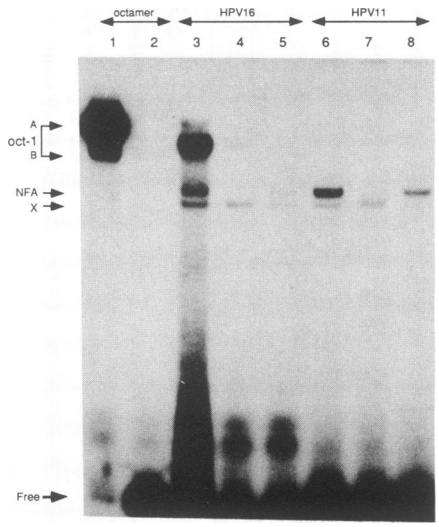

FIG. 9. The NFA motif of HPV-16 binds oct-1 as well as the potentially novel factor NFA. Lane 1: band shift and homologous competition (lane 2) of an octamer motif oligonucleotide; bands A and B are both derived from binding of the oct-1 factor (B owing to recognition of the octamer target site, A owing to recognition of a flanking heptamer site). Lanes 3 to 5: shift of the HPV-16 NFA oligonucleotide without competition (lane 3), homologous competition (lane 4) and heterologous competition (lane 5) with the octamer oligonucleotide. Lanes 6 to 8: shift of the HPV-11 oligonucleotide without competition (lane 6), homologous competition (lane 7) and heterologous competition (lane 8) with the octamer oligonucleotide. Shift and competition suggest that the octamer oligonucleotide has little affinity to the NFA factor, whereas the HPV-11 site has no affinity for the oct-1 factor. All experiments were done with HeLa nuclear extracts.

experiment by Cripe et al. (12). Although NF1 sites participate in the activation of the cell type-specific enhancer, they do not seem to bind a factor different from the bona fide adenovirus NF1 site.

A motif with flanking binding sites for NF1 and oct-1/NFA is involved in enhancer function. Outside of and 3′ to the 91-bp segment, the HPV-16 enhancer contains a motif typical for the enhancers of at least six genital HPVs, namely a nonpalindromic NF1-binding site that is spaced by exactly 2 bp from a consensus element that we termed NFA (11). In HPV-16 the NFA site has the sequence ATGCAATT (lower strand, positions 7739 to 7732), which is similar to the octamer sequence, the binding site for the ubiquitous oct-1, or the lymphocyte-specific oct-2 factor, ATGCAAAT. The consensus for this NFA motif diverges, however, in other papillomaviruses such as HPV-6 and HPV-11, in which it is ATGCTTTT (11).

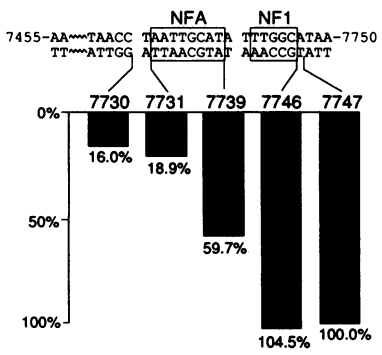

FIG. 8. Partial loss of enhancer activity during stepwise deletion of an NF1 and a flanking NFA motif characteristic of genital papillomavirus enhancers. Clone d7747 was set as a reference point (100%), after it was found to have a nearly unchanged activity relative to the 400-bp enhancer, although it was deleted for a segment of 107 bp, which contained one NF1- and one AP-1-binding site. CAT activities were determined after transient transfection of HeLa cells.

To examine the contribution of this element to enhancer function, we created *Bal* 31 deletions from the 3′ side of the 400-bp fragment, which ended at positions 7747, 7746, 7739, 7731, and 7730. The first two, d7747 and d7746, left the NF1 and NFA motifs intact, d7739 completely deleted the NF1 site but left the NFA site intact, and d7731 and d7730 deleted the NFA motif. To avoid distance effects in the functional tests, we cloned these mutants into ptkCATdH/N in inverted orientation such that the natural 5′ end of the enhancer at position 7454 was fused to the 5′ end of the thymidine kinase promoter-CAT fusion gene. This was done to place the deletions at a constant distance of about 280 bp from the 5′ side of the test promoter.

Figure 8 shows that deletion of the NF1 site decreases enhancer function by a factor of 2, and loss of the NFA as well as of the NF1 site decreases enhancer function by a factor of 6. We conclude that these two motifs contribute to the HPV-16 enhancer function.

We next examined some of the properties of the factors that binds the NFA motif. Figure 9 shows a band shift with oligonucleotides representing the bona fide octamer motif, the HPV-16 motif, and the HPV-11 NFA motif. The octamer oligonucleotide gives two bands typical for sites that contain the octamer as well as the heptamer motif (32). The HPV-16 oligonucleotides shows one of these bands, namely the one which is obtained with oct-1-binding sites that have only the

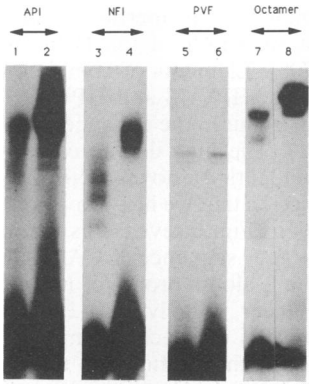

FIG. 10. Band shift of HPV-16 AP-1, NF1, and PVF oligonucleotides and an oligonucleotide representing the octamer-binding site with human MRHF fibroblast (lanes 1, 3, 5, and 7) and HeLa nuclear extracts (lanes 2, 4, 6, and 8). Relative to the PVF shift, which was fortuitously of similar intensity to either extract, fibroblast extracts gave generally a weaker signal. Qualitative differences were observed only for the NF1 oligonucleotide, which, in the absence of any degradation of the AP-1, PVF, and oct-1 factors, may represent qualitative differences without changes of binding specificity. The apparent quantitative difference between lanes 7 and 8 (oct-1 octamer/heptamer oligonucleotide) has to be interpreted as quantitative difference, since the upper band in lane 8 is indicative of binding to the heptamer site, which occurs only at high concentrations of oct-1.

octamer motif but not the flanking heptamer. This band was completely competed with the oct-1 binding-site oligonucleotide. This observation makes it very likely that the HPV-16 NFA motif binds the factor oct-1.

In addition, the HPV-16 oligonucleotide shows a band of higher mobility that was not observed with the oct-1 oligonucleotide and that was not completely inhibited by the oct-1 oligonucleotide. This same band, but not the oct-1 bands, is visible in the HPV-11 band shift. We conclude that the NFA motif binds the oct-1 factor in HPV-16, but that it also binds a possibly novel factor in HPV-16 and that it exclusively binds this novel factor in HPV-11. We term this factor NFA (NF1-associated factor), in keeping with the nomenclature that we proposed for the binding site. Since our functional studies are restricted to HPV-16, we cannot answer the question whether the observed reduction of enhancer activity is due to oct-1 or NFA, or both factors.

Qualitative and quantitative differences of factors involved in HPV-16 enhancer function. Previous publications and the experiments reported here show that TEF-2, NF1, AP-1, and oct-1/NFA activate the HPV-16 enhancer selectively in epithelial cells, although they can be detected in DNase I protections in a similar manner in fibroblasts. However, these experiments were not designed to reveal potential qualitative or quantitative differences between these factors. This possibility was approached by using band shift experiments that compared the different properties of nuclear extracts from HeLa and MRHF cells (Fig. 10).

Qualitatively, identical band shifts were observed in both cell types with TEF-2, AP-1, and oct-1/NFA-binding sites from HPV-16. With awareness of the limited analytical power of this procedure, we propose that TEF-2, AP-1, oct-1, and NFA occur in each of these cell types as transcription factors with similar properties. This was also observed for NFA, which was visible only after longer exposures than the one shown in Fig. 10. Although band shifts cannot be reliably quantified, we observed that the TEF-2 band occurred in both cell types in comparable strength. Relative to this band, AP-1, NF1, and oct-1 gave a significantly stronger band shift in HeLa cells. We hypothesize that these quantitative differences may play an important role in the apparent cell type specificity.

The NF1-binding site gave only a weak signal with fibroblast extracts at the position corresponding to the strong band shifted in HeLa, but, more significantly, it shifted several bands of higher mobility. Since none of the other band shifts are indicative of proteolytic degradation, we believe that part of NF1 can occur in fibroblasts in a form qualitatively different from that in HeLa cells.

DISCUSSION

Transcription from the E6 promoter P97 of HPV-16 not only is regulated by products of the viral E2 gene by a feedback mechanism (see reference 47 and references therein), but also depends on an enhancer that is activated by cellular transcription factors. All genital HPVs share a similar structural organization of functional enhancer elements (11). The HPV-16 enhancer has been proposed to be selectively active in keratinocytes (13), whereas we had found a cloned 400-bp LCR segment (positions 7454 to 7854) to be active in HeLa cells but inactive in the mammary carcinoma line MCF-7 (25), even though both cell lines were keratinocytes. On reexamination of this observation (Fig. 2), we found that this clone has a reduced but still significant activity in MCF-7 cells, whereas a 232-bp subclone (positions 7524 to 7755) has an activity in MCF-7 cells similar to that in cervical carcinoma cells. The HPV-16 enhancer is thus, as proposed, generally active in keratinocytes, even in cells originating from tissues in which HPV infection does not occur naturally. The enhancer is also independent from the presence or absence of HPV genomes in the recipient cell and independent of the species of origin of the cells. Dissection of the two previous enhancer clones revealed that the cell type-specific function of the HPV-16 enhancer is retained by a 91-bp fragment (positions 7629 to 7719). We have shown this segment to be the smallest enhancer unit with significant function, possibly as a result of a particular combination of AP-1-, NF1-, and TEF-2-binding sites. Cripe et al. (12) had proposed a nearly identical segment as a core enhancer (positions 7631 to 7718). A 66-bp clone (positions 7611 to 7676) deleted for NF1 and TEF-2 binding does not show any function (Fig. 3); likewise, a fragment encompassing these two types of sites but none for AP-1 (positions 7675 to 7755) (11) was not functional as an enhancer. Deletions shown in this paper and published elsewhere (10) did not lead to a gain of ubiquitous enhancer activity. Therefore, we do not believe that a silencer element is involved in bringing about cell type specificity.

Synergistic activation by unlike factors is suggested by these deletions and also by point mutations of the minimal enhancer unit. Footprint experiments show that alternative mutations eliminated binding of NF1, TEF-2, or AP-1 without having significant influence on binding of the other factors. Nevertheless, most or all enhancer activity is lost upon mutation of either type of transcription factor-binding site. If one views the difference between mutant and wild-type minimal enhancers as a gain of function, it could also be argued that addition of AP-1, NF1, or TEF-2 sites to an unfunctional fragment containing the other two types of sites is creating an enhancer that is active in HeLa cells but still inactive in fibroblasts.

The experiments whose results are illustrated in Fig. 3 show that this minimal enhancer is not the only segment with elements critical for epithelial specificity, but that a fragment between positions 7524 and 7617 (no overlap with the fragment from positions 7629 to 7719) also exhibits this specificity. Interestingly, this fragment does not contain an AP-1 site but does contain two NF1 sites (fp2e and fp3e in reference 26). The activity of this fragment may depend on synergistic activation by these NF1 factors, with unidentified factors revealed by DNase I protections (12, 39, 56).

Another facet of our study was to elucidate qualitative and quantitative properties of the transcription factors that activate the HPV-16 enhancer. We had previously identified binding sites for NF1, AP-1, and glucocorticoid-progesterone receptors and assigned the terms NFA and PVF to two motifs that overlap in HPV-16 with footprints and appear as consensus elements in all genital HPV enhancers. The NFA motif in HPV-16 has the sequence ATGCAATT (positions 7739 to 7732), which is similar to the sequence ATGCAAAT, the binding site of the ubiquitous transcription factor oct-1, and the lymphocyte-specific factor oct-2 (32, 45). However, in other papillomaviruses the sequence diverges, e.g., to ATGCTTTT in HPV-11. The octamer motif of the heavy-chain immunoglobulin enhancer forms two bands with the nuclear extracts designated A and B in Fig. 9, and the HPV-16 NFA oligonucleotide led to a specifically inhibited shift that coincided with band B. We conclude that this HPV-16 sequence binds the factor oct-1. The lack of band A was expected: it is known that this shift is due to the low-affinity binding of a second oct-1 protein to sites that have in proximity to the octamer sequence a heptamer consensus element (32, 45), a sequence present on the octamer oligonucleotide but absent from the HPV-16 NFA motif.

In addition to the oct-1 shift, the HPV-16 oligonucleotide shows another higher-mobility shift that is barely visible with the octamer oligonucleotide. Although this band may be specifically inhibited by the homologous oligonucleotide, it is only partially inhibited by the octamer oligonucleotide. Interestingly, the HPV-11 NFA oligonucleotide shows this same high-mobility shift, but no oct-1 binding. Similar findings (data not shown) were obtained with the potential HPV-31 NFA sequence CTGCAATC (11). These experiments indicate that in HPV-16 and HPV-11 the NFA site binds a factor different from oct-1, whose binding site specificity diverges from the octamer motif. We propose to continue to use the name NFA for this factor. This term NFA (for NF1 associate) had been coined in reference to the constant distance of the NFA motif from a single NF1 site. On examination of oligonucleotides containing the HPV-16 NFA site as well as the flanking NF1 site, we did not detect interactions between oct-1 and NF1 (10). However, owing to the intensity of the oct-1 band, these experiments did not allow the study of potential interactions between NFA and NF1, a question that must be readdressed by using the NFA site of HPV-11. In a similar manner, HPV-11 should be used in future experiments to reexamine whether the functional contribution of this motif as revealed by our deletions is brought about by the oct-1 or the NFA factor, or both of them. In light of the widespread occurrence of this NFA-NF1 motif and the absence of oct-1 binding in HPV-11, NFA is likely to bring about a function important for genital HPV biology. Possibly, NFA is identical to a factor recently studied by Royer et al. (48) that is supposed to shuttle between the nucleus and the cytoplasm in a cell cycle-dependent manner. NFA may be a tool of HPV to couple its transcription to the cell cycle.

We also examined the properties of the factor that binds the motif AGGCACATAT of the HPV-16 enhancer (positions 7699 to 7708). Elements of complete consensus occur in the enhancers of HPV-18 and HPV-33, and elements of partial consensus occur in the enhancers of HPV-6, HPV-11, and HPV-31 (11). The high degree of conservation of this motif seemed to identify a novel factor involved in papillomavirus transcription that we had provisionally termed PVF. The experiments in Fig. 6 reveal this factor to be the same one that binds the sequence CCACACCC, termed the CACA box of the β-globin promoter (15), or the GT-1 motif of the SV40 enhancer, namely TEF-2 (20). The positions of bands and the results of specific competitions suggest that TEF-2 binds with highest affinity to the globin CACA box and the HPV-16 motif AAGCACATAT in the 5′ part of the LCR and with lower affinity to the SV40 GT-1 motif and the HPV-16 motif AGGCACATAT in the HPV-16 enhancer. Differences in intensity and capacity for inhibition of band X in Fig. 6 leave open the possibility that an additional undefined factor with specific or nonspecific DNA-binding properties recognizes these oligonucleotides.

Interestingly, the related element AAGCACATAC occurs in HPV-16 five times downstream of the 3′ end of the L1 open reading frame. A functional examination of this latter segment has not revealed any enhancer function (10). This finding is not surprising since TEF-2 had been previously characterized as a factor which does not include enhancer activity after polymerization of its binding site but, rather, needs heterologous factors for cooperation (20).

The simplest explanation for cell type-specific transcription of any particular gene would be if a transcriptional element was found to bind a factor from nuclear extracts of only those cells that support promoter function but was unable to bind a factor in other cells. Unexpectedly, this plausible regulatory mechanism is rarely found: an example is the dependence of the hepatitis B virus enhancer on factors dependent on the differentiation stage of liver cells (55). There is no hint that this type of mechanism is operative in HPV-16 enhancer activation as no overt cell-specific element was found: DNase protection experiments revealed identical footprints with keratinocyte as well as fibroblast extracts. More significantly, a fragment of the 400-bp HPV-16 enhancer (91-bp segment) retained the cell specificity of the larger fragment, and function of this fragment was wholly dependent on the ubiquitous factors AP-1, NF1, and TEF-2, as there was no enhancer activity with loss of binding of these factors.

More often, cell type-specific transcription has been found to be induced by a factor with specificity for a site that is normally recognized by a ubiquitous factor. Examples are the B-cell-specific oct-2 factor, which activates the immunoglobulin G enhancer B cell specifically although it binds to the same site as the ubiquitous oct-1 factor (32, 45). A similar mechanism may exist in the liver-specific albumin promoter, whose D-site is bound by c/EBP, DBP, and LAP, which exert different functions (see reference 16 and references therein).

Since our data supported a view that specificity of the HPV-16 enhancer occurs under the influence of *cis*-responsive elements that bind ubiquitous factors, we used band shift experiments to examine possible cell type-specific differences between factors responsible for these ubiquitously observed DNase protections. No qualitative differences could be found for AP-1, TEF-2, oct-1, and NFA (data not shown for NFA). These factors may therefore occur in similar, if not identical, form in keratinocytes and fibroblasts. It is possible, however, that small differences derived from differential splicing, posttranslational modifications, or

the use of alternative subunits such as those of the *jun* and *fos* gene family would evade our analytical tools but result in functional differences.

For NF1, we observed only a shift of low intensity with fibroblast extracts corresponding to the strong shift obtained with HeLa extracts, whereas several complexes occur with fibroblasts at higher mobility. These complexes were observed with two independent nuclear extract preparations from actively dividing human MRHF fibroblasts. Some of the NF1 factor population in human fibroblasts might therefore occur in a form different from the one found in HeLa cells, a possible correlation with functional differences. Our experiments do not rigorously exclude the possibility that these different forms of NF1 originated through proteolytic degradation. However, such a degradation would have to be selective for NF1, since no alteration of AP-1, TEF-2, or oct-1 was seen. NF1-CTF mRNAs that varied as a result of alternative splicing have been observed (51), and cell type-specific differences of the NF1 factor population have been reported (29), but neither of these phenomena has yet been correlated with functional differences. Posttranscriptional down-regulation of NF1 transcription factor activity has been recently proposed as a mechanism involved in modulating feline leukemia virus promoter function (44).

Alternatively, contributions to cell type-specific functions may derive from quantitative cell type-specific difference which lead—in particular through synergism of factors that occur in different quantities—to apparently qualitative differences. A concentration-affinity concept was put forward (31) after proof that a combination of concentration of factor and varying affinity of the binding site was decisive whether or not a particular element was functional. Low-affinity binding sites require a higher concentration of factor to function. If such a mechanism is operative for the HPV enhancer, it might explain the somewhat surprising observation that 34 of 35 NF1-binding sites in the enhancers of six genital HPVs occur as nonpalindromic half-binding sites (27), which are believed to have a lower affinity for the dimeric NF1-CTF factor relative to palindromic elements (30). To get quantitative estimates of transcription factors, we made an effort to quantify band shifts, being aware of the problem that parameters such as contaminations of the nuclear contents with cytoplasm may alter factor concentrations in an uncontrolled manner. Fortuitously, the factor TEF-2 yielded shifts of similar intensity with HeLa and MRHF extracts. Relative to this baseline, the concentrations of AP-1, NF1, and oct-1 were significantly increased in HeLa cells relative to fibroblasts. A recent proposal for an epithelial cell-specific AP-1-binding site (41) may have to be reinterpreted in the light of the finding, in particular, since that study did not examine the protection of any bona fide AP-1 site differentially.

Several speculative aspects of HPV enhancer function remain recalcitrant to a satisfactory examination. First, transcription of the HPV genome in different layers of the epithelium is likely to be different from the situation in cervical carcinoma cell lines such as SiHa or CaSki. Second, cell type-specific transcription might be influenced by transcriptional coactivators that modulate communication between transcription factors and the transcription initiation complex. Recently published results have led to the inference that such a factor is involved in SpI-dependent transcriptional activation (46). Lastly, it is not known whether the in vitro DNA-binding studies of HPV enhancers are a valid model for the situation in vivo: a number of transcription factors, for example some involved in immunoglobulin G enhancer activation, seem to be ubiquitous but seem to bind their target sites only in B cells (35), possibly owing to regulation of the transport into the nucleus (40). Although one could perform in vivo footprint experiments of the HPV-16 enhancer in SiHa or CaSki cells, it may be impossible to define an appropriate negative control: a fibroblast line with transfected HPV genomes may not activate papillomavirus transcription for reasons other than lack of enhancer specificity.

In summary, we interpret our data such that keratinocyte-specific transcriptional activation is brought about mostly, if not exclusively, by means of transcription factor-binding sites that are recognized by ubiquitous factors. This may occur by a combination of synergism, varying factor concentrations, and qualitative differences of some factors that are not determined in experiments that address their sequence specificity.

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