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Transcriptional activation of human papillomavirus 16 by nuclear factor I, AP1, steroid receptors and a possibly novel transcription factor, PVF: a model for the composition of genital papillomavirus enhancers

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ABSTRACT

Human papillomavirus 16 (HPV-16), which is involved in genital carcinogenesis, contains an enhancer of transcription that is activated by cellular factors rather than by the viral E2 proteins. The activity resides on a 232 bp segment with 5 binding sites for nuclear factor 1 (NF1), 2 for AP1, and 1 for steroid receptors. Deletions and point mutations show that the constitutive enhancer and the steroid response depend on NF1 sites located 5' or 3' of a 65 bp fragment with AP1 sites that by itself shows little activity. Enhancement through a fragment with AP1 and NF1 sites is strongly reduced by mutation of the AP1 sites, or by mutation of the sequence AGGCACATAT. Sequence comparison and footprint analysis make it likely that this sequence binds a novel transcription factor which we call PVF. Fragments with one or several binding sites only for NF1, or AP1, or PVF exhibit little enhancement by themselves, suggesting the functional dependence of the HPV-16 enhancer on the cooperation of these factors. A comparison of our findings with the genomes and transcription factor binding sites of HPV-6, 11, 18, 31 and 33 lead us to propose a model of the composition of enhancers of genital papillomaviruses.

INTRODUCTION

Human papillomavirus-16 (HPV-16) and several related papillomaviruses infect mucosal epithelia and cause neoplastic lesions that may progress towards cancer (1). A segment of 850 bp of the 7904 bp genome of HPV-16 termed the long control region (LCR) does not contain genes but in analogy to the bovine papillomavirus-1 (BPV-1) probably contains several promoters as well as the replication origin (2). Several binding sites for the viral E2 proteins in the LCR mediate positive and negative transcriptional feedback regulation (2). We and others have shown that a segment of the LCR of HPV-16 mediates strong transcriptional enhancement independent of E2 protein binding sites (3,4). We have analysed a 232 bp fragment exhibiting maximal enhancer activity by DNase I protection experiments and identified 6 footprints that we termed fp2e to fp7e, 5 for the nuclear factor 1 (NF1) and 1 (fp4e) created by 2 neighbouring AP1 binding sites (5–7). A 106 bp segment of DNA encompassing the 2 AP1 sites and 2 NF1 sites showed in these studies partial protection at high concentrations of nuclear proteins through low affinity or low abundance factors that bind in addition to AP1 and NF1. One element in this region mediates response of the enhancer to certain steroid hormones through a binding site for progesterone and glucocorticoid receptors.

Different papillomaviruses have a similar general genomic organization, but seem to be quite divergent in details of their gene function as well as gene regulation. For example, a strong enhancer activated by cellular transcription factors has not yet been found in BPV-1. Surprisingly, however, all genital papillomaviruses contain such an enhancer (3,4,8–10), and it is built up of similar motifs, amongst them a cluster of NF1 sites, AP1 sites and glucocorticoid and progesterone response elements (GRE and PRE) (6,7). In this study, we used the example of HPV-16 to explore the contribution of these factors to the enhancer function. Deletion and point mutation experiments proved that the enhancer is dependent on a cooperative activation of these factors and looses most of its activity upon complete elimination of either the AP1 or the NFI sites. The enhancer is further activated by a possibly novel factor provisionally termed PVF that binds a sequence that is conserved multiple fold in the LCR of HPV-16. Our sequence analysis suggests that all genital papillomaviruses have assembled in their LCR a cluster of similar transcription factor binding sites possibly reflecting particular functional requirements and evolutionary conservation in spite of overall genomic sequence divergence.

MATERIALS AND METHODS

Fragments or oligonucleotides with potential enhancer activity were cloned into the vectors pBLCAT2 (11) and ptkCATdH/N (12). After transfection into mammalian cells, the chloramphenicole acetyltransferase gene (CAT) (13) in these plasmids is expressed from the Herpes simplex thymidine kinase promoter. ptkCATdH/N is identical to pBLCAT2 except for the deletion of a 216 bp NdeI/HindIII segment of prokaryotic origin

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which contains by chance binding sites for eukaryotic transcription factors that could modify a transcription analysis. Beyond the experiments reported here, we obtained qualitatively similar data with either vector, although the basic level of CAT expression is higher with pBLCAT2. Fragments were named by their endpoints in the HPV-16 genomic sequence. 7524/7755 was cloned as a DraI fragment and 7532/7653 as a RsaI fragment into the HincII site of pUC18 and then recloned as a HindIII-BamHI fragment into pBLCAT2. 7524/7673 and 7675/7755 were similarly cloned after blunt ending a HhaI site at position 7673 with Klenow polymerase. 7524/7617 and 7620/7755 were derived from the cloned DraI fragments, 7524/7755 by opening established vectors at a single DraIII site at position 7614 and deleting part of the HPV-16 fragment by cleavage at the HindIII or BamHI site respectively of pBLCAT2, blunt ending both ends with Klenow polymerase and fusing the circle by ligation.

All oligonucleotides were synthesized on a Pharmacia gene synthesizer and cloned in form of doublestranded sequences with XbaI ends into ptkCATdH/N and in one case into pBLCAT2 (7611/7676). Two mutant oligos were designed to eliminate the possibility of NF1 or AP1 binding (Fig. 3). For fp5e and fp6e, the TTGGC motif was changed to TGTTC. As to the AP1 site on the 5′ edge of fp4e, the motif TGAATCA was mutated to TCTAGAC, and for the AP1 site on the 3′ edge of fp4e, TGTGTCA was changed to GATGTAC. This latter sequence was chosen such as to maintain a glucocorticoid/progesterone response element, which overlaps in HPV-16 with this AP1 site (7). To test for a functional contribution of a factor that leads to DNase I protection of the sequence AGGCACATAT (5), this sequence was modified to AGTGTATCTC. Bal31 deletions were done following established protocols (14). The Bal31 generated fragments were cloned into ptkCATdH/N. All plasmids were sequenced by the dideoxynucleotide termination reaction (15). Transfection of HeLa cells by electroporation and CAT assays followed established protocols standardized in our lab (7). Each result reported in this paper has been obtained by independently transfecting at least 6 tissue culture dishes of HeLa cells with at least 2 independent preparations of the same plasmid. The different constructs were electroporated together within the same experiment to equalize conditions. Steroid responses were determined as published *(7)*.

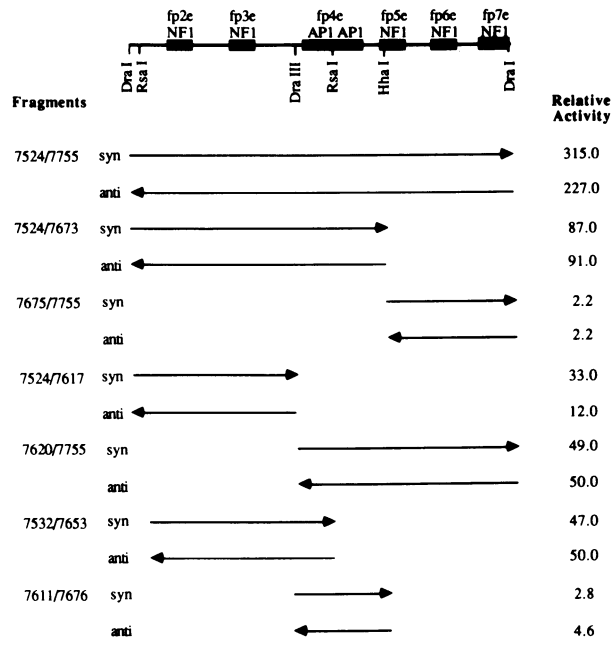

Figure 1. Localization of enhancer activity on a segment from the long control region of HPV-16. The upper part of the panel represents a 232 bp Dra I fragment containing the transcriptional enhancer with 5 NF1 sites (fp2e, 3e, 5e, 6e and 7e) and 2 AP1 sites (both in fp4e) (5, 7). Fragments were tested for induction of CAT activity after cloning into pBLCAT2 and electroporation into HeLa cells. The left column of the panel gives the endpoints of fragments in the genomic sequence of HPV-16, the right column the induction ratio (pBLCAT2: relative activity = 1, 1.81 pmole/min/mg protein). Fragments were tested in both orientations. The analysis shows that functional modules of the enhancer reside on non-overlapping fragments, and that sequences of overlap (7611/7676) bearing little activity can be activated through elements on either side (7524/7673 and 7620/7755). In general, fragments with lower activity contain only NF1 or AP1 sites, while fragments with high activity have both types of sites.

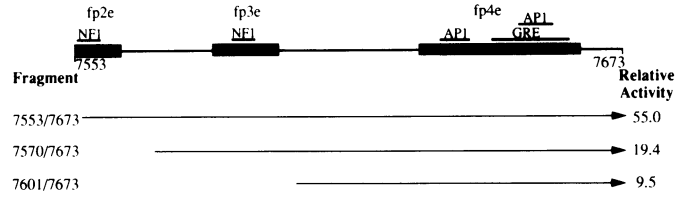

Figure 2. Two deletion mutants show the involvement of NF1 in transcriptional enhancement through functional decrease under progressive loss of NF1 binding sites. The relative activity of the mutants were obtained when compared to the basal activity of ptkCATdH/N (test vector without insert) which had been assigned the value of 1. (= 0.96 pmole/min/mg protein).

RESULTS

Functional elements of the HPV-16 enhancer are spread over at least 200 bp

We have published the identification of an E2 protein independent enhancer of HPV-16 on a 400 bp fragment located upstream of the major early promoter P97 (16). Strongest enhancer activity could be assigned to a 232 bp segment (genomic positions 7524 to 7755) with five binding sites for NF1 (initially termed fp2e, fp3e, fp5e, fp6e and fp7e) and binding sites for 2 AP1 factors (fp4e) (7, and Chan et al. manuscript in prep.). To localize elements relevant for determination of enhancer function 6 segments of the 232 bp fragment were inserted into pBLCAT2 for enhancer stimulated expression of the CAT gene. Fig. 1 documents the enhancer activity of each fragment in HeLa cells. Some of these fragments do not overlap and show that independent segments of the enhancer contain part of the total activity. Weak activity (2.2 to 12 fold and in 1 case 33 fold stimulation) is associated with those fragments which contain only NF1 sites (7524/7617 and 7675/7755), or AP1 sites (7611/7676). Strong stimulation (47 to 91 fold) is observed with fragments containing binding sites for both NF1 and AP1 (7524/7673, 7532/7653, 7620/7755). These strong stimulations were consistent in all cases with both syn and anti orientations of the segments. We conclude from these data that functional elements of the enhancer are spread over at least 200 bp. These elements influence the function of the enhancer in cooperative rather than additive manner.

Stepwise deletion of 2 NF1 sites from the 7524/7673 segment leads to a stepwise reduction of enhancer activity

Fragment 7524/7673 (87 and 91 fold induction) differs from fragment 7611/7676 (2.8 and 4.6 fold induction) by the presence of the 2 NF1 sites fp2e and fp3e. To test whether a deletion of these 2 NF1 sites leads to concomitant decrease of enhancer

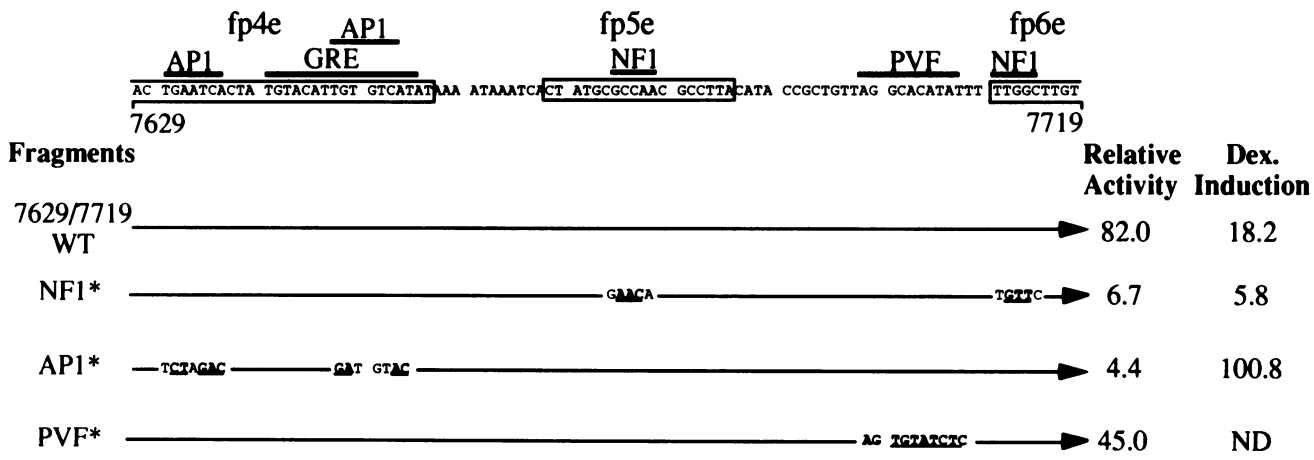

Figure 3. Loss of enhancer function of a 91 bp segment, the smallest identified sequence with significant enhancer activity, through mutation of either 2 NF1 sites (NF1*), or 2 AP1 sites (AP1*), or the PVF site. All fragments were cloned into ptkCATdH/N and tested for induction of CAT activity after electroporation into HeLa cells. Relative activity 1 was assigned to ptkCATdH/N and corresponds to a CAT activity of 0.56 pmole/min/mg protein. The rightmost column gives the additional activation by dexamethasome, a glucocorticoid. (ND = not done). These data point toward cooperativity between the glucocorticoid receptor and NF1 and possibly toward competition for the same binding site between the hormone receptor and AP1.

activity, we tested 3 mutants obtained by Bal31 deletion for enhancer activity in ptkCATdH/N. Fragment 7553/7673, retaining the 2 NF1 sites, led to 55 fold induction. Fragment 7570/7673, deleted for 17 bp including the NF1 site fp2e, induces CAT expression 19.4 fold and 7601/7673 that has in addition lost the NF1 site fp3e, only 9.5 fold (Fig. 2), a number reflecting the similarity of this deletion mutant with fragment 7611/7676.

An enhancer core segment with 2 AP1 and 2 NF1 sites looses most of its activity through mutations in either of these 2 types of binding sites

To obtain further clarification of our observation that the HPV-16 enhancer is activated through cooperation of NF1 and AP1 factors, we functionally tested an oligonucleotide with the 2 AP1 sites of fp4e and 2 NF1 sites located in 3′ position. This 91 bp oligonucleotide (7629/7719) was chosen since it still stimulated CAT expression 82 and 88 fold (syn and anti orientation, syn orientation shown in Fig. 3). It represents the smallest segment identified in this study with significant activity. Mutations designed such as to exclude the binding for either the 2 NF1 factors or the 2 AP1 factors retained only 8% and 5% of this activity, respectively (Fig. 3).

Glucocorticoid responsiveness of the HPV-16 enhancer is partially dependent on the presence of NF1, but not on the presence of AP1 sites

It has been reported that transcription factors bound to DNA close to a site recognized by a glucocorticoid receptor are cooperatively required to mediate the hormonal transcription stimulation, particularly if the steroid receptor binding site is remote from the regulated promoter (12). The glucocorticoid responsive element of HPV-16, which has the sequence TGTACATTGTGTCAT, is part of fp4e and overlaps with one of its 2 AP1 sites (TGTGTCA) (7, and Chan et al., manuscript in prep.). To test whether the AP1 sites or the NF1 sites on the 7629/7719 fragment are required for the HPV-16 enhancer glucocorticoid response, we measured the inducibility by dexamethasone of CAT expression from test plasmids carrying this insert. The data in Fig. 3 show that the wild type fragment leads to 18.2 fold induction by the hormone, the NF1 mutants

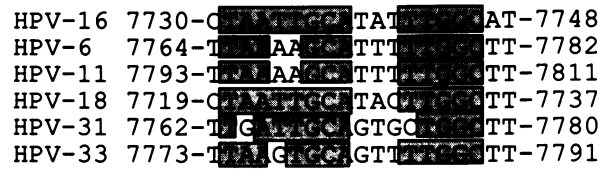

Figure 4. Homologies to a DNase I footprint in HPV-16 suggestive of a heterodimer between NF1 (right half) and an associated factor (NFA, NF1 associated factor).

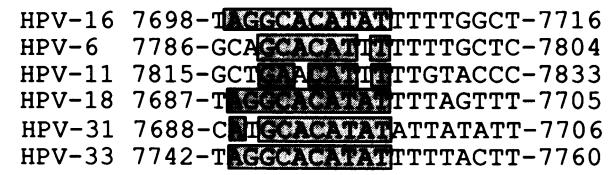

Figure 5. Binding sites of the proposed transcription factor PVF (papillomavirus enhancer associated factor). This sequence occurs in 6 positions in the HPV-16 long control region, one of these sites located within the HPV-16 enhancer. In HPV-16, all 6 sequences lead to footprints in DNase I protection experiments (5). As shown above, a mutation in this sequence leads to a decrease of the HPV-16 enhancer function. We assume this factor is funtionally relevant, since sequences with homology to the HPV-16 PVF binding site in association with a cluster of NF1 binding sites and the NFA site occur in the enhancer of all genital papillomaviruses (Fig. 6.).

only to 5.8 fold. We conclude that in HPV-16 NF1 is cooperating with the glucocorticoid receptor in transcriptional induction. Surprisingly, the AP1 mutants show a 100 fold induction by dexamethasone. We believe this observation is due to the overlap of the 3′ AP1 site of fp4e with the GRE resulting in steric hindrance of binding of the 2 transcription factors (7). The AP1 mutation hence creates a more accessible GRE. Indeed, when an oligonucleotide representing only the GRE TGTACATTGTGTCAT was cloned into ptkCATdH/N and examined in HeLa cells, it led to a 3 fold stimulation of CAT expression in a dexamethasone dependent way while the oligonucleotide TGTACATGATGTACT led to 40 fold stimulation. The question whether these point mutations rather

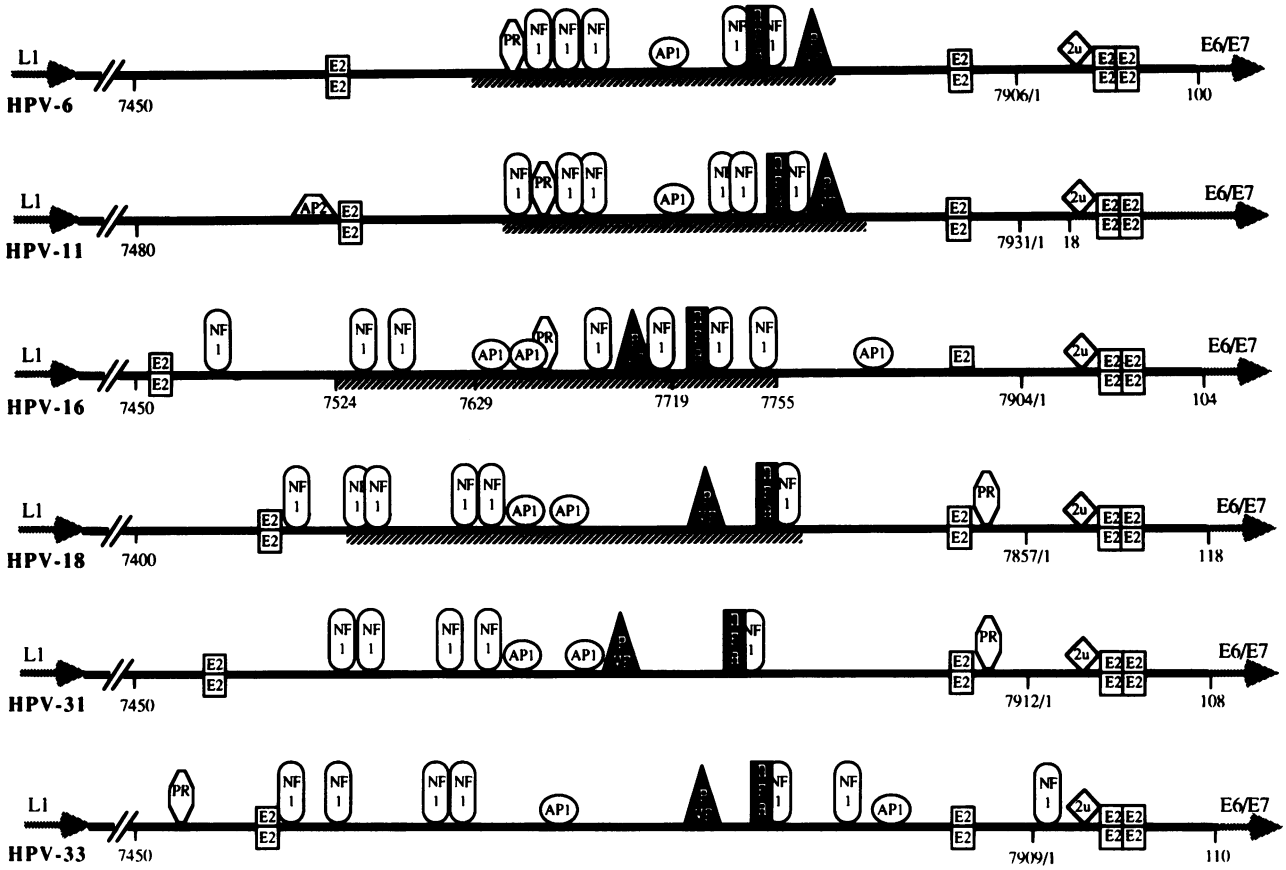

Figure 6. Organization of transcription factor binding sites in the enhancer/promoter region of 6 genital papillomaviruses. With a length of 550 bp the bar represents two thirds of the long control region of each virus from a position numbered an the left side to the position of the E6 ATG, on the right side. Fragments published (5,8,9,10) to contain enhancer activity are underlined with a dashed bar. The exact position of all potential binding sites for nuclear factor I (NF1), AP1, AP2, glucocorticoid and progesterone receptor (PR) in HPV-6, 11, 16, 18 and 33 has been published (6,7), and has been experimentally verified for HPV-11, 16 and 18. For HPV-31, all TTGGC or CTGGC sequences have been termed NF1, and 2 sequence elements with homology to TGAGTCA were indicated as 'AP1'. Binding sites for PVF and NFA are those documented in Fig. 4 and 5. Binding sites for the viral E2 protein followed the original publications of the genomic sequence of each virus. Two E2 boxes represent the palindromic sequence $ACCN_6GGT$. In HPV-16, one of these palindromic carries a mutation (single box). The distal promoter element fp2u (2u) has been published (5).

increase the affinity for the glucocorticoid receptor or eliminate the likely competitive binding of AP1 to this sequence element was not followed in more detail, since it was judged to be peripheral to the aspects addressed in this line of our research.

A possibly novel transcription factor, PVF, with multiple binding sites in all genital papillomavirus LCRs contributes to the enhancer function

We previously observed DNase I protections in the LCR of HPV-16 of an at least five times repeated decamer sequence outside the enhancer which has the consense AAGCACATAC (fp2l, fp4l, fp5l, fp6l and fp7l in ref. 5). The related sequence AGGCACATAT in the enhancer is protected by nuclear extracts at high protein concentration (5). To test whether this protein binding sequence is functionally relevant, we constructed an 7629/7719 enhancer test vector with a mutation in the 90 mer insert that eliminated this consensus. Fig. 3 includes the data obtained with this mutant showing that the enhancer has in the likely absence of this binding site lost 45% of its activity. We conclude that a factor that contributes to the enhancer function is binding to this site. Since the sequence bound by this factor is not reminiscent of any published enhancer element (17), we provisionally call this factor papillomavirus enhancer associated factor, *PVF*.

DISCUSSION

The involvement of papillomaviruses in human genital oncogenesis demands for a detailed analysis of their molecular biology. Understanding transcriptional regulation is an experimental handle to understand the etiology of these viruses in cervical cancer. As to the regulation of transcription of these viruses, research has concentrated on aspects of their E2 gene system, a complex means of feedback regulation (for references see 2, 18). Our group is undertaking a detailed analysis of the cellular transcription factors binding to papillomavirus promoters/enhancers to understand basic and regulated gene expression. 6 out of 23 DNase I protected sequences mapped in the HPV-16 LCR occur in a segment of 232 bp with strongest enhancer activity (5). 5 of these 6 footprints involve NF1 bound to non-palindromic TTGGC motifs, a feature common to all sequenced human papillomaviruses (6). The fragmentations, deletions and mutations of the enhancer studied here offer a quantitative explanation for the redundancy of these elements, namely a contribution of several if not all of the NF1 sites to the enhancer function. Further stimulation occurs by AP1, a family of transcription factors derived through dimerization of the protein products of the fos and the jun gene family (19). AP1 is at the end of certain intracellular signalling pathways and is a tool to modulate gene expression under the stimulation of second

messengers (20). Under our test conditions, namely in the presence of serum containing cell culture medium, AP1 mutations reveal a strong constitutive activation of the enhancer by this factor. For fragments of the HPV-16 enhancer that contain both AP1 and NF1 sites we determined a multiple fold rather than a sum of the activity of fragments that contain only either type of transcription factor binding site, a quantitative fact referred to as cooperativity. A similar relationship has been proposed as to the need of glucocorticoid receptors for transcription factors bound to flanking binding sites (12), an observation confirmed here for HPV-16.

This composition of enhancer elements does not seem to be an idiosynchrasy of HPV-16, since we observed in a footprint analysis of DNA segments from the LCR of HPV-11 and 18 similarly clusters of NF1 binding sites associated with 1 or 2 protections on likely AP1 binding sites (6). In HPV-11 and 16, a GRE/PRE is closely, in HPV-18 distantly linked to these enhancer elements (7).

Considering that additional enhancer elements may have evaded our previous interpretations of published DNase I-protections, we scrutinized our published data for HPV-16 and compared our findings with the sequences of the 5 genital papillomaviruses HPV-6, 11, 18, 31 and 33 (21–25).

Two sequences caught our attention which had been previously pointed out to be elements of extensive homology between HPV-16 and 18 (10). One of it includes in HPV-16 the footprint fp7e, a NF1 site protected on the lower strand over 12 bp beyond the normal limits of NF1 footprints. Sequence inspection of the 6 papillomavirus LCRs reveals that in each virus the NF1 cluster contains a single NF1 site associated with a homology to the sequences 5′ of the TTGGC motif of fp7e (Fig. 4). Provisionally, we designate this factor NFA for NF1 associated factor, since the constant distance between the NF1 and the NFA motif may reflect the ability of NF1 to dimerize with a heterologous rather than a homologous subunit (26).

While the introduction of NFA is based only on footprint analysis and sequence comparison, we have identified an additional DNase I protected sequence that is functionally involved in transcriptional enhancement. Just like NFA, this element is located in a segment of extensive homology between the enhancers of HPV-16 and 18 (10). This homology contains between the 2 NF1 sites fp5e and fp6e the sequence AGGCACATAT, protected at high concentrations of nuclear proteins. A related sequence, AAGCACATAC, is repeated several times 500 bp 5′ of this element and gives rise to defined footprints, without being associated with NF1 sites. Upon mutation of this binding site in a HPV-16 enhancer core segment to a randomly modified sequence element, the enhancer looses 45% of its activity (Fig. 3). Sequence comparison suggests a similar element in the enhancer of all genital papillomaviruses. Since a consensus sequence of these elements does not seem to be strongly related to any published transcription factor binding site (17), we propose the functional participation in all genital papillomavirus enhancers of a common factor, that we would like to call papillomavirus enhancer associated factor, PVF (Fig. 5).

A recently published segmentation of the HPV-11 enhancer (27) is suggestive of modules functionally similar to HPV-16. The 5′ half of the HPV-11 enhancer as represented in Fig. 6 by a dashed line (10) had been termed CEII and was shown to exhibit reduced enhancer activity. With 2 NF1 sites termed by the authors DEII, and an AP1 site (6), this module is comparable to our clone p7524/7673 with a similar activity profile. The other module of HPV-11, CEI, is not functional by itself but cooperates with CEII, similar to the HPV-16 clone p7675/7755. Both HPV-11 CEI and HPV-16 p7675/7755 contain binding sites for 2 NF1 factors and the newly proposed factors PVF and NFA.

Fig. 6 summarizes all sequence elements: restriction fragments of HPV-6, 11, 16 and 18 containing documented binding sites for NF1 and AP1 and likely binding sites for NFA and PVF had been published to contain transcriptional enhancer activity (3, 4, 8, 9, 10, 27). Similar segments in HPV-31 and 33 carry the same sequence elements, which probably induce the same function. Fig. 6 includes the location of proven or likely steroid response elements (7), binding sites for the viral E2 proteins (2) and the distant promoter element fp2u, common to all genital papillomaviruses (5).

This model of the apparently homogenous make-up of the elements of enhancers of genital papillomaviruses is based on sequence analysis of all these viruses and on footprint and functional data from HPV-11, 16 and 18. Without doubt, this model will have to be further refined. Among additional elements may be silencers suggested by our experiments to be present in HPV-16: The data presented above could be reproduced in MCF-7 cells, an epithelial cell line in contrast to HeLa free of HPV gene products. Several clones with sequence elements outside the 232 bp enhancer studied in this paper show little activity in MCF-7 and may contain an element responsible for cell-type specificity as reported previously (3).

We could not find this association of sites in the sequences of HPV-1, HPV-8, BPV-1 or the cottontail rabbit papillomavirus, although they contain several NF1 sites. Future experimental analysis of the functional parameters associated with each element will hopefully resolve the question whether these enhancers evolved in this particular formation which was then retained, and whether each particular element contributes a module necessary for particular aspects of the papillomavirus life cycle.

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