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# Numerous Nuclear Proteins Bind the Long Control Region of Human Papillomavirus Type 16: A Subset of 6 of 23 DNase I-Protected Segments Coincides with the Location of the Cell-Type-Specific Enhancer

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**The long control region of the human papillomavirus type 16 genome is 856 base pairs (bp) long. It contains a cell-type-specific enhancer, a glucocorticoid response element, and sequences mediating the response to the viral gene products of open reading frame E2; all three regulate the promoter P97. We mapped binding sites of *trans*-acting proteins relevant for the cell-type-specific enhancer and other *cis*-acting elements by DNase I footprint experiments with nuclear extracts from HeLa cells. Throughout the human papillomavirus type 16 long control region 23 footprints protect 557 of 900 bp. Nine footprints fall into a 400-bp segment that was previously identified to contain the cell-type-specific enhancer. Variations of the protein concentration in the footprint reaction do not affect six of these nine footprints. At high protein concentrations, three footprints fuse to a 106-bp protected region, suggesting that this segment specifically binds several proteins of lower affinity or abundance. Unexpectedly, extracts from human MCF7 and mouse 3T3 cells, in which the enhancer is inactive, give footprints identical to those obtained with HeLa extracts. Seven footprints contain the sequence 5′-TTGGC-3′. Footprint competition experiments suggest that factor NFI binds to these seven motifs. Competition with cloned oligonucleotides in transfections suggests that these elements contribute to the enhancer function. Subcloning identifies a 232-bp fragment between positions 7524 and 7755 as sufficient for full enhancer activity. Several of the six footprinted elements on this segment may cooperate functionally, since subclones of this region show decreased or no cell-type-specific enhancer function.**

Human papillomavirus type 16 (HPV-16) is the most frequently encountered papillomavirus type associated with cervical cancer and is considered to play a causative role in the genesis of this malignancy. Like numerous other HPV types, it is found in human epithelial cells of diverse origin. However, its tissue tropism seems to be biased toward epithelial cells of the female and male genital regions (for reviews, see references 43, 57, and 61).

In recent years, it has become clear that tissue tropism of many viruses can be mediated through genetic elements that direct viral gene expression. The enhancer and promoter of a virus may be active only in particular cell types that contain an appropriate supply of transcription factors (30, 40, 41, 44, 48, 63). Recently, we (20) and others (10) identified such an enhancer in the genome of HPV-16. This enhancer activates linked promoters in cells derived from a cervical carcinoma (HeLa) but not in cells derived from a breast tumor (MCF7) (20), and it is functional in various human keratinocytes but not in cells of other differentiation types (10). A possibly homologous enhancer element had been found in HPV-18 (19, 60, 62) and in HPV-11 (26).

The cell-type-specific enhancer of HPV-16 had been located to a 400-base-pair (bp) segment between positions 7454 and 7854 within the 856-bp long control region (LCR). The LCR spans from the termination codon of the open reading frame (ORF) L1 to the ATG of ORF E6 (20). This enhancer activates *cis*-linked heterologous promoters and, as shown for the closely linked glucocorticoid response element (GRE) (20), is likely to stimulate the homologous promoter P97. P97 is 147 bp downstream from the right end of the 400-bp segment and is the only promoter documented so far in HPV-16 that is relevant to transcription of the HPV-16 early genes such as ORF E6 and E7 (55). P97 activity is further modulated by physiological factors such as progesterones and phorbol esters (W. K. Chan and H.-U. Bernard, manuscript in preparation) and in cells that express proteins derived from the papillomavirus ORF E2 by the E2 *trans*-activator protein and the short E2 repressor protein (10, 45). Three $ACCN_6GGT$ core-binding sites, which are recognized by these proteins (1, 24, 26, 31, 39, 45, 56), are centered 40, 56, and 544 bp upstream of P97 (52).

At this time, no other *cis*-regulating elements within the LCR of HPV-16 have been described. However, the similar genomic structure of bovine papillomavirus type 1 (BPV-1) suggests that the LCR of HPV-16 contains genetic elements that have been described so far only for BPV-1. These are two additional promoters for early-gene expression, a promoter for late-gene expression (3), and elements necessary for viral replication (34, 46, 58, 64). In contrast to the detailed analysis of papillomavirus gene products that mediate feedback regulation of *cis*-responsive elements on the papillomavirus genome, very little information exists about cellular transcription factors that interact with papillomavirus genomes.

To further our understanding of the cooperation between cellular *trans*-acting factors and *cis*-acting elements on the papillomavirus genome, we have performed DNase I protection (footprinting) experiments (16) throughout the HPV-16 LCR with nuclear extracts from human HeLa and MCF7 and mouse 3T3 cells. We document that 557 of 900 bp between

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genomic positions 7100 and 97 are protected against DNase I cleavage, resulting in 23 footprints. Several of these elements are located within a segment that gives enhancer function. We propose that seven elements bind the same factor, most probably nuclear factor I (NFI), as judged by footprint competition in vitro. This first documentation of the large number of elements that bind nuclear factors within the LCR of any papillomavirus will form a framework and a map to direct the functional analysis of *cis*-acting elements which are relevant for transcription and replication of HPV-16 and most probably other types of papillomaviruses.

## MATERIALS AND METHODS

Plasmid pURR-SVE-cat-i was made available by T. Cripe and L. Turek, and its sequence has been published previously (plasmid i in reference 10, Fig. 2). Sequences of all other plasmids have been published (20), or the construction of the plasmids is described here. The oligonucleotides are from B. Li, Institute of Molecular and Cell Biology, Singapore, or from Applied Gene Technology Services, Heidelberg, Federal Republic of Germany. Sequences of the LCR of HPV-16 were cloned as subcloned fragments or oligonucleotides into the *Hin*cII or *Xba*I sites of pUC18 and pBLCAT2 (33).

Human HeLa and MCF7 cells and mouse 3T3 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Nuclear extracts were prepared by the method of Dignam et al. (14) as modified by Wildeman et al. (64a).

Footprint reactions (16) were carried out by using 10 μl of buffer containing 100 mM KCl, 4 mM spermidine, 5 mM $MgCl_2$, 0.25 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol, and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9). The nuclear proteins were incubated in this buffer with 100 ng of poly(dI-dC) for 15 min on ice, and then 1 to 2 ng (5,000 to 10,000 Cerenkov cpm) of asymmetrically $^{32}P$-labeled probe fragment was added. Incubation was continued for 15 min on ice. The samples were digested with DNase I (at 25 ng/μl) for 90 s at 25°C and treated with phenol-chloroform and ether and DNA was recovered by two ethanol precipitations. Electrophoresis of the samples together with chemically cleaved DNA (35) was done on 0.4-mm-thick denaturing 6% or 8% polyacrylamide gels.

For footprinting competition experiments, synthetic oligonucleotides were hybridized as described by Kadonaga and Tjian (29) and used directly in the binding reaction; 10 to 70 ng was added to the binding reaction mixture 5 min before the labeled DNA fragment, and all further reactions were carried out as described above. The HPV-16 oligonucleotides represent segments as described in the Results section; the adenovirus NFI-binding site was represented by the oigonucleotide 5'-AATTCTTATTTTGGATTGAAGCCAA TAATCG-3' (13).

DNA fragments for footprint analysis were prepared from subclones of the HPV-16 genome in pUC18. Radioactive labeling with Klenow polymerase at the natural *Eco*RI site at position 7454 permitted the analysis of the upper strand toward ORF L1 and the lower strand toward P97. pUC18 clones of segments of the LCR extending 5' from 3' borders at position 7282 (a natural *Dra*I site), 7854, or 160 (20) were opened, labeled at restriction sites of the plasmid polylinker, and used for analysis of sequences on the upper strand 5' to these three sites. The lower strand between positions 7756 and 160 was examined after a *Dra*I-*Bam*HI fragment from pHPV-16-14 (20) was cloned into pUC18 opened at *Hin*cII and *Bam*HI. This construct was labeled at the *Hin*dIII site.

Cell cultures were transfected with a Bio-Rad Gene Pulser with capacitance extender at 960 μF and 250 V. Cells were grown to less than 80% confluency, trypsinized, and washed twice with phosphate-buffered saline. Then, $3 \times 10^6$ cells in 600 μl of phosphate-buffered saline were mixed with DNA in 200 μl of phosphate-buffered saline and left for 10 min on ice before electroporation. Five micrograms of DNA was used for testing the activity of chloramphenicol acetyltransferase (CAT) expression plasmids, and 22 μg of DNA was used for in vivo competition experiments. The DNA was always ethanol precipitated and redissolved in phosphate-buffered saline. In competition experiments, 2 μg of CAT expression vector was mixed with either 20 μg of pUC18 (base line, no competition) or 4 μg of competitor plasmid plus 16 μg of pUC18 (representing a 3-fold molar excess of the competitor) or 20 μg of competitor plasmid (representing a 15-fold molar excess of the competitor). After electroporation, cells were plated into 10 ml of Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and harvested after 28 h. Cell survival before harvesting was 30 to 50%. Cells were lysed by being freeze-thawed three times, and soluble extracts were tested for CAT activity as described previously (22).

## RESULTS

**Protein-binding motifs within the DNA segment that contains the cell-type-specific enhancer of HPV-16.** A segment from the LCR of HPV-16 enhances transcription from the simian virus 40 (SV40) early promoter and the herpes simplex virus type 1 thymidine kinase promoter (*tk* promoter) in a cell-type-specific way (10, 20). In HPV-16 this enhancer is functionally distinct and physically separated from the E2 protein-dependent enhancer, an element common to many papillomaviruses. We assume that the HPV-16 enhancer exerts its function by binding nuclear protein factors as shown for the enhancers of numerous cellular and viral genes (for reviews, see references 21 and 53). As a prerequisite for a functional map of the HPV-16 enhancer, we decided to map the DNA sequences which bind nuclear proteins by DNase I protection (footprinting) studies. This was done with nuclear extracts from HeLa cells, a cell line that activates the HPV-16 enhancer. Nuclear extracts from human MCF7 and mouse 3T3 cells were included in this study on the assumption that these cells, which do not activate the HPV-16 enhancer, may lack some nuclear factors and hence give a different footprint profile from that of HeLa extracts. A 400-bp fragment of the HPV-16 LCR, from the *Eco*RI site at position 7454 to a polylinker at position 7854 (20), was terminally labeled at either end, digested with DNase I without or after incubation with nuclear extracts (see Materials and Methods), and run on an 8% denaturing polyacrylamide gel next to an A+G sequence lane. Figure 1 documents nine footprints, which we designate fp1e to fp9e. Each of these footprints identifies the binding of at least one cellularly encoded factor to this segment of the HPV-16 genome. This experiment was reproduced several times with protein concentrations of 2 μg/μl and with extracts from numerous independent preparations. fp1e, fp2e, fp3e, fp6e, fp7e, and fp9e always showed similar borders and similar intensity, whereas fp4e, fp5e, and fp8e varied strongly in intensity. To follow up on this observation, in some experiments we increased the concentration of nuclear protein in the DNase I protection reaction from 2 to

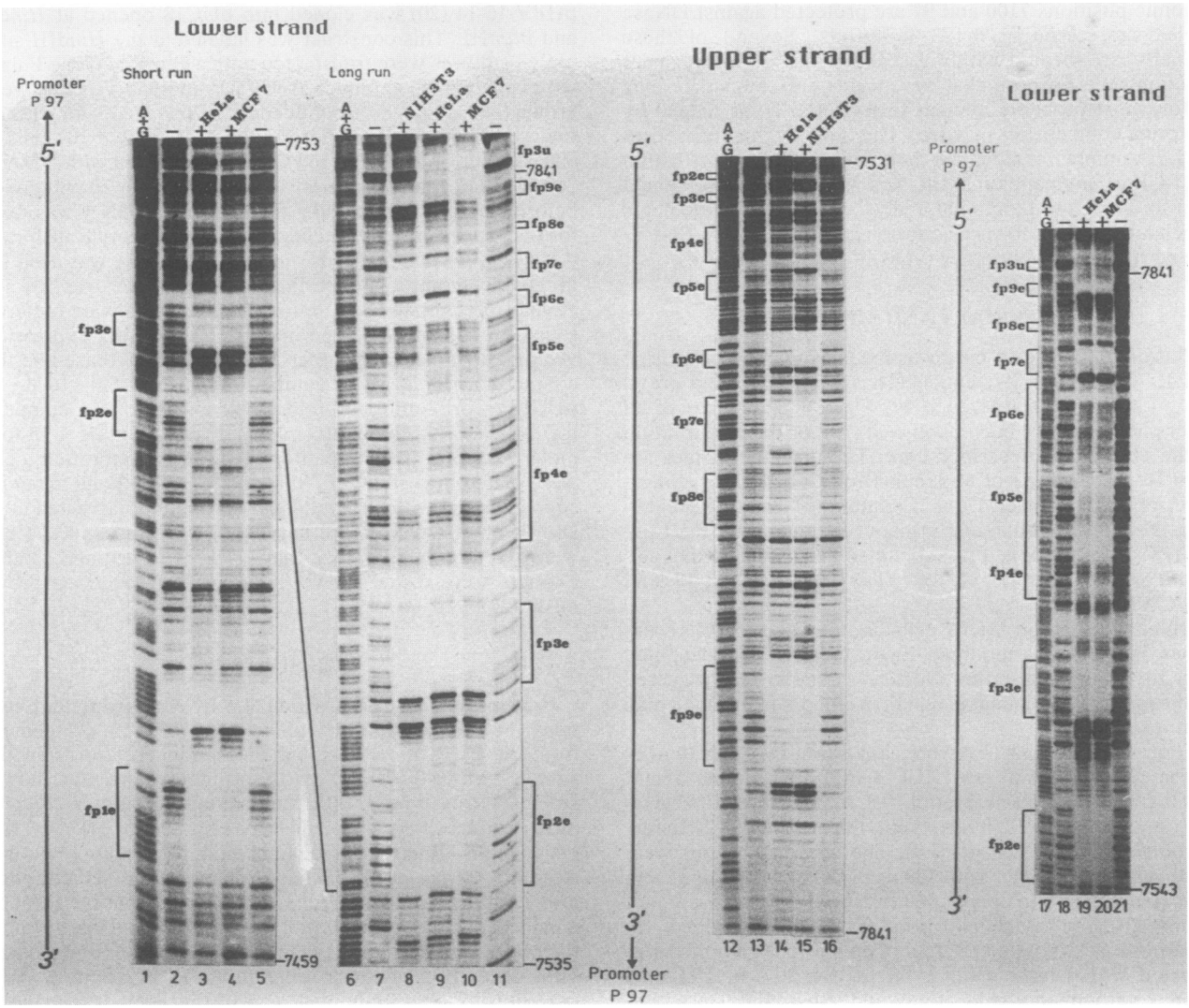


FIG. 1. DNase I protections by nuclear extracts from human HeLa and MCF7 cells and mouse 3T3 cells on a 400-bp fragment containing the cell-type-specific enhancer of HPV-16. Symbols: −, DNase I-sensitive sites in the absence of nuclear proteins; +, DNase I-sensitive sites in the presence of nuclear extracts. The source of the extract is indicated next to each lane. A+G sequencing lanes are shown next to the DNase I digestions. Sequence positions are indicated by numbers at top and bottom of each gel. The nomenclature chosen for the footprint is given next to the brackets along the gel. The ends of the brackets identify minimal footprint borders as defined by the location of clearly protected bands rather than by the positions of flanking enhancements. In numerous gels involving several independent extract preparations, no difference was identifiable between HeLa and MCF7 lanes. The protection of the upper strand with MCF7 extracts was as shown for HeLa extracts (lane 14). Two differences between the extracts of both human cells and those of mouse 3T3 cells are visible. Sometimes DNase I-sensitive sites between fp9e and fp3u were protected by HeLa or MCF7 but not by 3T3 proteins (compare lanes 7, 8, and 9), and the borders of fp7e differed under the influence of 3T3 extracts. We did not follow up on this, since it did not correlate with functional differences. Independent of the source of the extract, the regions designated fp4e, fp5e, and fp6e varied strongly with small variations in the concentration of nuclear extracts (2 to 10 μg/μl). Extremes are shown in this figure: in lanes 8, 9, 10, 14, and 15, these footprints are marginally visible, whereas all other elements are well protected; in lanes 19 and 20, these regions are fused in a 106-bp virtually completely protected segment, whereas patterns outside this segment did not vary significantly.

10 μg/μl (Fig. 1, lanes 19 and 20). Although fp1e, fp2e, fp3e, fp7e, and fp9e were not significantly changed, fp4e, fp5e, and fp6e fused to a single 106-bp footprint between positions 7612 and 7717. We conclude that this segment specifically binds nuclear factors which may be lower in abundance or affinity than the factors responsible for fp1e, fp2e, fp3e, fp6e, fp7e, and fp9e.

In all experiments no clear difference between the footprints obtained with HeLa extracts and those from MCF7 extracts could be observed. Two differences were observed between footprints by mouse 3T3 nuclear extracts and the two human nuclear extracts, but they did not correlate to known functional differences (see Fig. 1 Legend). Figure 2 shows a compiled map of these footprint experiments and those described in subsequent paragraphs.

**Seven motifs of the HPV-16 enhancer segment have related sequence elements and bind the same factor, most probably NFI.** Four footprints, fp1e, fp2e, fp3e, and fp7e, exhibit the common sequence element 5′-TTGGCA-3′, fp6e contains the sequence 5′-TTGGCT-3′, and the two weak footprints, fp5e and fp8e, contain the sequences 5′-TTGGCG-3′ and 5′-TTGGCC-3′ (Fig. 2). These are consensus sequences

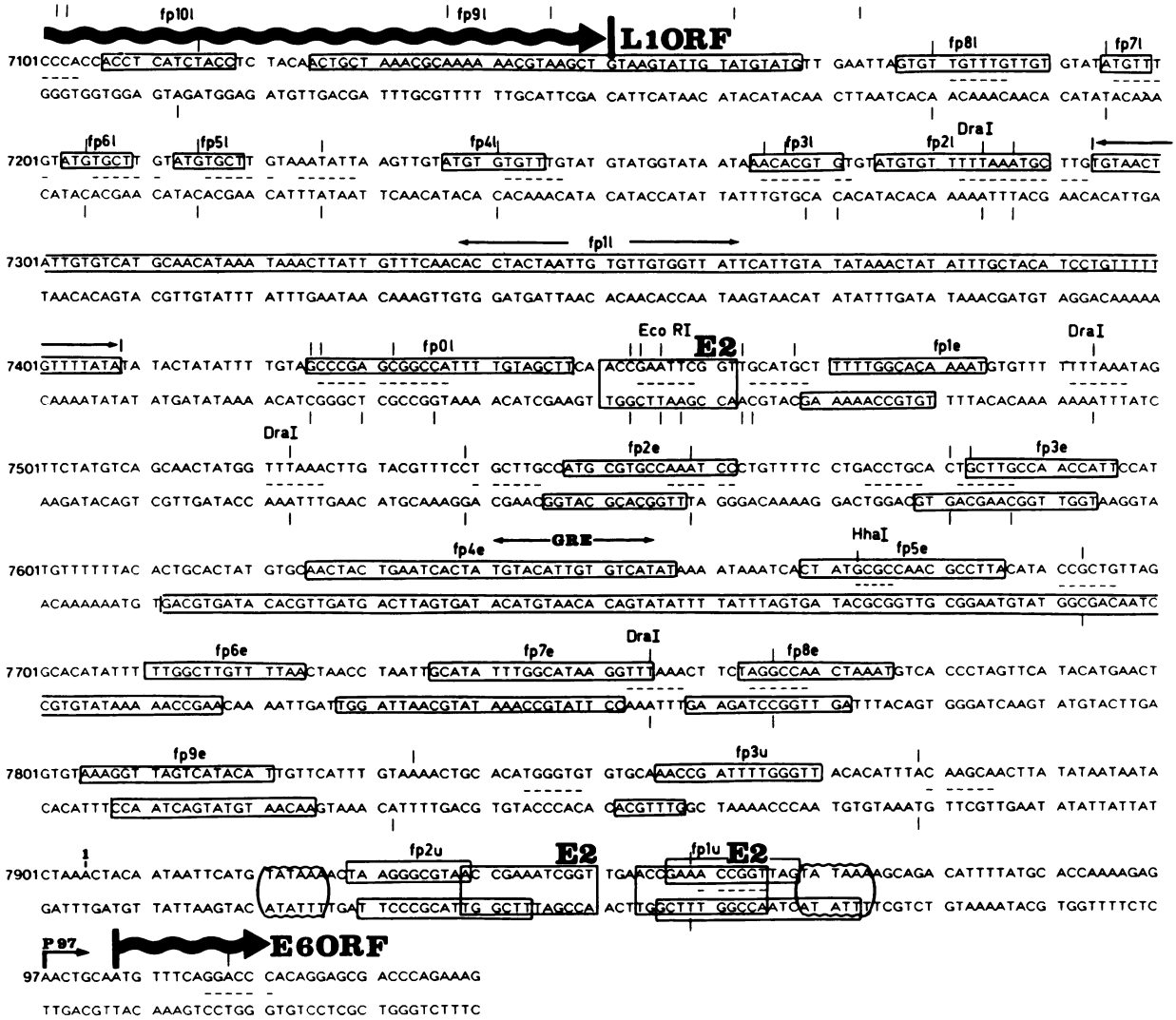


FIG. 2. Cumulative representation of footprints by HeLa nuclear extract in the LCR of HPV-16 between the 3′ end of ORF L1 and the 5′ end of ORF E6 (⇝). Both strands were analyzed 3′ of the *Eco*RI site at position 7454, whereas only the upper strand was analyzed 5′ of this position. All footprints are represented by boxes around clearly protected DNase I-sensitive bands. The promoter P97, two TATA box homologies (positions 17 and 65), and three homologies to papillomavirus E2 protein-binding sites are included. The figure shows restriction sites mentioned in this paper. We had originally identified the cell-type-specific enhancer between the *Eco*RI site at position 7454 and a polylinker cloned to position 7854. The fragment includes a binding site for the GRE at positions 7641 to 7655). All footprints 3′ of the *Eco*RI site were obtained with HeLa and MCF7 extracts except fp1u, which could be visualized only with MCF7 extracts. On the upper strand, the segment between positions 7612 and 7724 shows fp4e, fp5e, and fp6e as defined from gels obtained with lower nuclear protein concentrations; on the lower strand, a 106-bp segment of complete protection at increased concentration of nuclear proteins is shown. Most of the data used in creating this map can be verified from Fig. 1, 6, and 7.

recognized by the factor NFI (see references 13 and 27 and references therein). This suggests that this factor or a factor with a related recognition sequence produces seven footprints in HPV-16. To test this possibility, we performed DNase I protection experiments in the presence of double-stranded 20- and 18-bp oligonucleotides corresponding to positions 7577 to 7596 and 7709 to 7729 whose sequences include fp3e or fp6e. Increasing amounts of the fp3e oligonucleotide eliminate fp2e, fp3e, fp6e, and fp7e (and possibly fp5e and fp8e) but not fp9e (Fig. 3A). fp1e, which is not visible in Fig. 3A, was eliminated in a similar fashion (data not shown). Identical results were obtained in competition experiments with an fp6e oligonucleotide or with an oligonucleotide representing the prototype adenovirus NFI-binding site (13) (data not shown). Conversely, a footprint on the cloned adenovirus NFI-binding site was eliminated by oligonucleotides representing the HPV-16 elements in the same way as by the authentic adenovirus NFI oligonucleotide (Fig. 3B). Beyond this, a double-stranded oligonucleotide spanning fp9e eliminates only fp9e (and possibly fp4e; see Discussion), not fp2e, fp3e, fp6e, or fp7e. We conclude that a common protein, most probably NFI, is involved in the induction of these seven footprints but not in the induction of fp9e.

**Are the TTGGC motifs functionally relevant for the HPV-16 enhancer?** To test whether the TTGGC motifs contribute to the enhancer function, we decided to perform in vivo competition experiments. To do this we used two different constructs, which have the gene coding for the enzyme chloramphenicol acetyltransferease (CAT) (22) under the

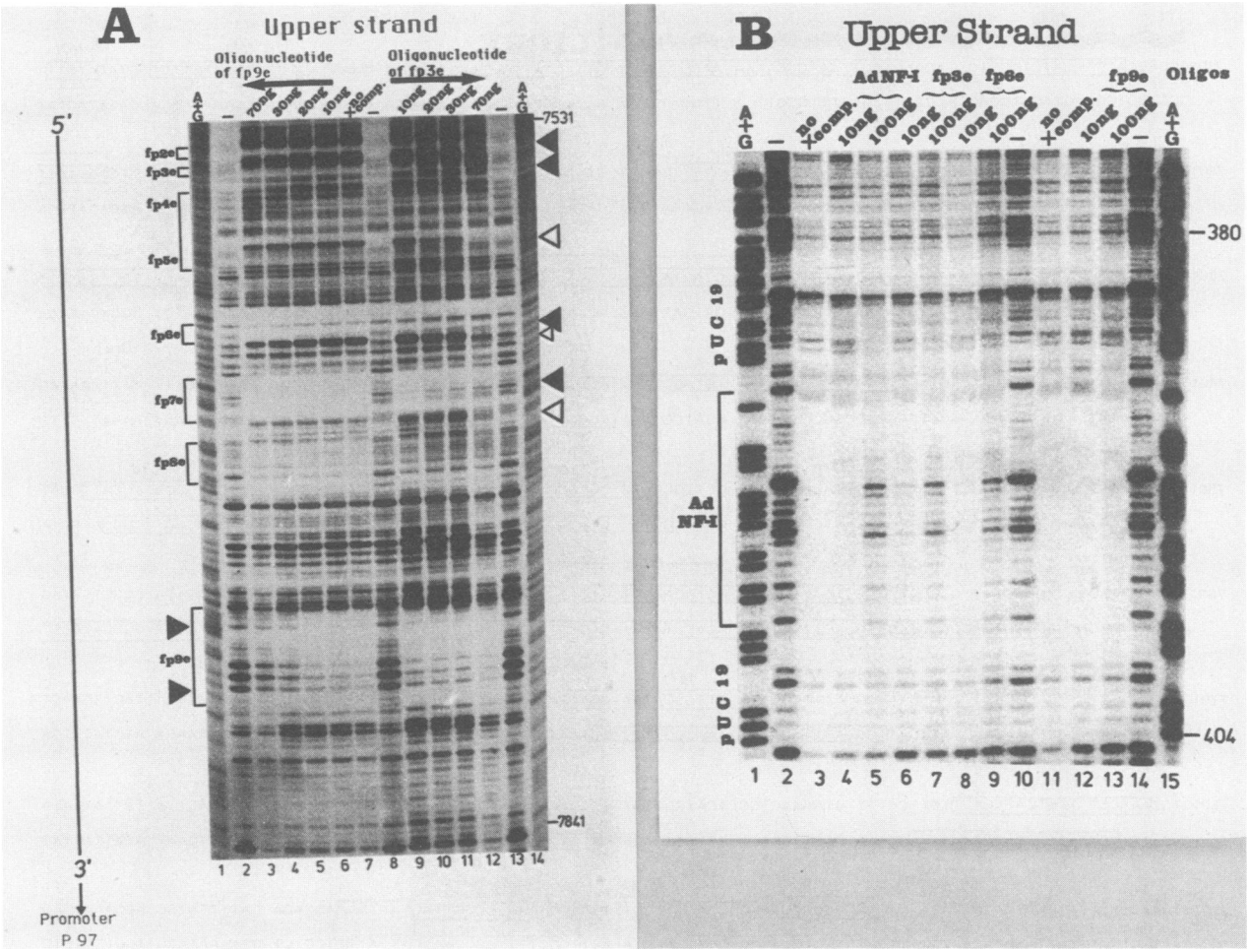


FIG. 3. Competition for footprints. (A) Under the influence of increasing concentrations of the fp9e oligonucleotide, only fp9e (and possibly fp4e) disappears (lanes 3 to 7), whereas under the influence of the fp3e oligonucleotide, fp2e, fp3e, fp5e, fp6e, and fp7e but not fp9e (lanes 9 to 12) disappear. (B) A footprint on the cloned palindromic adenovirus NFI-binding site is eliminated by increasing concentrations of the homologous oligonucleotide representing fp3e or fp6e but not by the fp9e oligonucleotide. The adenovirus sequence was cloned as an oligonucleotide into the *Eco*RI site of pUC19. Reappearing DNase I-sensitive bands (◀) and disappearing enhancements at the flanks of footprints (◁) are indicated.

influence of the HPV-16 enhancer. In each plasmid, the enhancer activates a different heterologous promoter. In plasmid pHPV-16-400.2 (20), CAT expression occurs from the *tk* promoter, which contains a CCAAT box relevant for promoter function (36, 37). This CCAAT box may be activated by the same transcription factor NFI/CTF (27) as the motifs in the HPV-16 LCR whose functions we wanted to analyze. In pURR-SVE-cat-i (clone i in reference 10, Fig. 2), CAT expression occurs from the SV40 promoter which is independent of CCAAT-box binding factors. As a control plasmid we used pSV2CAT (22), in which CAT expression occurs through the SV40 enhancer–early-gene promoter, a transcription system independent of the factor NFI. To perform functional competition experiments for the TTGGC factor binding the HPV-16 enhancer in these expression vectors, we constructed the clones p877 and p878. These are pUC18-derived plasmids which contain five copies of the 20-bp oligonucleotide representing fp3e and six copies of the 18-bp oligonucleotide corresponding to fp6e, respectively. As a positive control, competition was monitored with plasmid pHPV-16-14, a construct containing the 610-bp HPV-16 sequence between positions 7454 and 160 (20). To avoid artifacts due to variations in the quantity of transfected DNA and to saturate the transfected cells with DNA sequences not representing NFI-binding sites, we always adjusted the amount of competitor plasmid (pUC18 with insert) to 20 μg with pUC18 (no insert).

Figure 4 documents the results of a competition experiment in which a 3- and a 15-fold molar excess of the competitor plasmids was used. Cotransfection with pHPV-16-14 decreased CAT expression from pHPV-16-400.2 and from pURR-SVE-cat-i by 70 to 80%, documenting competition for functionally relevant transcription factors. Competition for pSV2CAT-dependent expression by the same plasmid was reduced by only about 22%, making it likely that few if any transcription factors are shared by these two viral enhancers. Competition with p877 and p878 for CAT expression from either pHPV-16.400.2 or pURR-SVE-cat-i reduced CAT expression by 40 to 50%, whereas hardly any competition was observed for pSV2CAT. We infer from these data that one or several of the TTGGC motifs are contributing to the HPV-16 enhancer function.

**Localization of the HPV-16 enhancer.** In contrast to the function of the E2 protein-dependent enhancer of papilloma-

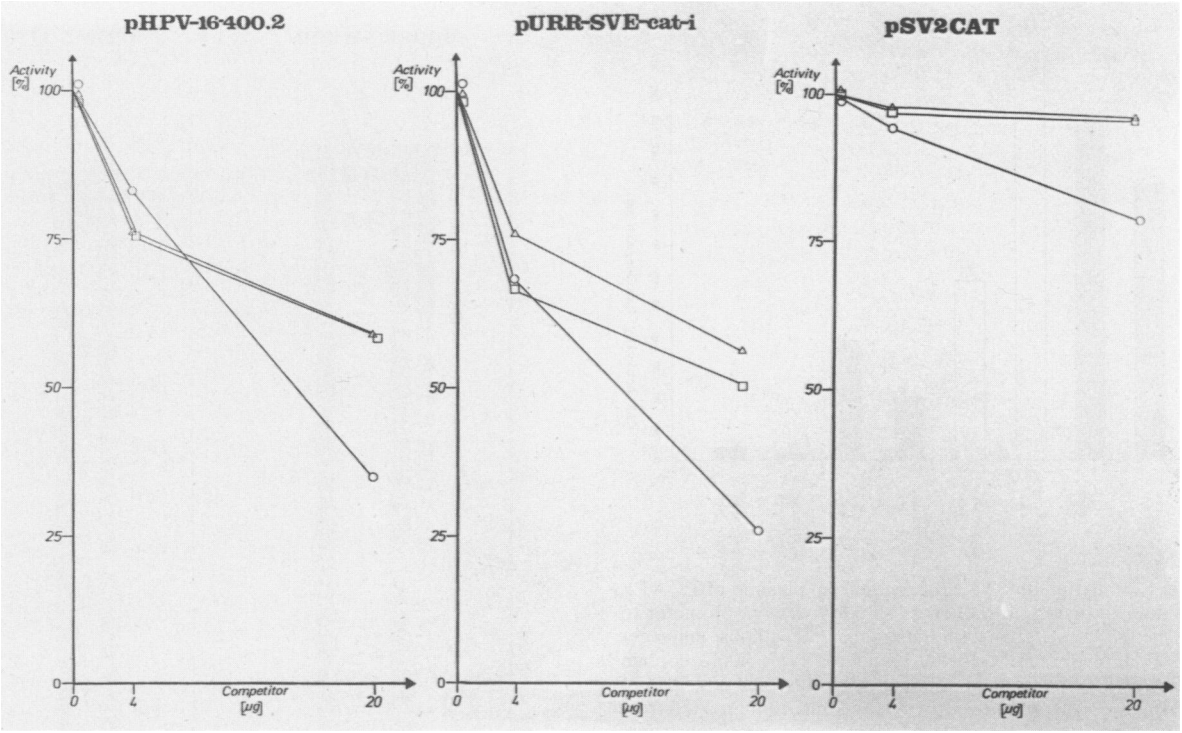


FIG. 4. Competition for expression of the CAT enzyme under the control of the HPV-16 cell-type-specific enhancer in HeLa cells. In pHPV-16-400.2, CAT expression occurs from fusion of a 400-bp segment of the HPV-16 LCR to the herpes simplex virus *tk* promoter. In pURR-SVE-cat-i, the whole LCR of HPV-16 has been fused to the SV40 early promoter (with the SV40 enhancer deleted). In pSV2CAT, the CAT gene is transcribed by the SV40 early promoter under the control of the SV40 enhancer. Competition with 4 μg of competitor plasmids represents a 3-fold molar excess; competition with 20 μg represents a 15-fold molar excess. The competitors were pHPV-16-14, a pUC18 construct with 610 bp of the HPV-16 LCR including the HPV-16 enhancer (○); p877, which is pUC18 with five 18-bp oligonucleotides inserted which include fp3e (△); and p878, which is pUC18 with six 20-bp oligonucleotides inserted that include fp6e (□). One hundred percent activity is the conversion of 17 pmol of chloramphenicol per min per mg of protein for pHPV-16-400.2, 8 pmol/min per mg of protein for pURR-SVE-cat-i, and 15 pmol/min per mg of protein for pSV2CAT.

viruses, enhanced transcription of cellular and most viral genes does not normally occur under the influence of a single protein recognizing a single DNA sequence motif but, rather, through cooperation of many such elements, as documented originally for the SV40 enhancer (66). It seems likely that the cell-type-specific enhancer of HPV-16 functions in a similar way and that only a detailed analysis of deletions and point mutations would reveal individual modules. To approach such a goal, we have cloned three fragments of the 400-bp enhancer segment (positions 7454 and 7854) into pBLCAT2 and tested for their functions as enhancers (Fig. 5). A 232-bp *Dra*I fragment (positions 7524 to 7755 [Fig. 2]) is a slightly stronger enhancer than the 400-bp fragment. It does not contain fp1e, fp8e, or fp9e, excluding a relevant positive function of these three motifs. Subdivision of this 232-bp fragment at a single *Hha*I site at position 7675 results in a 152-bp *Dra*I-*Hha*I fragment (positions 7524 to 7675) with fp2e, fp3e, and fp4e and its flanking protections. This fragment exhibits a reduced enhancer activity, and the complementing 80-bp *Hha*I-*Dra*I fragment (positions 7676 to 7755) with fp6e and fp7e is inactive. We conclude that several elements on both sides of the *Hha*I site are necessary to give the full enhancer function.

**DNase I protections 5′ and 3′ to the enhancer segment in the HPV-16 LCR.** A map of specific interactions between nuclear proteins and HPV-16 DNA is a useful prerequisite for experiments aimed toward an understanding of the regulatory functions of the LCR of this virus. Therefore, we extended our footprint analysis toward the identification of elements of DNA-protein interactions 5′ and 3′ to the 400-bp enhancer segment.

No function has yet been assigned to the region of HPV-16 between the *Eco*RI site at position 7454 and ORF L1 (Fig. 2). However, the homologous structural organization of all sequenced papillomavirus genomes suggests that similar functions may be found in HPV-16 as had been identified in bovine papillomavirus-1 (BPV-1). This region of the BPV-1 genome contains an origin of replication (34, 46, 64); a promoter (P1) at position 7186, which is involved in replication (3, 55); and a promoter relevant for late-gene expression (3). An ORF E6-dependent enhancer activity had been proposed for the genome of HPV-18 (19).

Figure 6 documents the footprints in this region of HPV-16 which we termed fp10l to fp0l. Interesting features of this analysis are the localization of fp10l and fp9l within ORF L1 and the overlap of fp7l, fp6l, fp5l, fp4l, and fp2l with a 10-bp repeated sequence (fp6l: 5′-GTATGTGCTT-3′). fp0l (data not shown) and the segment fp1l, which is protected over 119 bp at a concentration of 10 μg of nuclear protein per μl, may stem from low-abundance and/or low-affinity proteins, since small changes of the protein concentration led to disintegration of these protected regions into smaller footprints with poorly defined borders.

The genomic sequences of five types of human papillomaviruses which infect epithelia of the genitals (HPV-6, HPV-11, HPV-16, HPV-18, and HPV-33) (7, 8, 11, 50, 52) exhibit similarities upstream of the promoter corresponding to P97 in HPV-16 (see Fig. 8): namely, a TATA box supposedly

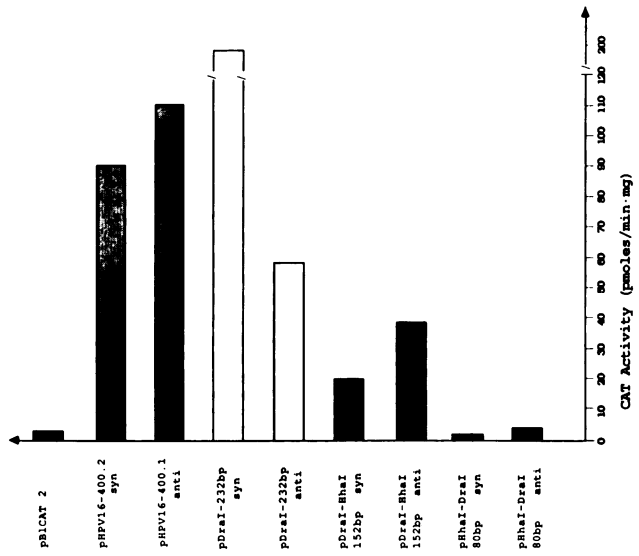


FIG. 5. Stimulation of CAT expression from plasmid pBLCAT2, which contains a fusion of the herpes simplex virus *tk* promoter to the CAT gene, by several subfragments of the 400-bp enhancer segment of HPV-16. pHPV-16-400.1 and pHPV-16-400.2 contain both orientations of a fragment between positions 7454 and 7854 of the HPV-16 LCR (20); pDraI-232bp syn and anti contain a subfragment between positions 7542 and 7755. Two further subclones show that the 152-bp 5′ segment of the 232-bp fragment still contains reduced enhancer activity, whereas the 80-bp 3′ segment is not functional. The reduction observed between the 232- and the 152-bp fragment may indicate that some enhancer functions are located on the 80-bp fragment.

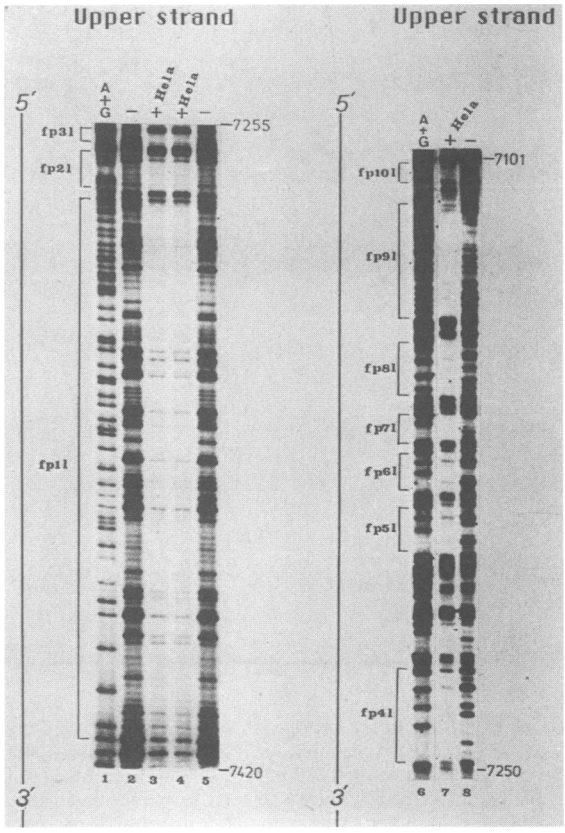


FIG. 6. DNase I protections by nuclear extracts from HeLa cells of sequences between ORF L1 and the *Eco*RI site at position 7454. The footprints were obtained by labeling either at the *Eco*RI site (position 7454 [left gel]) or in a polylinker cloned at the *Dra*I site (position 7284 [right gel]). The concentration of nuclear proteins in this footprint reaction was 10 μg/μl in this experiment. At 2 μg of protein per μg, the large segment fp11 was barely protected.

relevant for early promoter activation, two tandem palindromic E2 protein-binding sites upstream of this TATA box, a segment of 10 bp upstream of these two E2-binding sites with a large degree of interspecies homology, and a sequence reminiscent of a second TATA box which has not yet been observed to initiate transcripts. We cannot identify CCAAT - or SPI boxes in the HPV-16 promoter region as relevant for the transcription of many cellular or viral genes.

In the absence of the viral E2 protein, three footprints, termed fp1u, fp2u, and fp3u, occur in this region (Fig. 7). Interestingly, two of them overlap with the E2 protein-binding sites. fp2u overlaps with the above-mentioned sequence homology between five papillomavirus types upstream of the E2 protein-binding sites and may represent an HPV-specific distal promoter element (Fig. 8). fp1u was consistently seen only with MCF7 and 3T3 extracts but not with HeLa extracts. We are in the process of analyzing the functional relevance of this differential footprint. In some gels, protection of the P97-TATA box but not of the TATA box upstream of fp2u can be visualised.

## DISCUSSION

All papillomavirus genomes analyzed exhibit a homologous organization despite significant sequence diversity (18). Ten large ORFs in similar relative positions cover 90% of the approximately 8,000-bp circular double-stranded DNA genome. A segment encompassing roughly 10% of the genome has been termed the upstream regulatory region (URR) or LCR and does not exhibit large ORFs.

The analysis of transcripts from BPV-1 led to the identification of four promoters in the LCR of this virus (3, 59). All promoters face in the same direction, namely, away from ORF L1 (5′ end of the LCR) toward ORF E6 (3′ end of the LCR). A principal promoter for ORFs E6 and E7 is 5′ to the ATG of E6 at the 3′ end of the LCR (3). Homologous promoters have been found in various other papillomaviruses, and its equivalent in HPV-16 has been termed P97 (55). In tissues producing BPV-1 viral particles, transcripts derived from a promoter, PL, located on the 5′ side of the LCR are the only ones identified as containing the ORF L2 and ORF L1 necessary for the translation of viral capsid proteins. A BPV-1 promoter at position 7186 has been identified as important for expression of the ORF E1 (3, 58) and seems to be involved in regulating the replication origin of BPV-1 (34, 46).

Transcriptional analysis of papillomaviruses had concentrated on functions of proteins expressed from ORF E2. A protein derived from the complete ORF E2 can bind to the sequence 5′-ACCN$_6$GGT-3′, thereby acting on linked promoters in an enhancerlike fashion (1, 24, 26, 31, 39, 45, 56). A protein derived from the 3′ part of ORF E2 has similar binding properties but negatively regulates linked promoters, possibly by interfering with the binding of the large E2 protein (30). Only recently, interactions between cellular transcription factors and papillomavirus DNA have been demonstrated. One unidentified nuclear factor from HeLa cells binds 3′ to the promoter P1 that is relevant for BPV-1 replication (58), and the glucocorticoid receptor binds to the

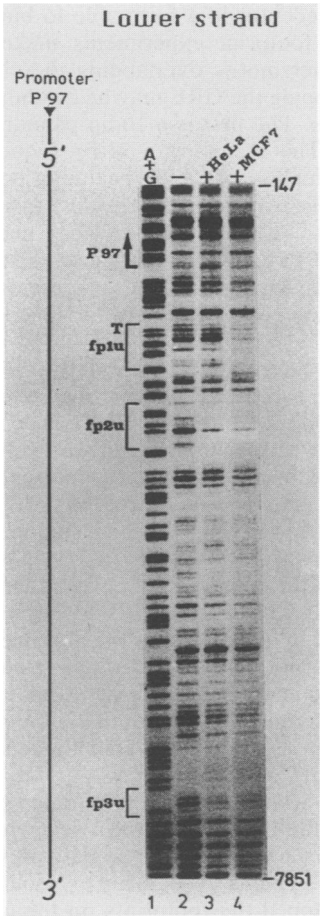


FIG. 7. Footprints between position 7850 and the promoter P97. This 150-bp segment of HPV-16, which may contain distal elements of P97, did not exhibit footprints as strong as those of other segments of the HPV-16 LCR. Three consistently observed weak protections are named fp1u, fp2u, and fp3u. Interestingly, the first two overlap with the papillomavirus E2 protein-binding sites. fp1u could not be visualized with HeLa extracts. The letter T indicates the position of the P97 TATA box.

GRE within the LCR of HPV-16, thereby regulating P97 (20).

To create a basis for an extensive functional analysis of HPV-16 *cis*-responsive elements activated by cellular proteins, we have identified sequences in the LCR which bind nuclear factors. This paper documents that more than 50% of the LCR of HPV-16 is protected by sequence-specific DNA-binding proteins, as judged by using the DNase I footprinting technique. The map of these DNA-protein interactions (Fig. 2) will serve as a valuable tool for pinpointing *cis*-acting elements within the HPV LCR that are relevant for gene expression and replication of HPV.

Toward this end, we have made an effort to identify functional elements of the cell-type-specific enhancer of HPV-16. This enhancer functions independently from HPV gene products such as those derived from ORF E2 (10, 20), but it is possible that the effect of this enhancer is overrun by ORF E2 products during persistent infection (10). P97-dependent transcription may be under the unmodulated activation of this enhancer in tumour cells, in which E2 proteins may be absent as a result of the disruption of the viral genome after integration into cellular DNA (51). We hope that the analysis of this enhancer will lead to the identification of transcription factors relevant for papillomavirus cellular tropism or even for the regulation of gene expression during epithelial differentiation, mechanisms possibly exploited by papillomaviruses.

Our DNase I protection experiments identified nine footprints (fp1e to fp9e) within a 400-bp fragment between position 7454 and 7854 that we used previously to test the enhancer function. fp1e, fp2e, fp3e, fp6e, fp7e, and fp9e were reproducible in numerous experiments, and their intensities and borders did not vary significantly with the source and the concentration of the nuclear extract. In contrast, details of fp4e, fp5e, and fp8e were dependent on the concentration of nuclear protein. Some of these variations are likely to represent the binding of low-affinity or low-abundance proteins which protect a 106-bp segment including fp4e, fp5e, and fp6e at high protein concentrations (see below).

Seven footprints, fp1e, fp2e, fp3e, fp5e, fp6e, fp7e, and fp8e, contain the sequence 5′-TTGGC-3′, which has been shown to be a recognition site for the transcription factor NFI/CTF (see reference 27 and references therein). The competition experiments documented in Fig. 3 led us to propose that NFI is the factor that recognizes these seven motifs on the HPV-16 DNA. The length of the protected sequences (12 to 18 bp) and the lack of a palindromic structure of these recognition sites suggest that this factor binds in HPV-16 as a monomer rather than as a dimer as is the case for its high-affinity palindromic binding sites (13, 27). Only fp7e extends the size range of the other six footprints by showing protection at the lower strand over 13 bp upstream of the TTGGC motif, suggesting the binding of a different factor close to the TTGGC motif on this strand (Fig. 1).

In the light of evidence that quite different proteins can bind to motifs seemingly similar to those recognized by NFI/CTF (15, 23), the exact nature of the factors bound to the HPV-16 motifs will have to be further evaluated by mutational and gel shift analysis. One of these seven sites, fp6e, covers the motif 5′-TTTGGCTT-3′, which was earlier identified as a consensus element between the promoters of several cytokeratin genes and of the LCR of different types of HPVs. This motif was suspected to have a role in cell-type-specific gene expression (5, 10). A role of this motif in specific gene regulation seems to us to be less likely, since we observed ubiquitous occurrence of factors that give identical-looking footprints on six other related sequences in the HPV-16 LCR.

NFI had initially been identified to participate in the

| | TATA-box ? | fp2u | E2 binding site | | E2 binding site | fp1u | TATA-box |
|---|---|---|---|---|---|---|---|
| HPV-16 | TCATG TATAAAAC | TAAGGGCGTA | ACCGAAATCGGT | TGA | ACCGAAACCGGT | TAGTATAAAA | |
| HPV-18 | TGTAG TATATAAA | AAAGGGAGTA | ACCGAAAACGGT | CGGG | ACCGAAAACGGT | GTATATAAAA | |
| HPV-33 | AAGTT TTAAAAAA | GTAGGGTGTA | ACCGAAAGCGGT | TCA | ACCGAAAACGGT | GCATATATAA | |
| HPV-6b | TCTTG GTTTAAAA | AATAGGAGGG | ACCGAAAACGGT | TCA | ACCGAAAACGGT | TGTATATAAA | |
| HPV-11 | TCTTA GTTTAAAA | AAGAGGAGGG | ACCGAAAACGGT | TCA | ACCGAAAACGGT | TATATATAAA | |

FIG. 8. Sequence comparison between a segment upstream of promoter P97 of HPV-16 and the corresponding sequences of other HPV types. Homologies in a segment that showed DNase I protection (fp2u) upstream of 5′ E2 protein-binding site 7 suggest a common factor binding to a potential distal promoter element (underlined letters). fp1u could not be visualized with nuclear extracts from HeLa cells. At least in these cells, the corresponding protein may not be required for P97 function (20).

replication of adenovirus DNA (42). Its involvement in gene regulation had been suggested to occur in the promoter of the mouse mammary tumour virus, in which glucocorticoid responsiveness seems to be dependent on a 5'-TGGCA-3' motif adjacent to the GRE (9, 38). A protein isolated from chicken cells was shown to be functionally homologous to NFI and was implicated in tissue-specific lysozyme gene expression (54). NFI may be involved in the regulation of the type 1 collagen promoter by transforming growth factor beta (47). As to viral enhancers, NFI interacts with several motifs in the complex cytomegalovirus immediate early promoter (25). In the cell-type-specific promoter of the albumin gene, NFI binds to a TGGCA motif, but interestingly, not to an adjacent CCAAT box. In this promoter, NF1 contributes to efficient function, whereas cell type specificity is dependent on adjacent elements (6, 32).

A similar situation may prevail in HPV-16. We have performed competition experiments for the function of the HPV-16 enhancer by using a 15-fold molar excess of plasmids containing five and six homologous TTGGC motifs. This competition reduced CAT expression from HPV-16 enhancer-dependent fusion genes by 40 to 50%, suggesting that some of the TTGGC elements contribute to the HPV-16 enhancer function. Polymerization of various oligonucleotides representing these HPV-16 motifs did not lead to artificial enhancers (data not shown), as is the case for SV40 enhancer motifs (48). This suggests the need for cooperation of additional elements in the HPV-16 LCR with the TTGGC elements.

To narrow the search for sequences relevant for the enhancer, we tested the function of subclones of the 400-bp fragment previously shown to contain the enhancer. A 232-bp fragment (position 7524 to 7755) gave slightly stronger enhancement of *tk* promoter-dependent expression of the CAT gene than the 400-bp fragment did. By *Hha*I cleavage, this 232-bp fragment can be split into a 152-bp fragment (positions 7524 to 7675) and an 80-bp fragment (positions 7676 to 7755). The 152-bp fragment contains reduced enhancer activity, whereas the 80 bp fragment is inactive.

We assume that several elements flanking the *Hha*I site at position 7675 have to cooperate for full enhancer function. This is also suggested by the observation that deletions of sequences close to this position gradually lose enhancer activity (10). Candidates for these elements are fp2e to fp7e, or the 106-bp segment (positions 7612 to 7717) protected only at increased protein concentrations. A correlation of the extent of DNase I protection with variations in protein concentrations has been observed for the albumin promoter (32). The 106-bp segment of HPV-16 that behaves in this way may be bound by numerous transcription factors whose binding sites may overlap and which may exclude one another. We have shown previously that a motif between positions 7641 and 7655 binds the glucocorticoid receptor, which exerts hormonal responsiveness to P97 and to heterologous promoters over a distance of more than 300 bp (20). To produce long-range effects, the glucocorticoid receptor may have to cooperate with a factor bound in proximity, as had been shown to be a prerequisite for the function of the GRE in the promoters of the mouse mammary tumor virus (9, 38), the tryptophan oxygenase gene (49), and the tyrosinaminotransferase gene (4). We do not know the nature of such a factor; however, a candidate may be API (2), which binds close to the GRE (positions 7630 to 7637) and makes the HPV-16 enhancer responsive to phorbol esters (Chan and Bernard, in preparation). In the absence of receptor activation by the hormone, extracts should not contain glucocorticoid receptor in a form able to bind to DNA (4). Therefore, our footprint experiments make it likely that factors bind other motifs overlapping the GRE. Three sequences overlapping the GRE may be candidates for protein recognition sites. The first is a 10-bp palindrome (positions 7636 to 7651). The second is a 21-bp sequence (positions 7646 to 7666) which is directly repeated at positions 7301 to 7321, where it is part of fp1l. Thirdly, fp4e contains two (positions 7637 to 7630 and 7658 to 7664), and fp9e contains one (positions 7816 to 7810) consensus sequence to a factor API-binding site. As shown above, an oligonucleotide representing fp9e seemed to compete for fp9e as well as fp4e (Fig. 3).

Our efforts to differentially footprint the HPV-16 enhancer did not indicate a cell-type-specific element. One possible explanation would be that a cell-type-specific protein not making contact with DNA but binding to a ubiquitous factor activates the enhancer, leading to different protein-DNA interactions in vivo and in vitro (4). Alternatively, two factors could bind the same element, only one factor conferring a cell-type-specific function, as was shown for the octamer motif of the enhancers of SV40 and immunoglobulin genes (12, 17, 65). Thirdly, this HPV-16 segment could be so densely covered by proteins that the absence of any particular factor does not lead to differences detectable by the footprint technique. A detailed analysis of this region is in progress to elucidate the nature of the DNA-protein interactions necessary for the cell-type-specific enhancer of HPV-16.

To determine whether the large number of nuclear proteins binding to the HPV-16 enhancer segment is an idiosyncrasy of a fragment that was functionally preselected and to pinpoint other elements recognized by cellularly encoded proteins, we mapped the footprints on the upper strand of the LCR 5' and on both strands 3' from the enhancer segment (Fig. 2, 6, and 7). One interesting outcome of this study is the presence of footprints within ORF L1. This may point toward elements relevant to replication as found in the BPV-1 genome within ORF L1 (34). Six footprints in this region overlap with repeats which also occur in the enhancer segment. One of these repeats which has the sequence 5'-GTATGTGCTT-3' in fp6l, correlates to fp7l, fp6l, fp5l, fp4l, and fp2l and is also protected in the enhancer segment (5'-ATATGTGCCT-3' positions 7708 to 7699). Part of fp1l is the sequence 5'-ATTGTGTCATGCAACATAAAT-3' (positions 7301 to 7321), which is repeated overlapping with the GRE in the enhancer segment (positions 7645 to 7665). Proteins binding to these sequences may functionally contribute to a variety of HPV-16 *cis*-acting elements.

Two footprints were observed immediately upstream of P97. fp1u and fp2u partially overlap with the papillomavirus E2 protein recognition sites, with fp1u observable only with MCF7 extracts. It will be interesting to study whether these factors cooperate or compete with E2 proteins and whether the apparent cell type specificity of fp1u is functionally relevant. Homologies between different papillomaviruses over the sequence covered by fp2u suggests the occurrence of a common distal promoter element. In HPV-16, this sequence is reminiscent of the binding site 5'-GGGCGG-3' for the factor SP1. However, the T at position 33 of HPV-16 and sequence variation between papillomaviruses make it unlikely that fp2u is due to binding of this factor (28, 29). Distal elements different from the frequently encountered CCAAT and SP1 boxes seem to be relevant for HPV-16 promoter function (Fig. 8).

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