Volume 17 Number 9 1989 Nucleic Acids Research

Clusters of nuclear factor I binding sites identify enhancers of several papillomaviruses but alone are not sufficient for enhancer function

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Received January 31, 1989; Revised and Accepted March 31, 1989

ABSTRACT

The long control region (LCR) of human papillomaviruses (HPV) encompasses 5-12% of the viral genome and contains an intricate network of cis responsive elements. In the LCR of seven unrelated HPV-types, namely HPV-1, 6, 8, 11, 16, 18 and 33, we have identified clusters of 4 to 7 5-TTGGC-3 motifs suggesting nuclear factor I (NFI) binding sites. We randomly selected 20 (out of a total of 38) of these motifs and showed that pure NFI from porcine liver protects virtually the same nucleotides as a factor present in crude HeLa nuclear extracts. The footprints obtained with HeLa extracts in the LCR of HPV-16 are eliminated in competition experiments by an oligonucleotide representing the palindromic adenovirus NFI binding site. Restriction fragments from the genome of HPV-11, 16 and 18, which contain this cluster of NFI binding sites associated with binding sites of unrelated transcription factors, function as transcriptional enhancers. In contrast, a fragment from HPV-8 exhibiting exclusively NFI binding sites, or polymerized NFI sites from HPV-16, are functionally inactive. NFI seems to be necessary but not sufficient for HPV enhancer activation.

INTRODUCTION

Human papillomaviruses (HPV) are strictly epitheliotropic viruses which cause proliferation in a restricted range of target cells : HPV-1 is found in flat warts of palms and soles, HPV-2 and HPV-8 in skin epidermis and HPV-6, 11, 16, 18 and 33 in mucosal lesions, mostly of the genitals. Scientific interest has concentrated on those types which induce lesions with a tendency to progress towards cancer, namely HPV-8, 16, 18 and 33 of those mentioned here (for reviews see 1-3).

The 7500-8000 bp circular double-stranded DNA genome of papillomaviruses contains a segment which is devoid of major open reading frames and encompasses 5-12% of the genomic length. This segment has been termed long control region (LCR) after accumulated evidence showed that it contains an intricate network of elements regulating transcription and replication (4-6 and references therein). One of these elements is a cell type specific

enhancer possibly involved in the epitheliotropism of HPVs that we and others detected in HPV-16 (7,8), and that seems to have an equivalent in HPV-6, -11 and -18 (9-11). We recently published a footprint analysis of the complete LCR of HPV-16, which led to the identification of 23 DNA segments that bind nuclear proteins (12). 7 of these 23 footprints contain the motif 5'-TTGGC-3', and all are located in the central third of the LCR that has an enhancer function. Footprint competition experiments with homologous oligonucleotides led us to propose that these 7 but not several other elements unrelated in sequence bind the same nuclear factor, which may be the nuclear factor I (NFI) as judged by sequence homology to known binding sites (13). In transfection competition experiments with HeLa cells, oligonucleotides representing the HPV-16 TTGGC motifs eliminated at least 50% of the HPV-16 enhancer activity proving a function of these motifs (12).

NFI was originally identified as a protein that binds the partially palindromic sequence 5'-TGGAN$_5$GCCAA-3' on the adenovirus genome with a function in its DNA replication (14,15). Subsequently, numerous related palindromic or monomeric sequence elements have been identified as sites of DNA/protein interaction being functionally involved in promoter and enhancer function (16-24), and subsequently the classical CCAAT-box of eukaryotic promoters has been shown to bind the same factor which was then termed NFI/CTF (25,26). Recently, several groups have identified various proteins binding this motif. Recognition site chromatography identified proteins seemingly identical in their binding site specificity but divergent in molecular weights (25-28). These proteins may be homologous and derived from the same gene (28) as it was shown for divergent NFI transcripts from HeLa cells (29). The picture is further complicated by the detection of additional proteins with similar DNA sequence specificities but strongly divergent affinities towards individual promoter sequences (30-32).

In the experiments leading to this publication, we wanted to accumulate arguments for the identity of the HeLa cell factor, which binds the 7 TTGGC motifs on the HPV-16 enhancer. Binding studies with purified NFI, footprint competition experiments and previously published in vivo competitions enabled us to propose that HeLa cell NFI binds and contributes to the function of the HPV-16 enhancer. The dependence on NFI binding sites may be a universal requirement of human papillomaviruses. Analysis of the sequence from seven out of nearly sixty human papillomavirus genomes (33-39) shows clusters of 4-7 normally non-palindromic TTGGC elements in

the LCR of each virus, which bind pure NFI as well as the corresponding factor from HeLa cells.

MATERIALS AND METHODS

The sequences of HPV-1, 6, 8, 11, 16, 18 and 33 have been published (33-39). A RsaI fragment from the LCR of HPV-8 between positions 7502 and 21 was inserted into the HincII site of pUC18 (40), labelled after cleavage at the EcoRI site of pUC18 next to position 21 of HPV-8 and recut with NarI in pUC18. An AluI fragment from the LCR of HPV-11 between positions 7219 and 7814 was inserted into the HincII site of pUC18, labelled at the HindIII of pUC18 site next to position 7814 of HPV-11 and recut with EcoRI. A fragment from the LCR of HPV-16 was cloned as an EcoRI/BamHI fragment between positions 7455 and 7854 or as a DraI fragment (pos. 7523 and 7755) into pUC18 and labelled and recut within the polylinker sequences (7). A RsaI fragment (pos. 7510 to 7740) from the LCR of HPV-18 was cloned into the HincII site of pUC18 labelled at the EcoRI site in the polylinker next to HPV-18 position 7746 and recut with NarI. In each case, labelling was done using the Klenow polymerase reaction. Nuclear extracts were prepared according to Dignam et al. (41) as modified by Wildeman et al. (42). Nuclear factor I (NFI) was purified from porcine liver to homogeneity as described (27).

Footprint reactions (43) were carried out in 10 µl buffer containing 100mM KCl, 4mM spermidine, 5mM $MgCl_2$, 0.25mM DTT, 0.1mM EDTA, 10% Glycerol, 10mM Hepes pH 7.9 and 50 µg protein (HeLa nuclear extracts) or 7 ng (pure NFI). The nuclear proteins were incubated in this buffer together with 100ng poly [dI-dC] for 15 min on ice followed by the addition of 1-2ng (5000-10,000 Cerenkow cpm) asymmetrically ^{32}P labelled probe fragment. Incubation was continued for 15 min on ice. The samples were digested with DNase I (25ng/µl) for 90 sec at 25°C, treated with phenol/chloroform and ether and DNA recovered by two ethanol precipitations. Electrophoresis, together with chemically cleaved DNA (44) was done on 6% or 8% denaturing, 0.4mm thick polyacrylamide gels.

For footprint competition experiments, 3 - 50 ng double stranded synthetic oligonucleotides were added 5 min before the labelled DNA fragment. All further reactions were carried out as described above. The adenovirus NFI binding site was represented by the doublestranded oligonucleotide 5'-AATTCTTATTTTGGATTGAAGCCAATAATCG-3' (15).

For the study of enhancer functions, the fragments from HPV-8, 11 and 16

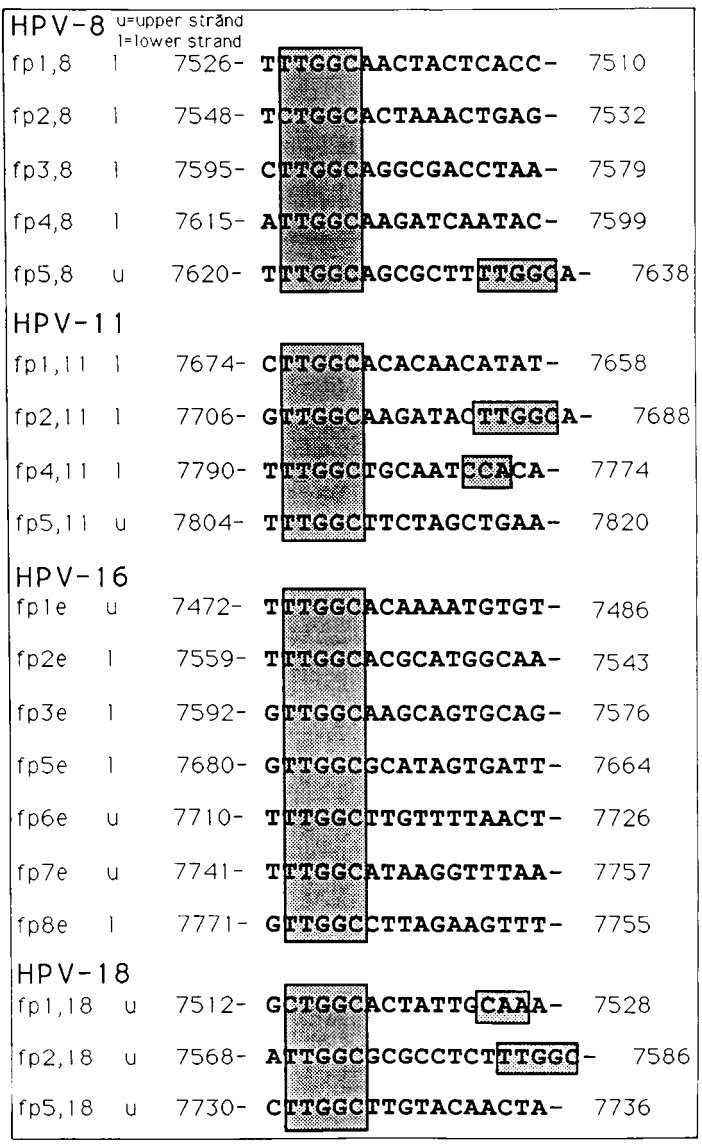

Table 1. Sequence of 22 nuclear factor I (NFI) binding sites in the long control region (LCR) of human papillomavirus (HPV) type 8, 11, 16 and 18. A search for 100% homology to the sequence 5'-TTGGC-3' identified 20 motifs in the 4 viral LCRs (Fig. 2). 18 of these 20 motifs (all except HPV-8 position 68 and HPV-18 position 7475) were analyzed with the DNase I protection technique. 17 of these 18 footprints showed similar footprints with pure NFI and with HeLa nuclear extract (Fig. 1, 3, 4), only HPV-8 fp4,8 showed significantly more protection with HeLa nuclear extract than with pure NFI. This list includes 2 additional NFI binding sites which were not identified in the sequence search but visible as footprints.

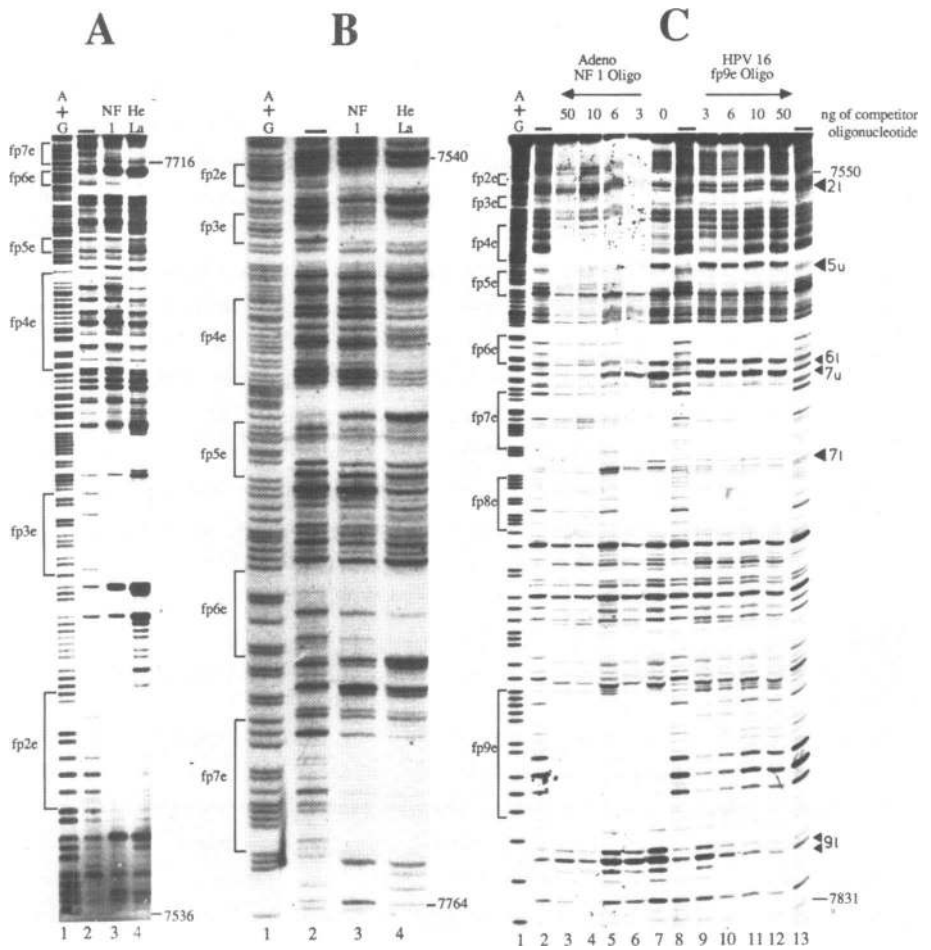

Fig. 1. DNase I protection analysis of an enhancer segment from the long control region (LCR) of human papillomavirus type 16 (HPV-16). A pure preparation of nuclear factor I (NFI) and HeLa nuclear extracts lead to similar footprints covering TTGGC motifs on the lower (A) and upper strand (B). fp4e is visible with HeLa extracts only. It does not contain a TTGGC motif and is derived from the overlapping binding of different transcription factors including 2 AP-1 factors (12). (C) Competitions for footprints. Right half: a 31 bp oligonucleotide representing fp9e eliminates the footprints fp4e and fp9e, probably by competing for binding of AP-1. Left half: an oligonucleotide representing the adenovirus NFI binding site eliminates fp2e, fp3e, fp5e, fp6e, fp7e and fp8e but not fp4e and fp9e. Triangles point to positions, where the disappearance of enhancements which flank a footprint is more diagnostic than the disappearance of the footprint. u and l refer to upper and lower enhancements flanking footprints.

described above were cloned in both orientations into pUC18, retrieved from this vector in form of HindIII-BamHI fragments and inserted into pBLCAT2, cut with HindIII and BamHI (45), to give the vectors pHPV-8-LCR1 (sense orientation), pHPV-8-LCR2 (antisense orientation), etc. pHPV-18-LCR1 and 2 contained in the BamHI site of pBLCAT2 a BamHI fragment from HPV-18 (positions 6229 to 119), encompassing the RsaI core enhancer fragment of this virus (11 and see above).

For transfection of HeLa cells, (a cell line derived from a human cervical carcinoma), or T47D cells ((46), a cell line derived from a human mamma carcinoma), we electroporated (47) the cells using a Biorad Gene Pulser. HeLa cells were harvested 20 h, and T47D cells 40 h after transfection. Cell extracts were prepared by freeze-thawing, and CAT assays were performed using 50 µg protein and 30-60 min incubation at 37°C for HeLa extracts, or 125 to 250 µg protein and 90 min incubation for T47D cells. The CAT activity was determined as percent conversion of chloramphenicol and is given in pmole conversion per min and mg protein (48).

RESULTS

7 elements of the cell type specific enhancer of HPV-16 bind purified NFI factor

A fragment representing 400bp of the 850bp long control region (LCR) of HPV-16 functions as a cell type specific enhancer (7) and exhibits 9 footprints with HeLa nuclear extracts, that we termed fp1e to fp9e (12). 2 of these footprints, fp4e and fp9e, bind a complex assortment of factors including steroid receptors and AP-1, while the other 7 footprints originate from the binding of a single type of factor to 7 non-palindromic TTGGC motifs (table 1). We have shown this by footprint competition experiments with oligonucleotides representing homologous sequences (12). One of these 7 motifs has the extended sequence TTTGGCTT which had been suspected to be a motif peculiar to keratin genes (49), confering epithelia specific gene expression to keratinocyte specific papillomaviruses (8). To test whether some or all of these 7 motifs bind NFI or novel transcription factors we performed comparative footprint experiments with HeLa nuclear extracts and pure NFI from porcine liver. Fig. 1A and B shows fp2e, fp3e, fp5e, fp6e and fp7e, which cover TTGGC motifs, protected by the NFI preparation on both strands in a manner indistinguishable from crude HeLa nuclear extracts. fp1e and fp8e are not visible on the gel shown here, but behaved like the other 5 TTGGC motifs. In contrast, fp4e, which contains

overlapping binding sites for AP-1 (50), steroid receptors and further unidentified transcription factors (7,12) is visible with HeLa extracts only.

Footprints on the NFI binding sites of the HPV-16 enhancer are competed by the adenovirus NFI binding site

To characterize further the sequence requirements of the HeLa factor that binds the HPV-16 TTGGC motifs, we performed footprint competition experiments with a doublestranded oligonucleotide representing the palindromic NFI binding site on the adenovirus genome. Fig. 1C documents that increasing concentrations of the adenovirus sequence eliminate fp2e, 3e, 5e, 6e, 7e and 8e but not fp4e and fp9e, which do not contain the TTGGC motif. In these experiments, the molar excess of the competitor was approximately 100 fold. This excess is sufficient to eliminate NFI binding to proven NFI binding sites, but insufficient to eliminate divergent factors such as CB1 or CB2 which recognize related sequences (31).

To complement our observations, we could eliminate fp4e and fp9e, likely AP-1 binding sites, with an oligonucleotide representing fp9e, without affecting the footprints on 6 TTGGC motifs (Fig. 1C). Taken together we interpret our results such that the footprints on the TTGGC motifs of the HPV-16 enhancer originate from a protein indistinguishable in its protection pattern and its affinity from NFI.

A cluster of TTGGC motifs occurs in the long control region of all sequenced human papillomaviruses

We have previously shown by in vivo competition experiments that the enhancer of HPV-16 depends functionally on proteins binding to its TTGGC motifs (12). We asked whether HPV-16 contains these elements fortuitously in the context of its different transcriptional elements, or whether they occur also in unrelated human papillomaviruses. The genomic sequences of HPV-1, 6b, 8, 11, 16, 18 and 33 (33-39) were available to us and we analyzed them for the occurrence of potential NFI binding sites. Searching the 7 genomes with the sequence TTGGC, we identified (in 47000bp (additively)) a total of 98 motifs. 60 of these were spread more or less evenly over those 90-95% of the genomes which code for proteins (data not shown). We take this as a statistical occurrence of possibly irrelevant homologies. However, 38 elements occurred in clusters of 4 to 7 more or less central in a 300bp segment of the LCR of each virus (Fig. 2). This means, that they occurred nearly twenty times more frequently in this segment than in segments of equal size elsewhere in the genome. We would like to hypothesize that these clusters of potential NFI binding sites are

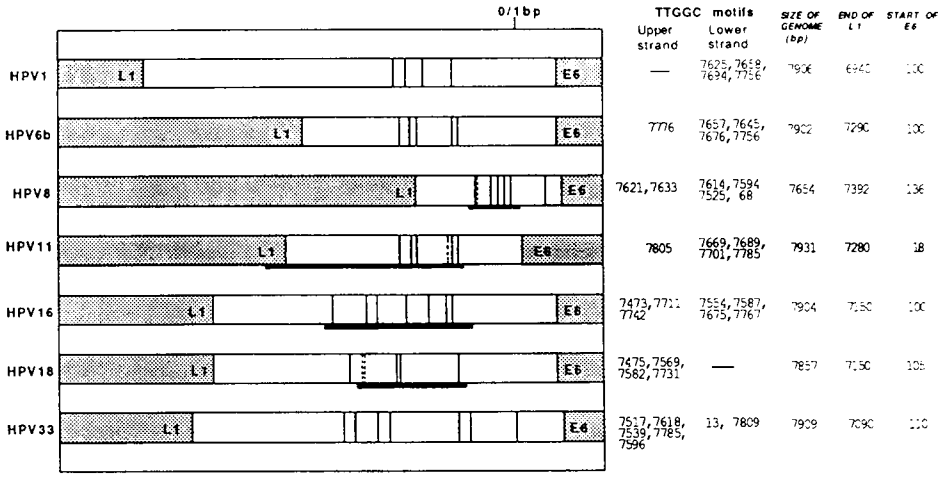

Fig. 2. Schematic representation of the NFI binding sites within a 1400 bp segment spanning the long control region (LCR) of 7 human papillomaviruses. The open reading frames L1 and E6 are indicated by stippled areas, the LCRs are shown in white. Transcription in several HPV-types has been shown to occur from a promoter upstream of E6 towards the right. TTGGC motifs are shown as vertical bars, additional NFI sites slightly divergent in sequence are shown by stippled bars. Strong horizontal lines indicate the 4 restriction fragments analyzed in this paper. The position of TTGGC motifs is given to the right of the figure, the number identifying the first T (upper strand motifs) or A (lower strand motifs). For complete sequence information, see table 1.

functionally relevant, possibly for the activation of the transcriptional enhancer.

Fragments from the LCR of HPV-8, HPV-11 and HPV-18 show similar footprints with pure NFI and HeLa nuclear extracts on all TTGGC elements.

To support our hypothesis, we decided to test whether these TTGGC motifs indeed bind transcription factors and whether this occurs in a similar fashion with HeLa nuclear extracts and pure NFI. To do this, we analyzed fragments from the LCRs of HPV-8, HPV-11 and HPV-18 after cloning into pUC18 and pBLCAT2 (45) (see MATERIALS and METHODS).

The fragment from HPV-8 exhibits 5 footprints termed fp1,8 to fp5,8 (Fig. 3A). fp2,8 is a NFI binding site protecting the sequence CTGGCA (lower strand, position 7541 to 7546), which could not be recognized in the sequence search. The other 4 footprints protect the 5 TTGGC motifs at positions 7525 (fp1,8), 7594 (fp3,8), 7614 (fp4,8) and 7621 plus 7633 (fp5,8) identified in Fig. 2 and table 1. Differences between pure NFI and

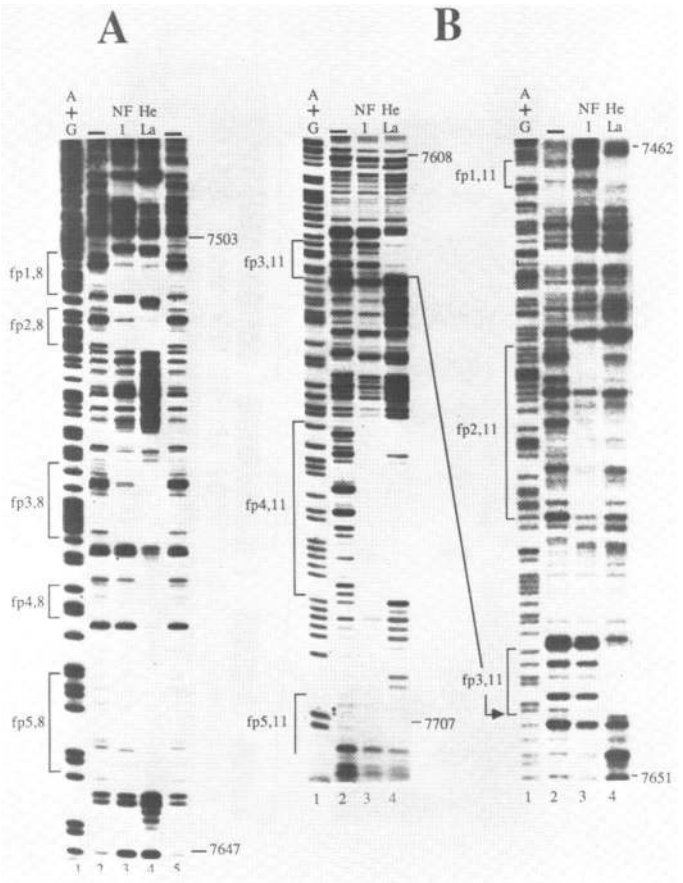

Fig. 3. DNase I protection analysis of segments with TTGGC clusters from the LCR of HPV-8 (A) and HPV-11 (B). fp1,11 and fp3,11 can be identified with HeLa extracts only and may stem from the binding of factor AP-2 and AP-1. The other 8 footprints protect NFI binding sites and show similar protection with pure NFI and HeLa extracts. For differences in the protection around fp4,8 see the result section of this paper.

HeLa extract in the protection of fp4,8 and fp5,8 may stem from steric hindering of different sized NFI proteins (24-29) binding to 3 TTGGC motifs within a 28 bp segment. Beyond this difference, we did not observe footprints within the HPV-8 fragment on sequences other than TTGGC motifs by HeLa extracts as we did in HPV-16 (see above) and in HPV-11 and HPV-18 (see below).

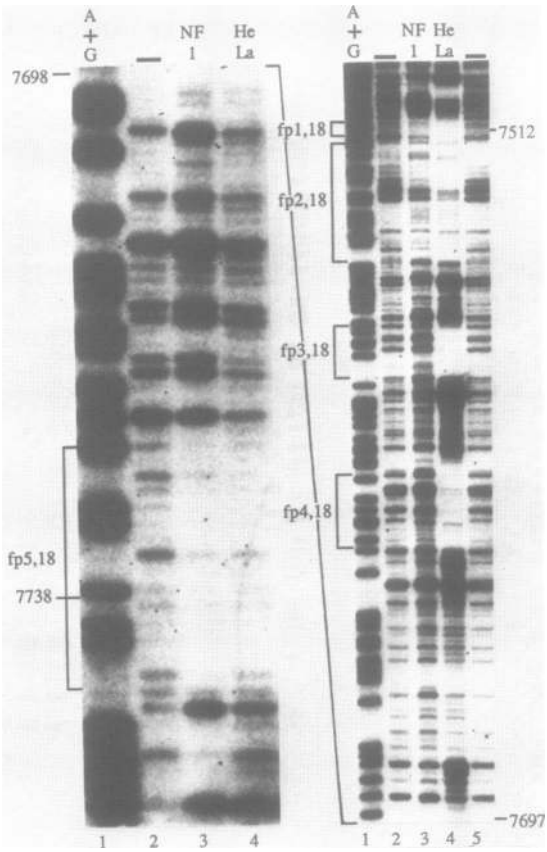

Fig. 4. DNase I protections by NFI and HeLa nuclear extracts within a fragment with enhancer activity from HPV-18. fp1,18, fp2,18 and fp5,18 are similar in NFI and HeLa lanes and coincide with TTGGC motifs. fp3,18 and fp4,18 are visible with HeLa extracts only and visualize the binding of unidentified transcription factors, as it is also possible for the upper segment of fp2,18.

In the fragment from HPV-11, we mapped the footprints fp1,11 to fp5,11 (Fig. 3B). fp1,11, visible with HeLa extracts but not with NFI, covers the sequence TACCCCCCCCACT (position 7574 to 7587), a hypothetical AP-2 binding site (51). fp2,11 identifies three tandemly repeated TTGGC motifs at positions 7669, 7689 and 7701. This segment also binds the glucocorticoid and progesterone receptor, which cannot be detected with the extracts used here (12, and our unpublished data). fp3,11, specific for HeLa extracts, overlaps the sequence TGCATGACTAAT (position 7732 to 7743), a potential

AP-1 binding site (50). fp4,11 identifies the TTGGC motif at position 7785 and occurs at the partial palindrome TGGATTGCAGCCAA. fp5,11 covers the TTGGC motif at position 7805, but is poorly visible due to the lack of DNase I sensitive bands in this region. It should be mentioned that the sequence of HPV-6 is very similar to that of HPV-11 in this region (34,35). Consequently, similar results would have been obtained with that closely related virus.

The HPV-18 fragment shows the footprints fp1,18 to fp5,18 (Fig. 4). fp1,18 stems from the binding of NFI to a CTGGCA motif at position 7513 to 7518, and fp2,18 from the protection of 2 TTGGC motifs at position 7569 and 7582. Protection between fp1,18 and fp2,18 suggests the dimeric binding of NFI to the partially palindromic motif fp1,18 (table 1) as well as the binding of additional HeLa factors. fp3,18 and fp4,18 are visible with HeLa extracts only. They cover the sequences ATTAGTCATTTTCCT (position 7617 to 7631), and GCTTGCATAACTA (position 7644 to 7656) the former possibly including an AP-1 binding site. fp5,18 protects the TTGGC motif at position 7731. A DNase I protection analysis of this HPV-segment had been published (52) but the authors identified only fp3,18 and fp4,18 and not the 4 NFI binding sites.

In summary (table 1 and Fig. 2), 20 TTGGC motifs in the LCRs of HPV-8, 11, 16 and 18 exhibit with pure NFI and with HeLa extracts a similar footprint pattern. Two additional NFI binding sites occur at the sequence CTGGC. 3 TTTGGCTT motifs, namely fp5,11 (HPV-11), fp6e (HPV-16), and the element at the HPV-18 position 7475 (table 1, footprints not shown) showed identical protections with NFI and HeLa extracts and are unlikely to differ qualitatively from the other TTGGC motifs. Only 1 or 2 out of these 22 NFI binding sites occur in the form of palindromes. The fragments from HPV-11, 16 and 18 each exhibit at least 2 additional unrelated footprints, probably in each virus including an AP-1 binding site.

HPV-DNA-segments containing the clusters of NFI binding sites exhibit cell-type-specific enhancer activity

All fragments described above were tested in pBLCAT2 (45) for their ability to stimulate expression of the chloramphenicol acetyltransferase gene (CAT) through the Herpes simplex virus thymidine kinase promoter. We have previously published that the HPV-16 enhancer gives a 50 up to 300-fold stimulation of transcription (7). The HPV-11 and HPV-18 enhancers have been described and localized on the fragments analyzed in our study (10,11). With our constructs, we found approximately 5 fold enhancement of

Plasmid	CAT-activity (pmoles/min and mg protein) HeLa	T47D
pBLCAT2	29.7	8.4
pHPV-8-LCR1	28.5	N.D.
pHPV-8-LCR2	31.5	N.D.
pHPV-11-LCR1	144.1	18.6
pHPV-11-LCR2	120.7	3.5
pHPV-16-LCR1	410.8	15.9
pHPV-16-LCR2	364.8	8.2
pHPV-18-LCR1	129.1	2.8
pHPV-18-LCR2	321.0	4.9
pORFEXCAT	748.1	112.0

Table 2. CAT activity from HPV-LCR containing vectors after electroporation of HeLa and T47D cells. HeLa cells are derived from a cervical carcinoma and exhibit activation of the cell-type-specific enhancer of human papillomaviruses. This enhancer is inactive in T47D cells, which are derived from a human mammary tumour. The enhancer of a cytomegalovirus immediate early gene, present in pORFEXCAT (53), is active both in HeLa and T47D cells.

CAT expression for HPV-11 constructs and up to 11 fold for HPV-18 constructs. The HPV-8 fragment whose footprint analysis revealed only NFI but no other transcription factor binding sites, did not stimulate CAT expression from pBLCAT2 at all (table 2).

DISCUSSION

The data presented in this paper followed our published observation that a cell type specific enhancer of the human papillomavirus-16 (HPV-16) contains 7 TTGGC motifs which suggest non-palindromic nuclear factor I (NFI) binding sites (12). One of these elements was part of the longer sequence TTTGGCTT, which had been proposed to mediate specific gene expression in epithelial cells (8,45). However, in our hands all 7 motifs were protected in footprint experiments in a similar way by an unidentified factor from HeLa cells (12).

This paper describes that all seven motifs are bound by pure NFI from porcine liver in the same way as by the nuclear factor from HeLa cells, and the footprints are eliminated by competition with the adenovirus NFI binding site. In conclusion, we propose that these motifs represent bona fide NFI binding sites.

As to sequence requirements, we see strong protection of fp1e, 2e, 3e and 7e, which contain the sequence TTGGCA, and with fp6e, which has the sequence TTGGCT. These are frequently found hexameric extensions of the TTGGC pentamer (13,17). In contrast, the in vitro binding affinity to fp5e and 8e seems to be less, both with pure NFI and HeLa extracts. These 2 elements contain the sequences TTGGCG and TTGGCC, respectively, rarely found in NFI binding sites (see table 1).

Clusters of similar sequences occur in other epitheliotropic human papillomaviruses (table 1 and Fig. 2). In HPV8, 11 and 18, just like in HPV-16, these sequences are bound identically by pure NFI and HeLa extracts. This similarity in the occurrence of transcription factor binding sites between different HPV-types is remarkable, since these viruses show (with the exception of HPV6/11) little sequence homology to one another in spite of a similar overall genome organization (4). The clustered occurrence of these NFI sites makes a related function in each virus likely. This function could be the enhancement of transcription, and 230 to 500 bp long restriction fragments from HPV-11, 16 and 18 containing these NFI clusters function as enhancers. These fragments contain two or several binding sites for unrelated transcription factors in association with the NFI clusters. In contrast to this, a fragment from HPV-8 contains, as judged by DNase I protection, exclusively NFI binding sites and does not function as an enhancer. This makes it likely that multiple NFI sites alone are not sufficient for strong transcriptional enhancement. Possibly, a factor not present in HeLa cells may have to contribute for HPV-8 enhancer activation, or the enhancer requires sites outside the tested restriction fragment.

In concordance with this failure to observe enhancer activity of the possibly pure cluster of NFI sites in this particular HPV-8 fragment is our finding that numerous vector constructs containing the HPV-16 NFI-binding sites fp2e, fp3e, fp6e or fp7e in the form of polymerized (2-7 copies), 18 to 29 bp long oligonucleotides in pBLCAT2, did not lead to any enhancer function (data not shown). We conclude that a cluster of NFI sites in the absence of binding sites for additional unrelated transcription factors does not suffice to establish an enhancer function.

At this time, we cannot resolve the enigma that the enhancer of HPV-16, and probably also those of HPV-11 and HPV-18, exhibit strong cell type specificity (7,8,10,11), while being protected in footprint experiments only by ubiquitous transcription factors, such as NFI and AP-1. Our future

research will have to concentrate on the question whether NFI occurs in the form of proteins identical in their DNA binding properties but differing in their transcriptional function. This possibility is suggested by molecular weight heterogeneity of NFI between different cell types and even within the NFI population occurring in HeLa cells (24-29). The observation of NFI heterodimers (31) could point towards a mechanism involving the binding of a DNA motif by one ubiquitous protein whose function is modified by formation of a complex with a specific factor.

ACKNOWLEDGEMENTS

The authors wish to thank Terence Chong and Gerd Klock for critical discussions, and Carol Wong for excellent typing of this manuscript.

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