Virology **272,** 40–49 (2000)
doi:10.1006/viro.2000.0363, available online at http://www.idealibrary.com on IDEAL®

A Functional NF-κB Binding Site in the Human Papillomavirus Type 16 Long Control Region

Véronique Fontaine, Els van der Meijden, Jos de Graaf, Jan ter Schegget,[1] and Linda Struyk

Department of Virology, AMC, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

Received September 2, 1999; returned to author for revision October 21, 1999; accepted April 14, 2000

By computer search, we identified one potential NF-κB binding site in the HPV16 long control region (LCR) at position 7554–7563 having two mismatches in comparison to the consensus NF-κB binding site of the Igκ L promoter. Bandshift experiments with nuclear extracts from HeLa cells or purified glutathione *S*-transferase–p65 fusion protein clearly demonstrated that NF-κB is able to bind to this region of the LCR. However, in comparison to NF-κB binding on a consensus probe, the affinity of NF-κB for this site is about 250-fold reduced. When mutations were introduced into this NF-κB binding site, the activity of the LCR was increased, strongly suggesting that NF-κB was acting as a transcriptional repressor in the context of the HPV16 LCR. In addition, overexpression of NF-κB p65 repressed the activity of the HPV16 LCR, strengthening this conclusion.

INTRODUCTION

Epidemiological and experimental studies have indicated that specific high-risk types of human papillomavirus (HPV), especially HPV types 16 and 18, contribute to the pathogenesis of cervical cancer (zur Hausen, 1996). The HPV E6/E7 early genes play a pivotal role in tumor development and also in the proliferation of cervical carcinoma cell lines. The abilities of E6/E7 proteins of high-risk HPV types to alter the function of cellular regulatory proteins such as the tumor suppressor proteins, p53 and pRb, are important progression factors contributing to chromosomal instability and the development of cell immortality. The regulation of HPV gene expression is complex and controlled by cellular and viral transcription factors. The transcription of the early region of HPV 16 is initiated at the p97 promoter and is regulated by the long control region (LCR). Many cellular transcription factors, including AP-1, C/EBPβ, nuclear factor 1 (NF-1), c-myb, Sp1, TEF-1, PEF-1, Oct-1, and YY-1, have been reported to either activate or inhibit the HPV 16 LCR (Chong *et al.,* 1990, 1991; Apt *et al.,* 1993; Tan *et al.,* 1994; Ishiji *et al.,* 1992; Sibbet *et al.,* 1995; Nürnberg *et al.,* 1995; O'Connor *et al.,* 1996; Struyk *et al.,* in press). Various immunoregulatory and hormonal factors, such as tumor necrosis factor (TNF), interleukin-1, interferons, steroid hormone, and retinoic acid, have been reported to modulate HPV transcription (Kyo *et al.,* 1994; Woodworth *et al.,* 1995; Bartsch *et al.,* 1992; Creek *et al.,* 1995; Khare *et al.,* 1996; Chan *et al.,* 1989; Chong *et al.,* 1990).

A transcription factor that plays a pivotal role in many cellular responses to environmental changes, such as virus infections, is NF-κB. NF-κB exists as homo- or as heterodimers of a family of structurally related proteins. Each member of this family possesses a conserved NH_2-terminal Rel homology domain, in which the DNA binding domain, dimerization domains, and the nuclear localization signal are located. To date, five proteins belonging to the NF-κB family have been identified in mammalian cells: p65/RelA, p50/p105, c-Rel, RelB, and p52/p100. NF-κB dimers are sequestered in the cytosol of unstimulated cells via noncovalent interactions with inhibitory proteins called IκBs. The specific interaction between the ankyrin repeats of IκBs and the Rel homology domain of NF-κB masks the nuclear localization signal of NF-κB and therefore prevents its translocation to the nucleus. Signals that induce NF-κB activity cause the phosphorylation of IκBs at Ser residues. This labels the IκBs for ubiquitinilation and subsequently for degradation, allowing NF-κB dimers to translocate into the nucleus and to regulate gene expression (May and Gosh, 1998; Stancovski and Baltimore, 1997; Woronicz *et al.,* 1997). NF-κB can be activated by cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) and by pro-apoptotic and necrotic stimuli such as oxygen free-radicals and UV light (Baeuerle and Henkel, 1994). The activity of NF-κB is also regulated by its interaction with other transcription factors such as AP-1 and C/EBPβ. These two latter proteins belong to the bZIP class of DNA binding proteins characterized by a leucine zipper structure and an adjacent basic DNA binding domain. Their bZIP region has been reported to directly interact with the Rel homology domain of NF-κB (LeClair *et al.,* 1992; Stein *et al.,* 1993). Although the NF-κB/Rel transcription factors act generally as transcriptional ac-

[1] To whom correspondence and reprint requests should be addressed. Fax: +31 20 697 9271. E-mail: j.terschegget@amc.uva.nl.

0042-6822/00 $35.00

TABLE 1

Oligonucleotides Used for Bandshift

Oligonucleotide		Source
wtLCR7550	5′ GCG TGC CAA ATC CCT GTT TTC CTG ACC AC 3′ 3′ CA CGC A*CG GTT* TAG GGA CAA AAG GAC TGG 5′	
wtLCR7545	5′ GC CAT GCG TGC CAA ATC CCT GTT TTC C 3′ 3′ CG GTA CGC A*CG GTT* TAG GGA CAA AAG G 5′	
mutLCR7545	5′ GC CAT GCG TGC CAA ATg aCT GTT TTC C 3′ 3′ CG GTA CGC A*CG GTT* TAc tGA CAA AAG G 5′	
NF1 cons	5′ TTT TGG ATT GAA GCC AAT ATG ATA A 3′ 3′ AAA ACC TAA CTT *CGG TT*A TAC TAT T 5′	Santa Cruz Biotechnology
NF-κB cons	5′ GCT TCA GAG GGG ACT TTC CGA GAG C 3′ 3′ CGA AGT CTC CCC TGA AAG GCT CTC G 5′	Sen and Baltimore, 1986
wtLCR7836	5′ CAA CTG CAC ATG GGT GTG TGC AGG G 3′ 3′ GG GTT GAC GTG TAC CCA CAC ACG TC 5′	Dong *et al.,* 1994
NF-IL6 cons	5′ GGC GTC ACA **TTG CAC AAT** CTT AAT G 3′ 3′ G CAG TGT AAC GTG TTA GAA TTA CGG 5′	Santa Cruz Biotechnology
wtLCR7288	5′ GGG C**TT GTG TAA C**TA **TTG TGT CAT** G 3′ 3′ C **GAA CAC ATT** GA**T AAC ACA GT**A CGG 5′	Kyo *et al.,* 1993

Note. NF-κB binding sites are underlined (the consensus NF-κB binding site is GGGRNNYYCC). The point mutations in the NF-κB binding site are written in lowercase letters. NF-1 and (potential) C/EBPβ binding sites are written in italics and in boldface type, respectively.

tivators, they also have been reported as transcriptional repressors (Supakar *et al.,* 1995; Wissink *et al.,* 1997).

In this report, we have examined the involvement of NF-κB in HPV16 LCR activity. Our data indicate that NF-κB can bind the HPV16 LCR and acts as a transcriptional repressor in the context of the HPV16 LCR.

RESULTS

NF-κB binds the HPV16 LCR

To investigate whether NF-κB is able to bind to the HPV16 LCR, a computer search for potential NF-κB binding sites was performed. Several potential binding sites were found; one had only two mismatches in comparison to the consensus NF-κB binding site from the Igκ L promoter (GGGRNNYYCC; see Table 1). Bandshift experiments using double-stranded oligonucleotides from the HPV16 LCR (nt 7550 to 7576: wtLCR7550 probe) and nuclear extracts from HeLa cells, which were transfected with p65 in order to increase the NF-κB activity, showed that a complex similar to the complex with the consensus NF-κB probe can be observed (see Fig. 1, lanes 1 and 7). This complex is absent in the presence of a 60-fold excess of cold consensus NF-κB oligonucleotide (see Fig. 1, lanes 3 and 8) and is also slightly diminished by a 60-fold excess of cold wtLCR7550 oligonucleotide (see Fig. 1, lane 9). Furthermore, the addition of antibodies directed against p50 and p65 supershifts this complex, indicating that it contained immunologically related p50 and p65 (see Fig. 1, lanes 4, 5, 10, and 11). In contrast, antibodies directed against C/EBPβ were unable to supershift this complex (see Fig. 1, lanes 6 and 12). These results indicated that NF-κB can bind the potential NF-κB site at position 7554–7563. The heavy band below the NF-κB complex is not shifted by the addition of α-p50 and α-p65, indicating that this complex does not contain a protein that is immunologically related to p50 and p65. This complex is most probably due to nonspecific interaction, as we observed a complex with the same apparent molecular weight using different probes (see also Fig. 1) and a reduced intensity with competing unrelated oligonucleotides (data not shown).

In addition, as shown in Fig. 2, when using purified GST–p65 we observed a strong binding to the NF-κB consensus probe (exposure time 4 h) and a relatively weak binding to the wtLCR7545 probe (exposure time 4 days). The addition of an antibody directed against p65 supershifts the complex as observed with both probes (see Fig. 2, lanes 3 and 7). These findings furnish direct evidence for p65 binding to the HPV16 LCR.

In view of the weak binding affinity of NF-κB on the wtLCR probe compared to the consensus NF-κB probe, as observed in Figs. 1 and 2, we investigated the relative affinity of NF-κB for binding to this region of the HPV16 LCR. Electrophoretic mobility shift assay (EMSA) experiments using the NF-κB consensus probe and nuclear extracts obtained from TNFα-treated HeLa cells showed that the NF-κB complex can be better competed (approximately 250 times) by an overdose of the NF-κB consensus cold oligonucleotide than by the wtLCR7545 oligonucleotide (see Fig. 3). In contrast, an irrelevant oligonucleotide, comprising nt 7836–7856 from the HPV 16 LCR (wtLCR 7836), did not compete for binding to the NF-κB probe.

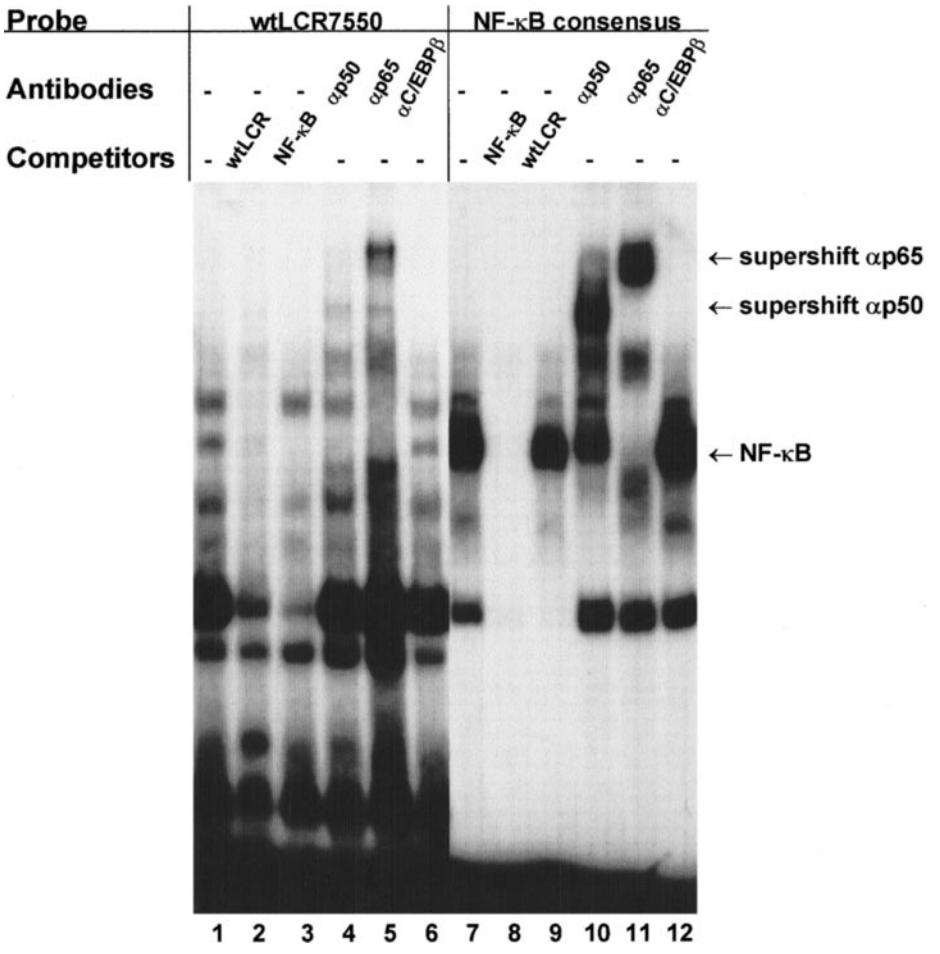

FIG. 1. NF-κB binds wtLCR7550 oligonucleotide. EMSA using the NF-κB consensus and wtLCR7550 probe and nuclear extracts from HeLa cells transfected with pRSVp65. Competition experiments were performed with a 60-fold molar excess of cold NF-κB consensus (see Table 1 for sequence; lanes 3 and 8) and cold wtLCR7550 oligonucleotide (see Table 1 for sequence; lanes 2 and 9). Supershift experiments were performed using antibodies directed against p50, p65, and C/EBPβ (lanes 4–6 and 10–12). The left part (lanes 1–6) was exposed for 20 h and the right part (lanes 7–12) was exposed for 6 h.

NF-κB represses the HPV16 LCR at position 7554–7563

To investigate the effect of NF-κB on the regulation of the HPV16 LCR, mutations were introduced in the NF-κB binding site of the LCR (mutLCR7545) (see Table 1). As shown in Fig. 3, we observed in EMSA experiments that NF-κB was not able to bind the mutated double-stranded oligonucleotide.

We studied the activity of pWtLCR-Luc and pMutLCR-Luc in two cell lines, HeLa and C33A cells. In EMSA, we observed that nuclear extracts of C33A, even after treatment with TNFα, contained very low levels of NF-κB DNA binding activity (see Fig. 4A). Furthermore, in transient transfections, we observed that chloramphenicol acetyl transferase (CAT) expression under the control of NF-κB promoter elements (pNF-κB–CAT construct) is very low in C33A cells treated with TNFα compared to HeLa cells treated with TNFα (see Fig. 4B). It seems therefore that HeLa cells are suitable for studying the effect of NF-κB on the HPV16 LCR while C33A cells could be used as control.

In transient transfected HeLa cells, which were untreated or treated with TNFα, the activity of pMutLCR-Luc is at least two times higher than the luciferase activity obtained with the pWtLCR-Luc construct (see Fig. 5A). To verify that the observed increase of luciferase activity was due to the introduced mutations in the HPV16 LCR, we assessed the luciferase activity driven by these two constructs in C33A. As expected, the pWtLCR-Luc and pMutLCR-Luc constructs exhibited a similar luciferase activity in C33A cells (see Fig. 5B). This latter result suggested that the increased luciferase activity observed in HeLa cells by introducing mutations in the NF-κB binding site at positions 7561 and 7562 of the HPV16 LCR was due to a specific inhibition of the negative NF-κB effect on the HPV16 LCR. These results strongly suggested that NF-κB acts as a repressor on the HPV16 LCR.

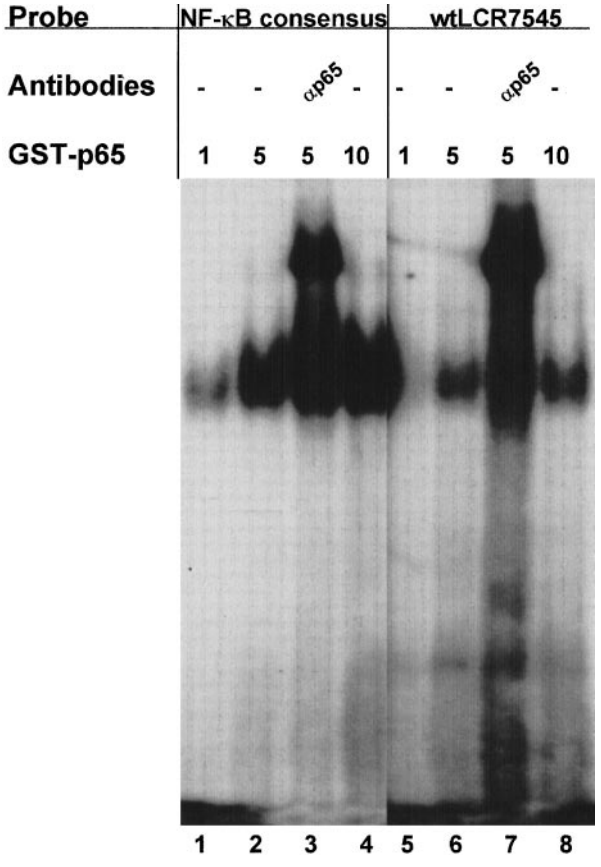

FIG. 2. Recombinant p65 binds wtLCR7545 oligonucleotide. EMSA using the NF-κB consensus and wtLCR7545 probe and purified GST–p65 fusion protein (1, 5, and 10 μg). Supershift experiments were performed using antibodies directed against p65 (lanes 3 and 7). The left part (NF-κB consensus probe) was exposed for 4 h, and the right part (wtLCR7545 probe) was exposed for 4 days.

To investigate the effect of an overexpression of p65 on the full-length HPV16 LCR, we transfected HeLa cells together with pRSV-p65 and pWtLCR-Luc reporter construct. As shown in Fig. 6, p65 represses the HPV16 LCR. A maximal inhibition (90%) was achieved using 1 μg of the pRSV-p65 plasmid. This result strengthened our previous observation that the HPV16 LCR is repressed by NF-κB.

The NF-κB and NF-1 transcription factors bind overlapping specific binding sites between nt 7545–7576 of the HPV16 LCR

Computer analysis searching additional DNA binding sites for transcription factors in the nt 7550–7576 HPV16 LCR was performed. Two overlapping binding sites were found: a potential C/EBPβ binding site containing one mismatch compared to the C/EBPβ consensus binding sequence (eighth nt from the 5′ end of this potential C/EBPβ binding site) and a half-consensus NF-1 binding site as previously reported by others (see Table 1) (Gloss *et al.,* 1989). To investigate whether C/EBPβ can bind this LCR, we performed bandshift experiments using a GST–C/EBPβ fusion protein. An oligonucleotide starting at nucleotide 7545 of the LCR was used to verify whether the negative results observed with α-C/EBPβ in Fig. 1 were not due to the short space between the C/EBPβ putative binding site and the end of the oligonucleotide (see Table 1). As shown in Fig. 7A, GST–C/EBPβ bound to the C/EBPβ consensus probe (lanes 1–4), but not to the nt 7545–7568 HPV16 LCR oligonucleotide (lanes 5–8). As shown in lane 4 of Fig. 7A, the complex binding to the C/EBPβ consensus probe was supershifted in the presence of anti-C/EBPβ antibodies, confirming that it contained a protein that is immunologically related to C/EBPβ. The formation of the complex is inhibited in the presence of a 60-fold excess of cold C/EBPβ consensus probe (see Fig. 7A, lane 2, and Fig. 7B, lane 2) or a 60- to 120-fold excess of a cold wtLCR 7288 oligonucleotide, containing four C/EBPβ binding sites as previously described (Kyo *et al.,* 1993) (see Fig. 7B, lanes 5 and 6) but not by a 60- to 120-fold excess of a cold wtLCR7545 oligonucleotide (see Fig. 7B, lanes 3 and 4) or a cold irrelevant LCR oligonucleotide (wtLCR 7836) (see Fig. 7A, lane 3). These results indicate that C/EBPβ does not bind to the 7545–7568 region.

Additionally, since this region appeared to contain a NF-1 binding site, we investigated the relative binding affinity of NF-1 to this region of the HPV16 LCR. EMSA experiments using the NF-1 consensus probe and nu-

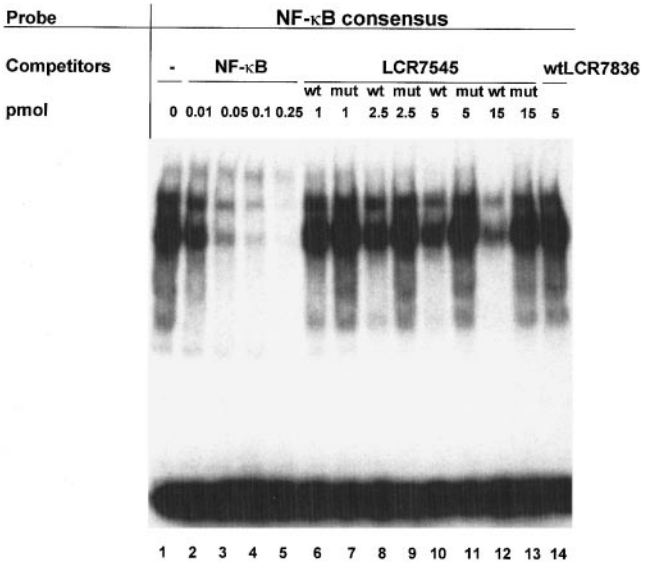

FIG. 3. NF-κB binds weakly to the wtLCR7545 oligonucleotide compared to the NF-κB consensus probe and NF-κB is not able to bind any longer to the mutLCR oligonucleotide. EMSA using the NF-κB consensus probe and nuclear extracts from HeLa cells treated with TNFα. Competition experiments were performed with 0, 0.01, 0.05, 0.1, and 0.25 pmol of cold NF-κB consensus oligonucleotide (lanes 1–5, respectively) or 1, 2.5, 5, and 15 pmol of cold wtLCR7545 or mutLCR7545 (lanes 6–7, 8–9, 10–11, and 12–13, respectively) and 5 pmol of a cold irrelevant oligonucleotide, comprising nt 7836–7856 from the HPV16 LCR (lane 14).

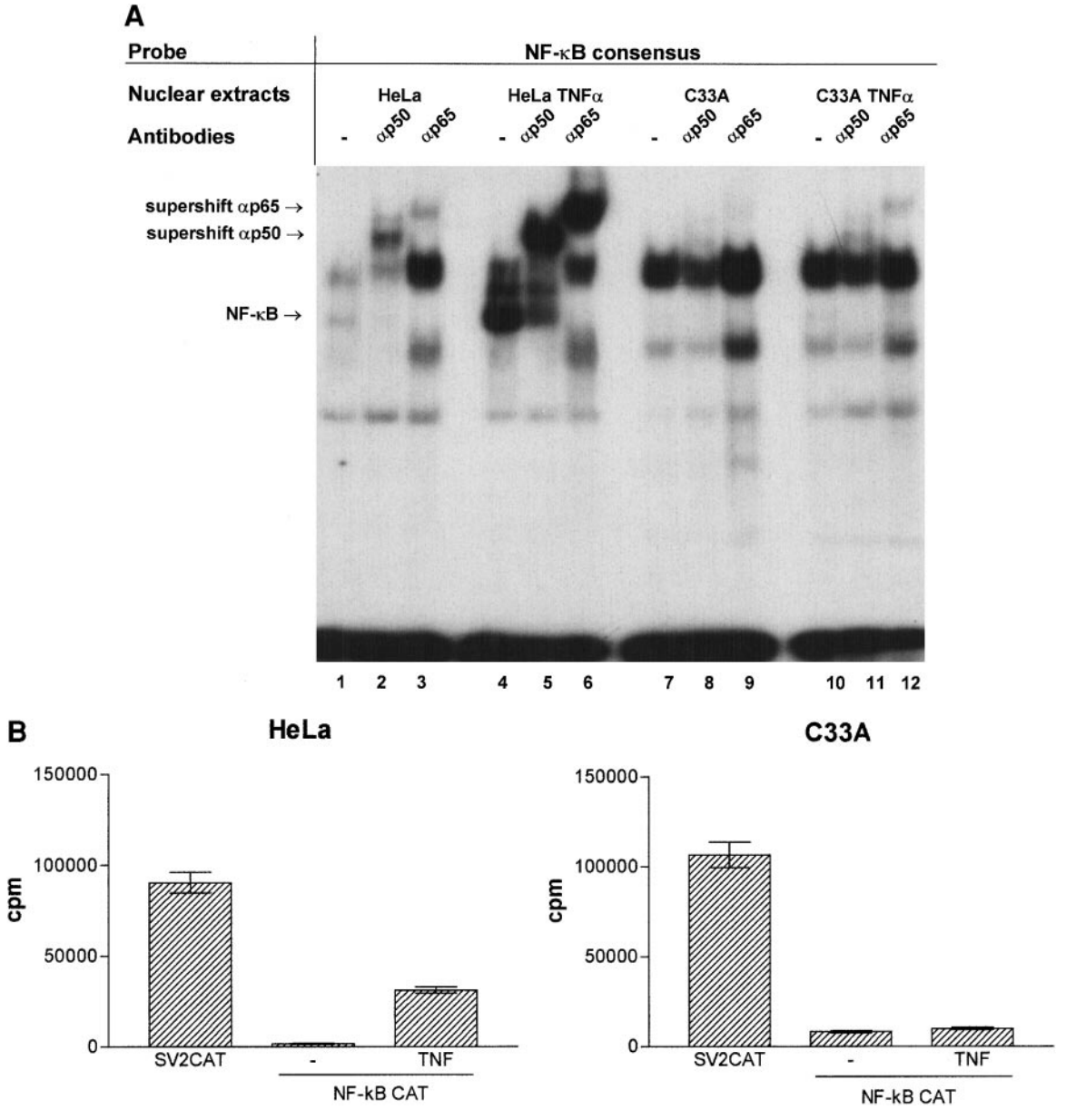

FIG. 4. (A) NF-κB activity in HeLa and C33A cell lines. EMSA using the NF-κB consensus probe and nuclear extracts of HeLa or C33A cells treated without or with TNFα. Supershift experiments were performed using antibodies directed against p50 and p65 and nuclear extracts of HeLa (lanes 2, 3 and 5, 6) and C33A (lanes 8, 9 and 11, 12) cells (untreated or treated with TNFα). (B) Transient transfection of pSV_2–CAT and pNF-κB–CAT plasmids in HeLa and C33A cells treated without or with TNFα. The conversion products were quantitated with a scintillation counter. At least three independent CAT assays were performed in triplicate, all showing similar results. The results of a representative CAT assay are shown. cpm, counts per minute.

clear extracts obtained from TNFα-treated HeLa cells showed that the NF-1 complex can be better competed (approximately 100 times) by an excess of the NF-1 consensus cold oligonucleotide than by the wtLCR7545 oligonucleotide (see Fig. 8A). We also observed in a competition experiment (Fig. 8A) and in direct NF-1/DNA binding (Fig. 8B) that NF-1 binds two times less efficiently to the mutLCR7545 oligonucleotide than to the wtLCR7545 oligonucleotide. These results suggested that the 2-nt mutations introduced in this region to abolish NF-κB binding seemed to affect also slightly the binding of NF-1. In contrast, the NF-κB consensus oligonucleotide did not compete for binding to the NF-1 probe.

DISCUSSION

In the present study, we have identified a NF-κB binding site in the HPV-16 LCR promoter, localized in a region exhibiting enhancer activity (Chong *et al.,* 1990). This site contains two mismatches in comparison to the consensus NF-κB Igκ binding site (Sen and Baltimore, 1986). Among the other HPVs, only HPV7, HPV59 and HPV66

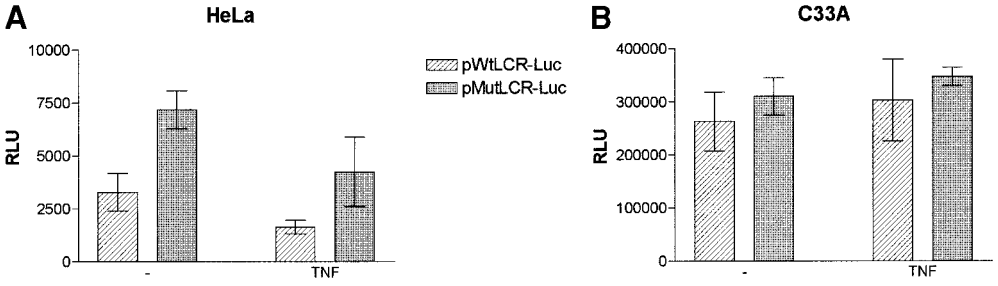

FIG. 5. NF-κB represses the HPV16 LCR activity. Transient transfection of pWtLCR-Luc and pMutLCR-Luc plasmids in HeLa (A) and C33A (B) cells treated without or with TNFα. At least three independent luciferase assays were performed in triplicate, all showing similar results. The results of a representative luciferase assay are shown. RLU, relative light unit.

contain a LCR with a potential NF-κB sequence also with two mutations in comparison to the NF-κB consensus sequence. HPV16 and HPV66 are the only high-risk genital HPV types containing a potential NF-κB binding site.

NF-κB can bind at position 7554–7563 of the HPV16 LCR as observed in EMSA experiments but with a much lower affinity than the consensus NF-κB binding site in the Igκ promoter. In addition, we observed in EMSA that NF-1 binds an NF-1 binding site overlapping the newly identified NF-κB binding site. This NF-1 site was previously reported to mediate an up-regulation of the LCR activity (Apt *et al.,* 1993; Chong *et al.,* 1990). When mutations were specifically introduced into the NF-κB site of the LCR, the binding of NF-1 to the mutated region was reduced by a factor of 2. However, the activity of the LCR was increased two- to threefold. Therefore the higher level of activity of the mutant LCR-luciferase construct is not due to the reduction of NF-1 binding. Furthermore, this was observed only in cells with a high level of NF-κB activity, such as in HeLa cells, and not in the C33A cell line, in which NF-κB activity cannot be induced by TNFα (see Fig. 4). This result suggests that NF-κB acts as a repressor in the HPV-16 promoter context. In addition, we observed that transient overexpression of p65 repressed the HPV16 LCR in HeLa cells (see Fig. 6). In contrast, overexpression of p50 had no effect on the expression of the reporter construct probably because p50 does not contain a transactivation domain (data not shown). Although NF-κB is more generally known as a transcriptional activator, it has been also previously reported to act as a transcriptional inhibitor on various promoters. Interestingly, NF-κB and the glucocorticoid receptor have been reported to repress each other's transcriptional activity (Wissink *et al.,* 1997). These observations suggest that the negative cross-talk between these two factors could result from direct protein–protein interaction and also from interference in interacting with cofactors.

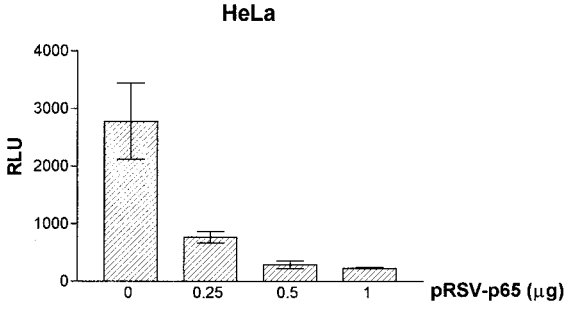

FIG. 6. Overexpression of NF-κB p65 represses the HPV16 LCR. Transient transfection of pRSV-p65 and pWtLCR-Luc plasmids in HeLa cells. At least three independent luciferase assays were performed in triplicate, all showing similar results. The results of a representative luciferase assay are shown.

Although we did not elucidate the mechanisms of HPV16 LCR repression by NF-κB, our results indicate that the NF-κB binding site at position 7554–7563 overlaps with a NF-1 binding site. Therefore, it is possible that NF-κB competes with NF-1 for DNA binding. Due to the weak binding affinity of NF-κB and NF-1 in this region, we were unable to study possible competition between these two transcriptional factors. It is also possible that NF-κB and NF-1 could compete for interaction with a coactivator, such as p300 or CBP. NF-κB was indeed reported to interact with p300/CBP coactivators (Gerritsen *et al.,* 1997; Perkins *et al.,* 1997) but, on the other hand, there is no evidence for direct interaction between NF-1 and these coactivators. However, various reports suggest that some members of the NF-1 family could interact directly or indirectly with the initiation complex machinery (Martinez *et al.,* 1991; Altmann *et al.,* 1994; Xiao *et al.,* 1994; Kim and Roeder, 1994).

Although a potential C/EBPβ binding site containing only one mismatch in comparison to the C/EBPβ consensus site is also present in the 7545–7568 fragment, we did not detect binding of C/EBPβ to this region in EMSA. Furthermore, although C/EBPβ was reported to interact with NF-κB we did not observe in supershift assays an interaction between these two factors in the context of nt 7545–7576 of the HPV16 LCR (see Fig. 1).

The pMutLCR-Luc, which does not bind NF-κB, is still repressed in HeLa cells by TNFα treatment (Fig. 5). This suggests that the effect of TNFα on the HPV16 LCR is not through NF-κB and that other transcription factors (like

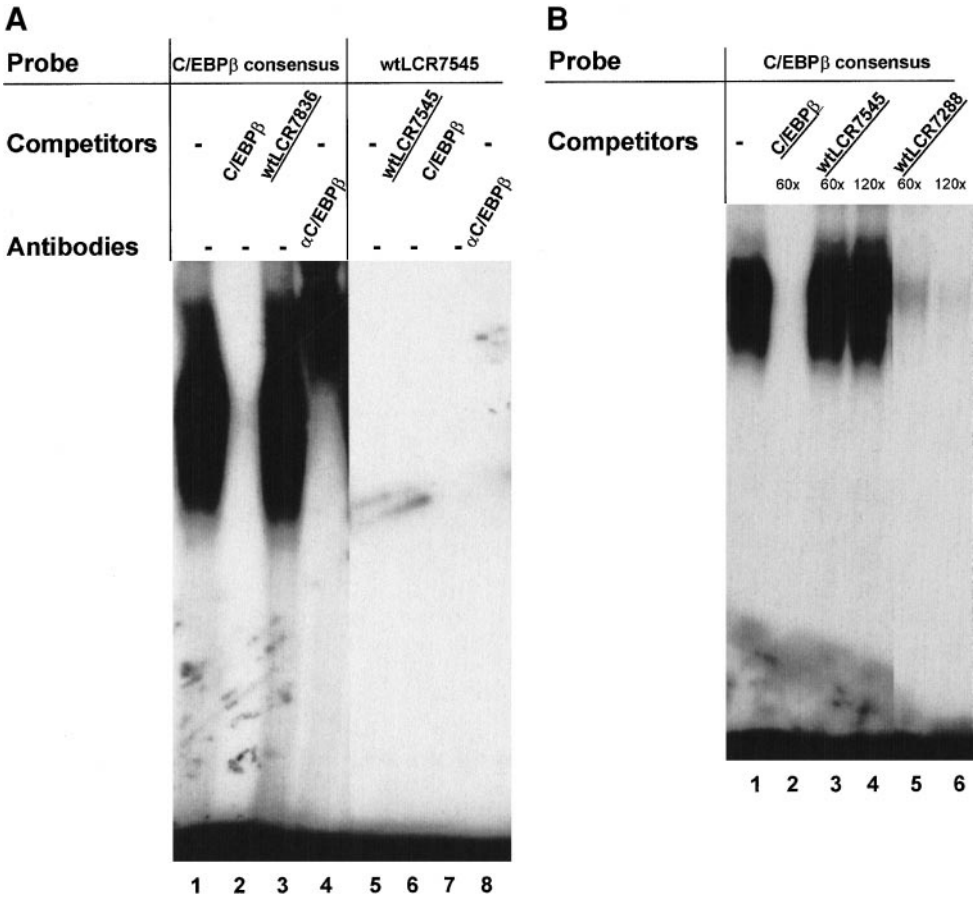

FIG. 7. C/EBPβ cannot bind the wtLCR oligonucleotide. EMSA using the C/EBPβ consensus and wtLCR7545 probe and purified GST–C/EBPβ fusion protein. Competition experiments were performed with a 60-fold molar excess of cold C/EBPβ consensus oligonucleotide (A, lanes 2 and 7; B, lane 2), a 60- and 120-fold molar excess of cold wtLCR 7288 oligonucleotide (containing four C/EBPβ binding sites) (B, lanes 5 and 6), a 60-fold (and 120-fold) molar excess of cold wtLCR7545 (A, lane 6; B, lane 3 and 4), or an irrelevant LCR oligonucleotide (wtLCR 7836) (A, lane 3). Supershift experiments were performed with antibodies directed against C/EBPβ (A, lane 4 and 8).

AP-1) are involved in HPV16 LCR inhibition by TNFα (Soto *et al.,* 1999). Alternative explanations are that the HPV16 LCR might contain additional NF-κB sites that were not detected in our computer search or that NF-κB could still bind the mutated NF-κB site in the context of the complete HPV16 LCR caused by a different higher order complex formation.

Our results clearly demonstrated that NF-κB can bind the HPV16 LCR and that the binding of this transcription factor at position 7554–7563 inhibits in HeLa cells the activity of the viral promoter. The possible implications of this finding for the viral life cycle must be evaluated in future experiments.

MATERIALS AND METHODS

Plasmid construction

The pWtLCR-Luc luciferase reporter gene was constructed by cloning the HPV16 LCR *Pst*I–*Ava*II (nt 7007–117) (Seedorf *et al.,* 1985) fragment in front of a luciferase gene in pUHC13.3 (digested with *Xho*I and *Kpn*I) (Gossen and Bujard, 1992). The HPV16 LCR luciferase construct with mutations in the NF-κB binding site (pMutLCR-Luc) was constructed by PCR amplification with two different primer sets (de Jong *et al.,* 1992). The first primer set included SP6-specific 5′ primer (5′ TTT AGG TGA CAC TAT AGA ACT C 3′) and HPV16 LCR-specific (7550–7576) 3′ primer (5′ GGT CAG GAA AAC AGT CCAT TTG GCA CGC 3′). The second primer set included HPV16 LCR-specific (7550–7576) 5′ primer (5′ GCG TGC CAA ATG ACT GTT TTC CTG ACC 3′) and T7-specific 3′ primer (5′ TAATACGACTCACTATAGGGAGA 3′). The two PCR fragments generated were pooled and subjected to a final PCR amplification with the SP6-specific 5′ primer and T7-specific 3′ primer.

The pGEX-C/EBPβ and pGEX-p65 plasmids were constructed by cloning a C/EBPβ *Eco*RI fragment (from pCMV − C/EBPβ) in pGEX5x-3 (Pharmacia Biotechnology) and a p65 PCR fragment with a *Bam*HI and an *Xho*I site in pGEX5x-3, respectively. All DNA constructs generated were checked by DNA sequence analysis. The

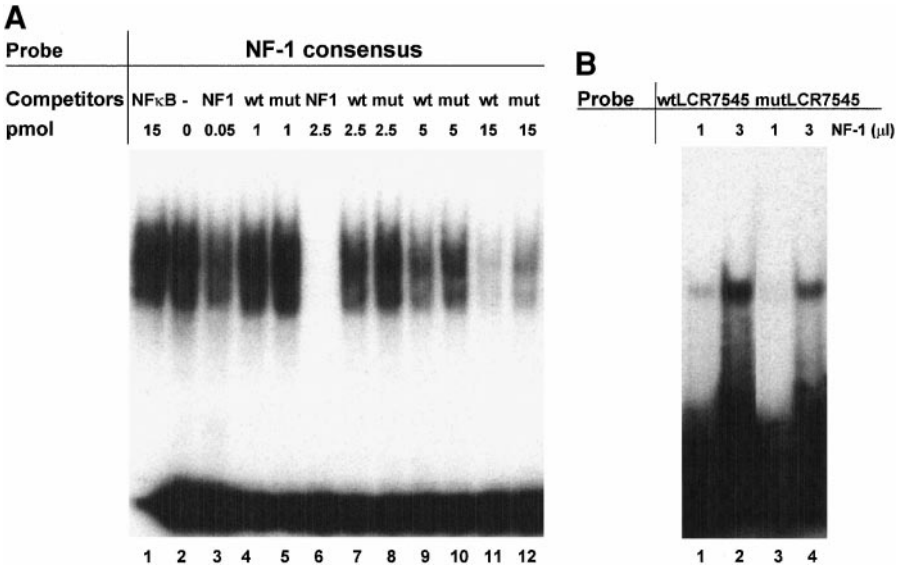

FIG. 8. NF-1 and NF-κB bind overlapping sites in the wtLCR oligonucleotide. (A) EMSA using the NF-1 consensus probe and nuclear extracts of HeLa cells treated with TNFα. Competition experiments were performed with 0, 0.05, and 2.5 pmol of cold NF-1 consensus oligonucleotide (lanes 2, 3, and 6, respectively) or 1, 2.5, 5, and 15 pmol of cold wtLCR7545 or mutLCR7545 (lanes 4–5, 7–8, 9–10, and 11–12, respectively) and 15 pmol of NF-κB consensus oligonucleotide (lane 1). (B) EMSA using the wtLCR7545 probe (lanes 1 and 2) or the mutLCR7545 probe (lanes 3 and 4) and NF-1 protein containing only the DNA binding domain (1 of 3 μl).

pNF-κB–CAT plasmid was kindly provided by N. Mukaida (Mukaida *et al.,* 1994).

The pRSVp65 plasmid was constructed by cloning the p65 *Xba*I (blunt ended)–*Hin*dIII fragment from the pCMV-p65 (Schmitz and Baeuerle, 1991) into the pRSVneo vector (Gorman *et al.,* 1984) digested with *Hpa*I and *Xba*I.

Cell cultures

The human cervical carcinoma cell lines HeLa and C33A (ATCC, Bethesda, MD) were grown in Dulbecco's modified Eagle's medium (Life Technologies Ltd., Paisley, Scotland) with 10% FCS, 0.1 mg/ml streptomycin, and 100 U/ml penicillin.

Nuclear extracts

Nuclear extracts were obtained from untreated HeLa and C33A cells, from HeLa and C33A cells treated for 15 min with 30 ng/ml TNFα, or from HeLa transfected with pRSV-p65. The nuclear extracts were prepared as described by Meijer *et al.* (1992).

Expression and purification of proteins from *Escherichia coli*

E. coli C600 was transformed with pGEX–C/EBPβ or pGEX–p65. GST–fusion proteins were produced and purified as previously described by Oude Essink *et al.* (1997).

The purity and identity of the proteins were determined by Coomassie brilliant blue staining of a SDS–polyacrylamide gel and by Western blotting with antibodies directed against C/EBPβ and p65 (Santa Cruz Technologies, Santa Cruz, CA). Bacterial recombinant NF-1 (DNA binding domain) (Dekker *et al.,* 1996) was a kind gift of J. Dekker and P. C. van der Vliet (University of Utrecht, The Netherlands).

Electrophoretic mobility shift assay

Double-stranded DNA oligonucleotides (2.5 pmol) (Table 1) were end-labeled with [γ-^{32}P]ATP using 2.0 μl T4 polynucleotide kinase (10 u/μl). Gel retardation assays were performed using equal amounts of nuclear extract proteins (5–10 μg), 5 × 10^4 cpm of DNA probe, 0.5 μg poly(dI–dC) (Pharmacia), 10 μg BSA, with or without cold competitors (in a 60- to 240-fold molar excess of labeled probe), in a final volume of 20 μl containing 10 mM HEPES, pH 7.9, 60 mM KCl, 1 mM EDTA, 2.5 mM $MgCl_2$, 5% glycerol, 1 mM dithiothreitol. For supershifts, 2 μl of anti-human NF-κB p50 (NFKB1), anti-NF-κB p65 (Rel A) (Rockland, Gilbertville, PA), or anti-C/EBPβ (C-19) (Santa Cruz Biotechnology) was added to the nuclear extracts at 4°C 1 h before the addition of the labeled oligonucleotide.

Binding reactions were performed for 25 min at 25°C, and the DNA–protein complexes were separated by electrophoresis in 4% acrylamide/bisacrylamide gels in 25 mM Tris–borate, pH 8.2, 0.5 mM EDTA for 2 h at 210 V.

DNA transfection, luciferase assay, and CAT assay

Approximately 5.0 × 10^5 cells per 60-mm dish were transiently transfected by the calcium phosphate method

(van der Eb and Graham, 1980). Five micrograms of pLCR-Luc (wild-type or NF-κB mutant GG → TC, 7554–7555) or 2.5 μg pNF-κB–CAT reporter constructs and 0.25, 0.5, or 1, μg pRSV-p65 were used. Twenty-four hours after transfection, fresh medium with or without TNFα (30 ng/ml) was added. After another incubation period of 24 h, lysates were prepared. Luciferase assays were carried out by the method of De Wet *et al.* (1987) and CAT assays were performed as described by Seed and Sheen (1988). All transient transfection experiments were performed in triplicate using different batches of DNA constructs.

ACKNOWLEDGMENTS

We thank Dr. J. Dekker and Dr. P. C. van der Vliet for providing us with recombinant NF-1. This research was supported by the Dutch Cancer Society Grants UVA 93-672 and UVA 94-719.

REFERENCES

Altmann, H., Wendler, W., and Winnacker, E.-L. (1994). Transcriptional activation by CTF proteins is mediated by a bipartite low-proline domain. *Proc. Natl. Acad. Sci. USA* **91,** 3901–3905.

Apt, D., Chong, T., Liu, Y., and Bernard, H. U. (1993). Nuclear factor I and epithelial cell-specific transcription of human papillomavirus type 16. *J. Virol.* **67,** 4455–4463.

Baeuerle, P. A., and Henkel, T. (1994). Function and activation of NF-κB in the immune system. *Annu. Rev. Immunol.* **12,** 141–179.

Bartsch, D., Boye, B., Baust, C., zur Hausen, H., and Schwarz, E. (1992). Retinoic acid-mediated repression of human papillomavirus 18 transcription and different ligand regulation of the retinoic acid receptor beta gene in non-tumorigenic and tumorigenic HeLa hybrid cells. *EMBO J.* **11,** 2283–2291.

Chan, W. K., Klock, G., and Bernard, H. U. (1989). Progesterone and glucocorticoid response elements occur in the long control regions of several human papillomaviruses involved in anogenital neoplasia. *J. Virol.* **63,** 3261–3269.

Chong, T., Apt, D., Gloss, B., Isa, M., and Bernard, H. U. (1991). The enhancer of human papillomavirus type 16: Binding sites for the ubiquitous transcription factors oct-1, NFA, TEF-2, NF1, and AP-1 participate in epithelial cell-specific transcription. *J. Virol.* **65,** 5933–5943.

Chong, T., Chan, W. K., and Bernard, H. U. (1990). 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. *Nucleic Acids Res.* **18,** 465–470.

Creek, K. E., Geslani, G., Batova, A., and Pirisi, L. (1995). Progressive loss of sensitivity to growth control by retinoic acid and transforming growth factor-β at late stages of human papillomavirus type 16-initiated transformation of human keratinocytes. *Adv. Exp. Med. Biol.* **375,** 117–135.

de Jong, J. J., Goudsmit, J., Keulen, W., Klaver, B., Krone, W., Tersmette, M., and de Ronde, A. (1992). Human immunodeficiency virus type 1 clones chimeric for the envelope V3 domain differ in syncytium formation and replication capacity. *J. Virol.* **66,** 757–765.

Dekker, J., van Oosterhout, J. A., and van der Vliet, P. C. (1996). Two regions within the DNA binding domain of nuclear factor I interact with DNA and stimulate adenovirus DNA replication independently. *Mol. Cell. Biol.* **16,** 4073–4080.

de Wet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R., and Subramani, S. (1987). Firefly luciferase gene: Structure and expression in mammalian cells. *Mol. Cell Biol.* **7,** 725–737.

Dong, X. P., Stubenrauch, F., Beyer-Finkler, E., and Pfister, H. (1994). Prevalence of deletions of YY1-binding sites in episomal HPV16 DNA from cervical cancers. *Int. J. Cancer* **58,** 803–808.

Gerritsen, M. E., Williams, A. J., Neish, A. S., Moore, S., Shi, Y., and Collins, T. (1997). CREB-binding protein/p300 are transcriptional coactivators of p65. *Proc. Natl. Acad. Sci. USA* **94,** 927–2932.

Gloss, B., Yeo-Gloss, M., Meisterernst, M., Rogge, L., Winnackerand, E. L., and Bernard, H. U. (1989). Clusters of nuclear factor I binding sites identify enhancers of several papillomaviruses but alone are not sufficient for enhancer function. *Nucleic Acids Res.* **17,** 3519–3533.

Gorman, C. M., Lane, D. P., and Rigby, P. W. (1984). High efficiency gene transfer into mammalian cells. *Philos. Trans. R. Soc. London Ser. B Biol. Sci.* **307** (1132), 343–346.

Gossen, M., and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. USA* **89,** 5547–5551.

Ishiji, T., Lace, M. J., Parkkinen, S., Anderson, R. D., Hangen, T. H., Cripe, T. P., Xiao, J. H., Davidson, I., Chambon, P., and Turek, L. P. (1992). Transcriptional enhancer factor (TEF-1) and its cell-specific co-activator activate human papillomavirus-16 E6 and E7 oncogene transcription in keratinocytes and cervical carcinoma cells. *EMBO J.* **11,** 2271–2281.

Khare, S., Kumar, K. U., Tang, S.-C., Pater, M. M., and Pater, A. (1996). Up-regulation of hormone response of human papillomavirus type 16 expression and increased DNA-protein binding by consensus mutations of viral glucocorticoid response elements. *J. Med. Virol.* **50,** 254–262.

Kim, T. K., and Roeder, G. (1994). Proline-rich activator CTF1 targets the TFIIB assembly step during transcriptional activation. *Proc. Natl. Acad. Sci. USA* **91,** 4170–4174.

Kyo, S., Inoue, M., Nishio, Y., Nakanishi, K., Akira, S., Inoue, H., Yutsudo, M., Tanizawa, O., and Hakura, A. (1993). NF-IL6 represses early gene expression of human papillomavirus type 16 through binding to the noncoding region. *J. Virol.* **67,** 1058–1066.

Kyo, S., Inoue, M., Hayasaka, N., Inoue, T., Yutsudo, M., Tanizawa, O., and Hakura, A. (1994). Regulation of early gene expression of human papillomavirus type 16 by inflammatory cytokines. *Virology* **200,** 130–139.

LeClair, K. P., Blanar, M. A., and Sharp, P. A. (1992). The p50 subunit of NF-κB associates with the NF-IL6 transcription factor. *Proc. Natl. Acad. Sci. USA* **89,** 8145–8149.

Martinez, E., Dusserre, Y., Wahli, W., and Mermod, N. (1991). Synergistic transcriptional activation by CTF/NF-1 and the estrogen receptor involves stabilized interactions with a limiting target factor. *Mol. Cell. Biol.* **11,** 2937–2945.

May, M. J., and Ghosh, S. (1998). Signal transduction through NF-κB. *Immunol. Today* **19,** 80–88.

Meijer, I., Boot, A. J., Mahabir, G., Zantema, A., and van der Eb, A. J. (1992). Reduced binding activity of transcription factor NF-κB accounts for MHC class I repression in adenovirus type 12 E 1-transformed cells. *Cell. Immunol.* **145,** 56–65.

Mukaida, N., Morita, M., Ishikawa, Y., Rice, N., Okamoto, S. I., Kasahara, T., and Matsushima, K. (1994). Novel mechanism of glucocorticoid-mediated gene repression. Nuclear factor-κB is target for glucocorticoid-mediated interleukin 8 gene repression. *J. Biol. Chem.* **269,** 13289–13295.

Nürnberg, W., Artuc, M., Nawrath, M., Lovric, J., Stüting, S., Moelling, K., Czarnetzki, B. M., and Schadendorf, D. (1995). Human c-myb is expressed in cervical carcinomas and transactivates the HPV-16 promoter. *Cancer Res.* **55,** 4432–4437.

O'Connor, M. J., Tan, S., Tan, C. H., and Bernard, H. U. (1996). YY1 represses human papillomavirus type 16 transcription by quenching AP-1 activity. *J. Virol.* **70,** 6529–6539.

Oude Essink, B. B., Back, N. K. T., and Berkhout, B. (1997). Increased polymerase fidelity of the 3TC-resistant variants of HIV-1 reverse transcriptase. *Nucleic Acids Res.* **25,** 3212–3217.

Perkins, N. D., Felzien, L. K., Betts, J. C., Leung, K., Beach, D. H., and Nabel, G. J. (1997). Regulation of NF-κB by cyclin-dependent kinases associated with the p300 coactivator. *Science* **275,** 523–527.

Schmitz, M. L., and Baeuerle, P. A. (1991). The p65 subunit is responsible for the strong transcription activating potential of NF-κB. *EMBO J.* **10,** 3805–3817.

Seed, B., and Sheen, J. Y. (1988). A simple phase-extraction assay for chloramphenicol acetyltransferase activity. *Gene* **67,** 271–277.

Seedorf, K., Krämmer, G., Dürst, M., Suhai, S., and Röwekamp, W. G. (1985). Human papillomavirus type 16 sequence. *Virology* **145,** 181–185.

Sen, R., and Baltimore, D. (1986). Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* **46,** 705–716.

Sibbet, G. J., Cuthill, S., and Campo, M. S. (1995). The enhancer in the long control region of human papillomavirus type 16 is up-regulated by PEF-1 and down-regulated by Oct-1. *J. Virol.* **69,** 4006–4011.

Soto, U., Das, B. C., Lengert, M., Finzer, P., zur Hausen, H., and Rösl, F. (1999). Conversion of HPV18 positive non-tumorigenic HeLa-fibroblast hybrids to invasive growth involves loss of TNF-α mediated repression of viral transcription and modification of the AP-1 transcription complex. *Oncogene* **18,** 3187–3198.

Stancovski, I., and Baltimore, D. (1997). NF-κB activation: The I κB kinase revealed? *Cell* **91,** 299–302.

Stein, B., Cogswell, P. C., and Baldwin, A. S. (1993). Functional and physical associations between NF-κB and C/EBP family members: A Rel domain-bZIP interaction. *Mol. Cell. Biol.* **13,** 3964–3974.

Struyk, L., van der Meijden, E., Minnaar, R., Fontaine, V., Meijer, I., and ter Schegget, J. Transcriptional regulation of human papillomavirus type 16 LCR by different C/EBPβ isoforms. *Mol. Carcinogenesis,* in press.

Supakar, P. C., Jung, M. H., Song, C. S., Chatterjee, B., and Roy, A. K. (1995). Nuclear factor kappa B functions as a negative regulator for the rat androgen receptor gene and NF-κB activity increases during the age-dependent desensitization of the liver. *J. Biol. Chem.* **270,** 837–842.

Tan, S. H., Leong, L. E., Walker, P. A., and Bernard, H. U. (1994). The human papillomavirus type 16 E2 transcriptional factor binds with low cooperativity to two flanking sites and represses the E6 promoter through displacement of Sp1 and TFIID. *J. Virol.* **68,** 6411–6420.

van der Eb, A. J., and Graham, F. L. (1980). Assay of transforming activity of tumor virus DNA. *Methods Enzymol.* **65,** 826–839.

Wissink, S., van Heerde, E. C., Schmitz, M. L., Kalkhoven, E., van der Burg, B., Baeuerle, P. A., and van der Saag, P. T. (1997). Distinct domains of the RelA NF-κB subunit are required for negative cross-talk and direct interaction with the glucocorticoid receptor. *J. Biol. Chem.* **272,** 22278–22284.

Woodworth, C. D., McMullin, E., Iglesias, M., and Plowman, G. D. (1995). Interleukin 1α and tumor necrosis factor α stimule autocrine amphiregulin expression and proliferation of human papillomavirus-immortalized and carcinoma-derived cervical epithelial cells. *Proc. Natl. Acad. Sci. USA* **92,** 2840–2844.

Woronicz, J. D., Gao, X., Cao, Z., Rothe, M., and Goeddel, D. V. (1997). IκB kinase-β: NF-κB activation and complex formation with IκB kinase-α and NIK. *Science* **278,** 866–869.

Xiao, H., Lis, J. T., Xiao, H., Greenblatt, J., and Friesen, J. D. (1994). The upstream activator CTF/NF-1 and RNA polymerase II share a common element involved in transcriptional activation. *Nucleic Acids Res.* **22,** 1966–1973.

zur Hausen, H. (1996). Papillomavirus infections—A major cause of human cancers. *Biochim. Bioph. Acta* **1288,** F55–F78.