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The E6/E7 promoter of extrachromosomal HPV16 DNA in cervical cancers escapes from cellular repression by mutation of target sequences for YY1

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Human papillomavirus type 16 (HPV16) induces squamous intraepithelial lesions of the cervical mucosa which may develop into invasive cancer. The expression of viral oncogenes in advanced neoplasias appears increased relative to the proliferating cell layers of low grade lesions raising questions about molecular mechanisms of deregulation of transcription. In a lymph node metastasis of a cervical cancer, we observed full-length HPV16 plasmids and molecules with a small deletion, which was mapped to the long control region (LCR). Both wild type and shortened LCR were amplified by PCR, cloned into the promoter test plasmid pBLCAT6 and sequenced to identify a 107 bp deletion from position 7794 to 7901 in the short LCR. CAT expression in cervical cancer-derived HT3, SiHa and CaSki cells appeared 5- to 6-fold increased under the control of the short LCR. This could be traced back to elevated levels of mRNA initiated at the viral oncogene promoter. A slight further increase in CAT expression was noted in the presence of the HPV16 E2 protein which is probably due to the deletion of one E2 binding site and consequent relief from E2 repression. Computer-assisted sequence analysis and band-shift experiments with purified YY1 protein and wild type or mutated oligonucleotides identified four binding sites for this cellular transcriptional repressor within the promoter-proximal segment of the HPV16 LCR, three of which were removed by the deletion. A LCR fragment comprising these YY1 binding sites was cloned in front of the heterologous thymidine kinase gene promoter and suppressed CAT expression 3- to 4-fold. This silencer activity was abolished by a mutation in the first YY1 binding site affected by the deletion. The LCRs of episomal HPV16 DNAs from three additional cases of cervical cancer were cloned and sequenced which revealed a naturally occurring point mutation in this very binding site in one case. The corresponding LCR showed a 4-fold enhanced promoter activity when compared with the HPV16 wild type LCR. This suggests that deletion or mutation of target sequences for YY1 represents a newly identified, repeatedly used strategy of HPV16 to escape from cellular repression.

Key words: cervical cancer/HPV16/transcriptional repression/YY1 protein

Introduction

Human papillomaviruses (HPV) induce dysplastic, squamous intraepithelial lesions (SILs) of the anogenital skin and mucosa, which may progress to cancer particularly at the uterine cervix (zur Hausen, 1991). The major oncoproteins of genital papillomaviruses are E6 and E7, which immortalize human keratinocytes and change their differentiation pattern in organotypic cultures. The E6 and E7 genes are continuously expressed in cervical cancer-derived cell lines and this has been shown to be necessary for the maintenance of their tumorigenicity in nude mice (von Knebel Doeberitz *et al.*, 1992). In the genital cancer-associated HPV types transcription of these genes is driven by one common promoter at the E6-proximal end of the non-coding, long control region (LCR) of the viral genome, called P_{97} in the case of HPV16 (Smotkin *et al.*, 1989). This promoter is stimulated by a keratinocyte-specific enhancer in the center of the LCR (Chong *et al.*, 1990, 1991; Cripe *et al.*, 1990) and by a binding site for the transcription factor Sp1 close to the TATA box (Gloss and Bernard, 1990). Binding of the viral E2 protein to three palindromic cognate sequences in the promoter proximal part of the LCR slightly represses the E6/E7 promoter, probably by hindering the access of Sp1 and the formation of the transcription initiation complex (Romanczuk *et al.*, 1990; Dostatni *et al.*, 1991; Thierry and Howley, 1991; Tan *et al.*, 1992). A more potent transcription-suppressing element depending on the cellular repressor YY1 was recently described close to the early promoter of HPV18 (Bauknecht *et al.*, 1992).

Messenger RNAs colinear with E6/E7 genes are hardly detectable by *in situ* hybridization in the basal epithelial layers of low grade HPV16-, 18- or 33-induced SILs but are prevalent in the more differentiated cells (Beyer-Finkler *et al.*, 1990; Dürst *et al.*, 1992; Stoler *et al.*, 1992). As the grade of neoplasia increases the viral oncogenes become comparatively strongly transcribed in basal cells and the mRNAs appear evenly distributed throughout the undifferentiated epithelium (Dürst *et al.*, 1992; Higgins *et al.*, 1992; Stoler *et al.*, 1992). This points to a derepression of the E6/E7 genes in proliferating cells which may contribute to tumor progression.

The DNA of HPV16 or 18 that persists mostly extrachromosomally in precancerous lesions frequently integrates into the genome of cancer cells (zur Hausen, 1991). It has repeatedly been shown that the disruption of the circular viral DNA due to integration destroys the E2 gene (Schwarz *et al.*, 1985; Matsukura *et al.*, 1986) thereby relieving the E2-dependent negative control of the E6/E7 promoter. Differences in viral gene regulation depending on the site of viral DNA integration into the host genome (zur Hausen, 1991) point to an additional role of surrounding cellular sequences in the deregulation of HPV transcription. The analysis of the lymph node metastases of cervical cancers suggested that the tumors are monoclonal with regard to the

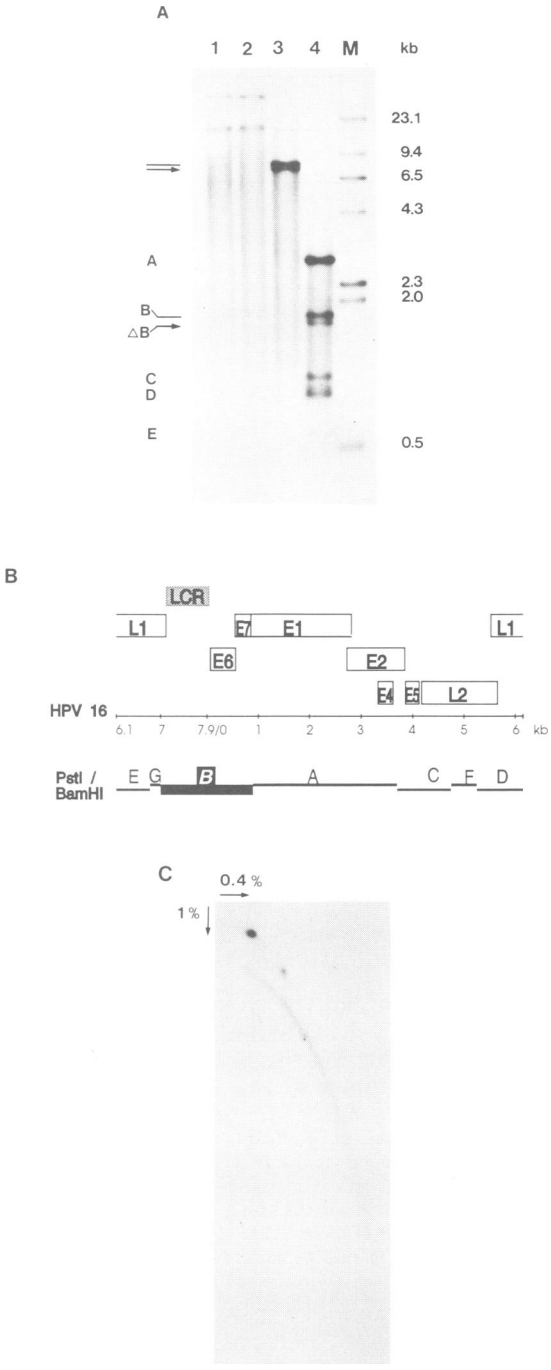

Fig. 1. Analysis of DNA extracted from a pelvic lymph node metastasis of a cervical carcinoma. (**A**) Southern blot hybridization of biopsy DNA with an HPV16-specific probe. Lane 1: uncleaved DNA; lanes 2–4: DNA digested with *Hin*dIII, *Bam*HI and *Bam*HI+*Pst*I, respectively; M: *Hin*dIII-cleaved ^{32}P-labeled λ DNA as molecular weight markers. Characteristic fragments of the *Bam*HI+*Pst*I digest of episomal HPV16 DNA are labeled to the left. The arrows point to additional bands beneath full-length HPV16 DNA (lane 3) and *Bam*HI−*Pst*I fragment B (lane 4) originating from HPV16 molecules with a deletion. (**B**) Localization of the LCR and open reading frames (E1–E7, L1 and L2) within the HPV16 genome and distribution of the *Bam*HI and *Pst*I cleavage sites. The *Bam*HI−*Pst*I fragment B, which is affected by the deletion, is highlighted as a black bar. (**C**) Two-dimensional agarose gel analysis. DNA was electrophoresed undigested in the first dimension in 0.4% agarose and in the second dimension in 1% agarose, and subjected to Southern blot hybridization. The two discrete spots above the trail of linear DNA represent circular episomal HPV16 DNA.

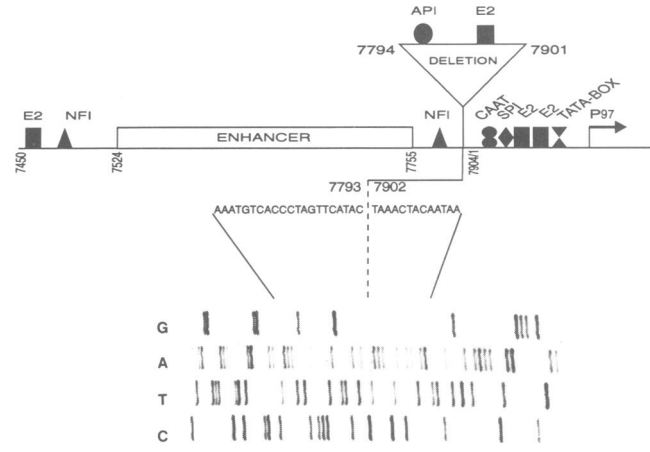

Fig. 2. Deletion in the LCR of extrachromosomally persisting HPV16 DNA from a lymph node metastasis of a cervical cancer. The sequencing gel defines a 107 bp deletion from position 7794 to 7901. The organization of the viral LCR upstream of the early promoter P_{97} is schematically presented in the upper part. The enhancer box summarizes numerous binding sites for transcription factors in the area of the major constitutive enhancer of HPV16, among others two AP1 and five NF1 sites (Chong *et al.*, 1990). Additional binding sites for NF1, AP1, SP1 (Gloss and Bernard, 1990) and the viral E2 outside this region are individually shown together with the CAAT- and TATA-box of P_{97}. Nucleotide positions according to Seedorf *et al.* (1985).

characteristic integration pattern of viral DNA (Fuchs *et al.*, 1989). This dates the integration event prior to invasion and may suggest that its contribution to the derepression of viral oncogenes plays an important role in tumor progression.

At least one-third of HPV16-positive cervical cancers contain only extrachromosomal viral DNA (Matsukura *et al.*, 1989; Choo *et al.*, 1987; Fuchs *et al.*, 1989; Cullen *et al.*, 1991). This raises questions about alternative possible ways of changing the transcription rate of viral oncogenes. One might think about mutations in the regulatory genes of the virus or in transcription control sequences of the LCR. We observed a short deletion in episomal HPV16 DNA from a lymph node metastasis of a cervical cancer (Girardi *et al.*, 1993), which was mapped to the viral LCR. This report describes the consequence of this deletion for P_{97} activity and thereby defines a strong negative transcription control element in the HPV16 promoter that is dependent on the cellular repressor YY1.

Results

Deletion of 107 bp from the HPV16 LCR stimulates gene expression

Total DNA from a lymph node metastasis of a cervical cancer was analyzed by Southern blot hybridization with an HPV16-specific probe. Uncleaved tumor DNA and a sample digested with *Hin*dIII, which does not cut HPV16 DNA, showed two bands in the high molecular weight range suggesting the presence of extrachromosomal viral DNA (Figure 1A). Two-dimensional gel electrophoresis in 0.4% or 1% agarose confirmed that they represent oligomeric episomes (Figure 1C). Cleavage of HPV DNA with *Bam*HI, which cuts this sequence only once, converted these bands into two new ones. The upper band corresponded to linear HPV16 DNA and the slightly lower one was suggestive of a small deletion in ~40% of the molecules. Digestion with *Bam*HI and *Pst*I led to the normal pattern of episomal HPV16

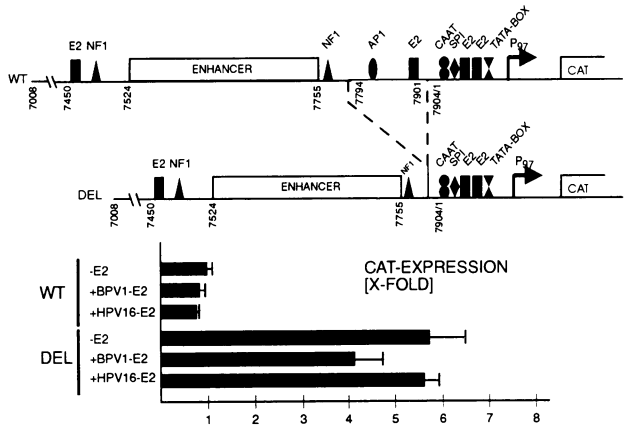

Fig. 3. Expression of CAT under the control of the wild type and spontaneously deleted HPV16-LCR. The upper part presents the structures of the HPV16-LCRs as cloned in the promoter test plasmids pLCR16-WT (WT) and pLCR16-DEL (DEL; see Figure 2). Lower part: comparison of the promoter activities of the wild type and the mutated HPV16-LCRs and the influence of homologous (HPV16) and heterologous (BPV1) E2 protein on the P_{97} activity. Error bars are based on at least five independent experiments.

DNA plus one additional band beneath the *Bam*HI−*Pst*I B fragment (Figure 1B). This band was interpreted as due to a ~100 bp deletion within the B fragment.

The HPV16 genome region presumably affected by the deletion in a part of the viral DNA molecules was amplified by PCR with oligonucleotide primers 1 and 2 flanking the LCR. We obtained two products with ~1 kb and 0.9 kb, which were cloned and sequenced. The 1 kb fragment showed the HPV16 prototype sequence (Seedorf *et al.*, 1985) except for point mutations at positions 7191 (G to T), 7494 (T to C) and 7519 (G to A). The A at position 7861 of the originally published sequence was missing and there was an additional A at position 7764 as noted before (Krajinovic and Savic, 1991; Chan *et al.*, 1992). The shorter fragment lacked sequences from position 7794 to 7901. This 107 bp deletion relative to the full-length LCR of this isolate removes binding sites for the cellular transcription factor AP1 and the viral regulator protein E2 (Figure 2). The shortened LCR was otherwise identical to the full-length LCR.

To look for the effect of the deletion on the activity of the early viral promoter P_{97}, which drives transcription of the oncogenes E6 and E7 in the context of the viral genome, we determined the amount of CAT enzyme expressed from pBLCAT6 under the control of the full-length (pLCR16WT) and shortened (pLCR16DEL) LCR (Figure 3). The constructs were transfected into HT3 cells, which represent an HPV DNA negative cervical carcinoma cell line. The deletion surprisingly led to a 5- to 6-fold increased CAT expression in spite of the loss of one AP1 binding site. A similar increase in CAT expression was observed in the HPV16-positive, cervical cancer derived cell lines SiHa and CaSki (Table I). Primer extension analysis of RNA from transfected HT3 cells confirmed that the increased amount of CAT enzyme is due to elevated steady-state levels of mRNA correctly initiated at the early viral promoter. The cap site turned out to be unchanged in the case of the shortened LCR (Figure 4).

The difference between the constitutive activities of pLCR16WT and pLCR16DEL could be expected to be affected by the viral regulator protein E2, which down-regulates transcription of genital papillomaviruses and is likely to be expressed in cancers with extrachromosomally persisting HPV16 DNA. We therefore tested pLCR16WT and pLCR16DEL in the presence of bovine papillomavirus (BPV)1 and HPV16 E2 expression vectors (Figure 3). BPV1 E2 protein led to a moderate reduction of CAT expression from both LCR vectors without changing the relation between pLCR16WT and pLCR16DEL. HPV16 E2 also repressed pLCR16WT but had no significant effect on pLCR16DEL; as a net result this led to a 7.5-fold increased CAT expression of pLCR16DEL relative to pLCR16WT. The slightly more pronounced difference between pLCR16WT and pLCR16DEL in the presence of HPV16 E2 is likely to be due to the deletion of one E2 binding site and consequent relief from E2 repression. However, the major functional effect of the deletion is constitutive, which may indicate that the deleted sequence contains an additional strong negative control element.

Table I. Activity of the HPV16 P_{97} in the context of wild type, shortened and point mutated LCR in various cell lines

Plasmids	Relative CAT activity (x-fold)[a]		
	HT3	SiHa	CaSki
pLCR16-WT	1	1	1
pLCR16-DEL	5.5	6.5	5
pLCR16-P	4	6	7

[a]Mean values from at least four independent transfection assays relative to the amounts of CAT expressed by pLCR16-WT.

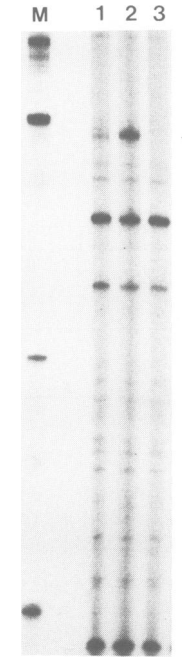

Fig. 4. Activity of the HPV16 P_{97} promoter in the context of the wild type and shortened HPV16-LCR. HT3 cells were transfected with pLCR16-WT (lane 1) and pLCR16-DEL (lane 2), and total cellular RNA was isolated (Chomczynski and Sacchi, 1987). RNA from untransfected HT3 cells served as negative control (lane 3). Primer extension analysis was carried out with a primer complementary to sequences at the 5′-end of the *cat* ORF. Specific extension products on the autoradiogram are marked by an arrowhead. Labeled *Hae*III fragments of ϕX174 DNA (M) were run in parallel as molecular size standards (234, 194, 118, 72 nt from top to bottom).

		5′ AANATGGNS	3′		YY1-Consensus-sequence
1	7787	5′ ACTAGGGTG	3′	7779	(rev.)
2	7791	5′ TACATGAAC	3′	7799	
3	7831	5′ CAAATGAAC	3′	7823	(rev.)
4	7834	5′ AAACTGCAC	3′	7842	
5	7840	5′ CACATGGGT	3′	7848	
6	7864	5′ AAAA.CGGT	3′	7857	(rev.)

Fig. 5. Sequence comparison of potential YY1 binding sites in the HPV16-LCR between positions 7779 and 7901. YY1 consensus binding site is according to Lee *et al.* (1992). The mismatches are underlined. (rev.) indicates reverse orientation.

A cluster of YY1 binding sites defines a transcriptional silencer

An analysis of the deleted DNA sequences showed that they had similarities to negative regulatory elements that bind the cellular transcriptional repressor protein YY1 (Lee *et al.*, 1992). Four potential binding sites lie within the deleted DNA segment, one is just disrupted by the deletion, and another is located upstream between positions 7779 and 7787 in reverse orientation (Figure 5). All sites show two mismatches with the consensus YY1 binding site and one gap had to be introduced in one site.

To test for binding of YY1, band-shift experiments were carried out with various oligonucleotides (Figure 6A) and affinity purified HIS−YY1 fusion protein. Oligonucleotide A, which contains potential binding sites 5 and 6, formed one shifted complex (Figure 6B) that could be prevented by competition with an unlabeled oligonucleotide containing a YY1 binding site (data not shown). The same shift was obtained with oligonucleotide B where site 6 was mutated by exchanging four nucleotides. In contrast, mutation of site 5 (oligonucleotide C) almost abolished binding activity indicating that this site is responsible for the observed YY1 interaction with oligonucleotides A and B. To test for binding of site 4 which overlaps with site 5, we used oligonucleotides D and E containing sites 4, 5 and 6. Site 5 was mutated in oligonucleotide E by exchanging five nucleotides as in oligonucleotide C. Both oligonucleotides formed one complex with YY1 suggesting that site 4 can bind the cellular protein but only one complex is formed if sites 4 and 5 are intact probably due to the overlap. Oligonucleotide F which represents site 3 was not shifted by YY1. PCR amplification product G, which contains potential binding sites 1, 2 and 3 formed two competable complexes. The strong, rapidly migrating band is likely to represent fragments with one bound YY1 molecule whereas the upper band will be due to complexes of YY1 with binding site 1 and 2. This was confirmed for site 2 by showing that the exchange of five nucleotides prevented the formation of the slowly migrating complex and significantly reduced the intensity of the lower band (Figure 6B, lane H). In summary, there are at least four YY1 binding sites in HPV16 DNA (displayed in black in Figure 6A), three of which were removed or destroyed in the naturally occurring deletion mutant from the cervical cancer.

To show that the cluster of YY1 binding sites acts as a true transcriptional silencer, HPV16 sequences between positions 7773 and 16 were cloned upstream of the thymidine kinase (*tk*) promoter of pBLCAT2. They reduced CAT expression 3- to 4-fold (Figure 7). When the second YY1

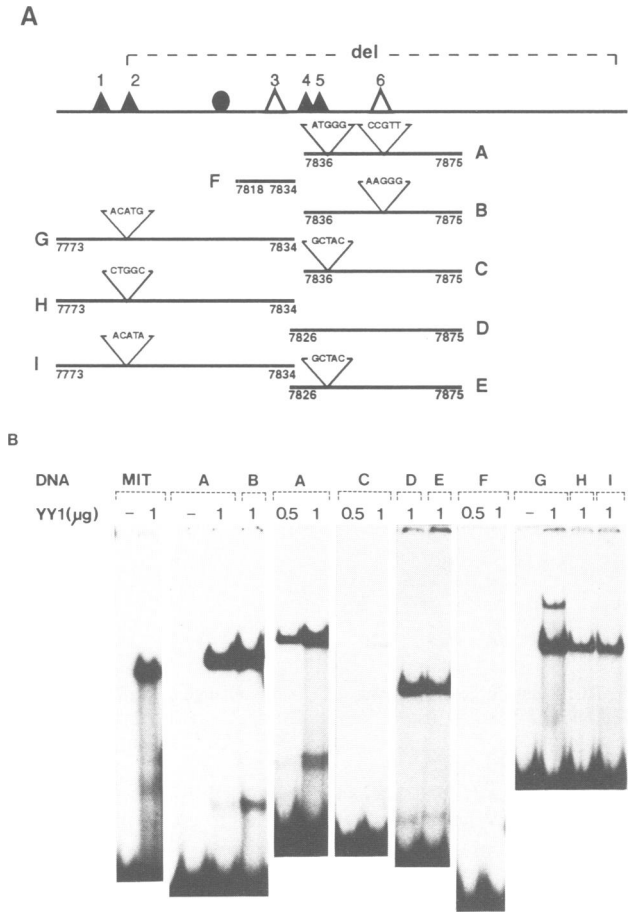

Fig. 6. (**A**) Localization of six potential YY1 binding sites (triangles) and one AP1 site (black oval) in the area of HPV16-LCR affected by a spontaneous deletion (del). The oligonucleotides (A, B, C, D, E and F) and the PCR amplification products (G, H and I) used for band-shift analyses of YY1 binding are presented beneath. Sequences of the wild type and mutated YY1 binding sites are displayed at the corresponding positions. True YY1 binding sites according to panel B are displayed in black whereas open triangles represent sequences that did not interact with YY1. (**B**) Band-shift analysis of HPV16-LCR subfragments with purified YY1 protein. MIT: oligonucleotide containing a bona fide YY1 binding site from a mitigator element of the major late promoter of adenovirus type 12 (Zock *et al.*, 1993). A to I: subfragments of the HPV16-LCR as defined in A.

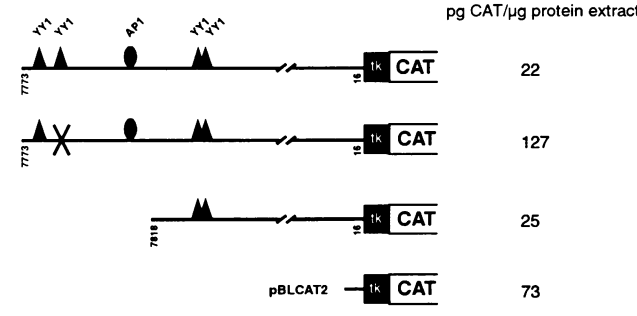

Fig. 7. Effect of HPV16 sequences on the activity of the *tk* promoter. The expression of CAT from individual constructs is given on the right. X: mutated YY1-binding site (see Figure 6A, oligonucleotide H).

binding site, which is destroyed by the deletion, was mutated as described for oligonucleotide H (Figure 6A), CAT expression increased 6-fold. In fact, it was even higher than

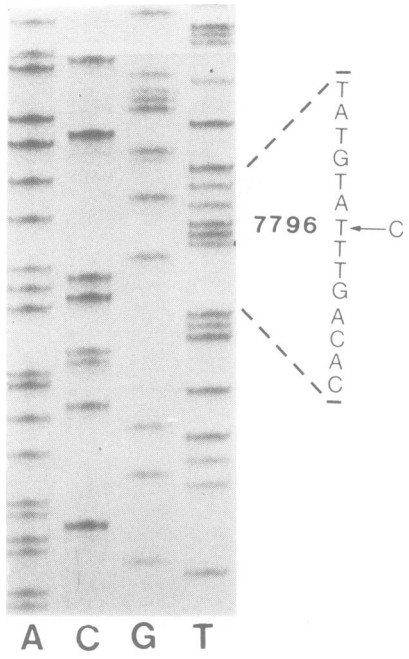

Fig. 8. Sequence gel identifying a C to T exchange at position 7796 of the lower strand of the HPV16 LCR from a cervical cancer.

with pBLCAT2, probably due to the stimulatory activity of the AP1 site. When this site was deleted to create pLCR16-B-*tk*CAT, the residual HPV16 sequences showed silencing activity again (Figure 7). This points to an intimate cross-talk of positive and negative control elements within this genome region of HPV16 leading to an overall negative effect under conditions tested in this study.

Mutation of an HPV16-YY1 binding site in an additional cancer

We were tempted to see if removal of target sequences for the YY1 protein represents a more widely used strategy of extrachromosomal HPV16 DNA in genital cancers to escape from intracellular control. Three additional cases of cervical cancer were available that contained within the detection limits of Southern blot hybridization only extrachromosomal HPV16 DNA (Fuchs *et al.*, 1989). The LCR sequences were amplified by PCR using primers 1 and 2 as described before and cloned into pBLCAT 6. Sequencing between HPV16 positions 7520 and 123 revealed wild type LCR in two cases except for the previously described changes at positions 7764 and 7861. However, one sequence showed a point mutation at position 7796 (G to A) within YY1 binding site 2 (Figure 8) and one T at position 7788 was deleted. To see if the transition affects YY1 binding we performed a band-shift experiment with PCR-amplified HPV16 sequences between positions 7773 and 7834 carrying the mutation at position 7796. The single nucleotide exchange affected YY1 binding in the same way as the more extensive engineered mutation of binding site 2 described above (Figure 6B, H and I). The mutated LCR was tested for P_{97} activity after cloning into the same plasmid background used for the analysis of pLCR16-WT and pLCR16-DEL. Plasmid pLCR16-WT served as reference. The mutated LCR led to a 4-fold higher CAT concentration (Table I). A comparable stimulation of CAT expression was observed in the cell lines SiHa and CaSki.

Discussion

About 40% of the episomal HPV16 DNA molecules in a lymph node metastasis of a cervical cancer showed a 107 bp deletion in the P_{97}-proximal part of the LCR. Restriction enzyme cleavage revealed no further deletions or insertions in the coding regions of the viral genomes. There were only a few nucleotide exchanges in the remaining part of the LCR relative to the HPV16 prototype sequence (Seedorf *et al.*, 1985) and these were also present in the full-length LCR of this isolate. They did not affect known binding sites for transcription factors and their number was in the range of sequence microheterogeneity observed with many HPV16 isolates (Chan *et al.*, 1992). For example, the transition at position 7519 is very prevalent and occurs in HPV16 DNAs from CaSki and SiHa cells.

The promoter activity of the shortened LCR could no longer be repressed by HPV16 E2 in contrast to that of the full-length LCR. This can be explained by the deletion of one E2 binding site in line with the observations of Thierry and Howley (1991), who noted a dependence of repression by the homologous E2 on the three promoter-proximal E2 binding sites of HPV18. Similar to this virus, two E2 cognate sequences of HPV16 apparently suffice to mediate repression by the heterologous BPV1 E2 because no difference was observed regarding BPV1 E2 repression of the full-length and short LCR. In the presence of HPV16 E2 the LCR with the deletion showed a 7.5-fold higher promoter activity than HPV16 wild type LCR. In a minor part this is due to the relief from homologous E2 repression, but another mechanism accounts for a constitutive 5-fold increase of promoter activity.

The LCR segment affected by the deletion could be shown to contain three binding sites for the cellular transcriptional repressor YY1. These sequences down-regulated basal level activity of the heterologous *tk* promoter and this silencer activity was lost as a result of a mutation that inactivated one YY1 binding site. Removal of the AP1 binding site from the HPV16 LCR segment revealed silencing activity in its promoter-proximal moiety (nt 7818–16) that contains the two other YY1 binding sites (Figure 7) but this is obviously too weak to override the stimulating effect of AP1 on its own. In the presence of AP1, the upstream YY1 binding site is absolutely essential for silencer activity. This suggests that the enhanced promoter activity of the shortened LCR is mainly due to the deletion of the target sequence(s) of the cellular YY1 protein. The increased promoter activity is not simply an effect of moving the enhancer closer to the promoter because inactivation of YY1 binding site 2 by a point mutation in the context of the full-length HPV16 LCR was sufficient to stimulate the activity of P_{97} in the same way as the deletion.

The regulation of viral gene expression by YY1-dependent silencer elements as defined in this study for HPV16 appears to be rather widespread among viruses that are able to establish persistent infections. It was noted before for the P5 promoter of adeno-associated viruses (Shi *et al.*, 1991) and for the *BZLF1* gene promoter of the Epstein–Barr virus (Montalvo *et al.*, 1991). The regulation of the *BZLF1* gene is particularly crucial as it holds a key position at the switch from latent to lytic infection. A YY1-dependent silencer was recently described in HPV18, which represents a high-risk HPV type like HPV16 (Bauknecht *et al.*, 1992). The map position of this silencer in the LCR close to the promoter-

proximal AP-1 site is similar to HPV16 except for the localization on the opposite site of the E2 binding palindrome of this area. The HPV16 sequences between positions 7868 and 16 corresponding to the HPV18 silencer were assayed in band-shift experiments and showed only negligible binding comparable to that of the mutated HPV16 oligonucleotide C (data not shown). This represents another example for slight differences in the exact constellation of protein recognition sequences between individual genital HPVs that were also noted for enhancer elements (Chong *et al.*, 1990). The sequences of the YY1 binding sites of HPV16 and 18 differ considerably. This would not be surprising in view of the unusually diverse binding specificity of this protein (Lee *et al.*, 1992) but is remarkable with regard to the close evolutionary relationship of genital papillomaviruses. From these findings it appears almost impossible to predict YY1 regulation of other papillomavirus types on the basis of a consensus sequence for the binding site. It will be necessary to test this experimentally, particularly for HPV types that are more distantly related.

Mutations of YY1 cognate sequences in two out of four episomal HPV16 DNAs from cervical cancers substantiate the biological significance of deleted or inactivated repressor binding sites for escape from cellular control. In fact we have observed a deletion of the two upstream YY1 binding sites of HPV16 in yet another case, where viral episomes revealed additional rearrangements in open reading frame E1 (X.P.Dong, F.Stubenrauch, E.Beyer-Finkler and H.Pfister, in preparation).

A PCR analysis identified the deletion described in this study in a second lymph node of the patient (Girardi *et al.*, 1993), which suggests that the metastasizing cancer was monoclonal with regard to the mutation as discussed before for viral DNA integration (Fuchs *et al.*, 1989). This assigns a crucial role in tumor progression to both these events.

We started this analysis of transcription control sequences of episomal HPV DNA by looking for alternatives to integration-mediated inactivation of E2 but one may speculate if YY1 target sequence mutation is restricted to episomal viral genomes. It may also play a role in cancers with integrated HPV16 DNA showing no evidence for E2 inactivation (Stoler *et al.*, 1992). Over and above that, the relief from YY1 repression appears to have a greater effect on P_{97} activity than relief from E2 repression. Both HPV-positive (HeLa: Bauknecht *et al.*, 1992; SiHa and CaSki: this study) and HPV-negative (HT3: this study) cervical cancer cells still reveal an active YY1 repression system. In the absence of evidence for somatic inactivation of YY1 genes there may be a more general role for viral mutations in YY1 binding sites to guarantee an enhanced expression of viral oncogenes, which are necessary to maintain the tumorigenic activity (von Knebel Doeberitz *et al.*, 1992).

Materials and methods

Isolation and analysis of biopsy DNA

The biopsies were collected at the University Hospital for Obstetrics and Gynecology in Graz, Austria, and kindly provided by F.Girardi (Fuchs *et al.*, 1989; Girardi *et al.*, 1993).

DNA from biopsy tissue was isolated according to the method of Shirasawa *et al.* (1988). Briefly, the specimen was minced in buffer containing 5 M guanidinium thiocyanate, 25 mM sodium citrate, 1% 2-mercaptoethanol and 0.5% sodium *N*-lauroyl sarcosinate, pH 7.3. The lysate was layered on a cushion of 5.7 M CsCl, 0.1 M EDTA, pH 7.0 and centrifuged at 32 000 r.p.m. for 22 h in an SW 55 rotor. The collected DNA was dialyzed against 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, incubated with proteinase K (100 μg/ml) for 2 h, extracted with phenol/chloroform and precipitated with ethanol.

For Southern blot analysis 10 μg aliquots of biopsy DNA were cleaved with the indicated restriction enzymes, separated on a 1% agarose gel and transferred in 1.5 M NaCl, 0.25 M NaOH onto Hybond N Nylon membrane. Two-dimensional gel electrophoresis of undigested DNA was performed in 0.4% agarose gels for 16 h in the first dimension and in a 1% agarose gel for 16 h in the second dimension. Both gels were run with 0.5 μg/ml ethidium bromide at 1.4 V/cm. Hybridization was carried out with nick-translated HPV16 DNA as described previously (Fuchs *et al.*, 1988).

Oligonucleotides and plasmid constructions

The following single-stranded (ss) and double-stranded (ds) oligonucleotides were used for PCRs and band-shift analysis [the nucleotide (nt) numbering of the HPV16 sequence is according to Seedorf *et al.* (1985)]: primer 1, ss: 5′-GGATCCGACCTAGATCAGTTTCCTTTAGGAC-3′ (nt 1–6 represent a *Bam*HI cleavage site to allow cloning of the PCR product obtained with this primer, and nt 7–31 represent HPV16 nt 7008–7032), primer 2, ss: 5′-GGATCCTCCTGTGGGTCCTGAAACATTGCAGT-3′ (nt 1–6 represent a *Bam*HI cleavage site, and nt 7–32 represent HPV16 nt 123–98), oligonucleotides A, B, C, ds (nt 7836–7875, with wild type sequence or mutated as shown in Figure 6A), oligonucleotides D, E, ds (nt 7826–7875, with wild type sequence or mutated as shown in Figure 6A), oligonucleotide F, ds (nt 7818–7834), primer 3, ss (nt 7773–7792), primer 4, ss (nt 7773–7810, mutated as shown in Figure 6A), primer 5, ss (nt 7773–7806, mutated as shown in Figure 6A), primer 6, ss (nt 16–7901), primer 7, ss (nt 7767–12), CAT primer (nt 185–156, pBLCAT6) and Ad12MLP-mitigator, ds (Zock *et al.*, 1993). All synthetic oligonucleotides were purchased from MWG-Biotech (Ebersberg, Germany) or Eurogentec (Seraing, Belgium).

The wild type, spontaneously deleted and point mutated HPV16-LCRs were PCR-amplified from biopsy DNA using primers 1 and 2, cleaved with *Bam*HI and cloned into the *Bam*HI site of the promoter test-vector pBLCAT6 (Boshart *et al.*, 1992) creating the constructs pLCR16-WT, pLCR16-DEL and pLCR16-P, respectively. To generate plasmids pLCR16-A-*tk*CAT, pLCR16-B-*tk*CAT and pLCR16-Amt-*tk*CAT, PCRs were carried out on pLCR16-WT template with the primers 3 + 6, F (upper strand) + 6, and 4 + 6, respectively. Amplifications were performed using Vent DNA polymerase (BioLabs, Beverly) according to the manufacturer's guidelines. The PCR products were phosphorylated and cloned into the *Xba*I site of the pBLCAT2 vector (Lukow and Schütz, 1987) blunt-ended by treatment with Klenow polymerase.

Cell culture and CAT assays

The human cervical carcinoma cell lines HT3 (Fogh *et al.*, 1977), SiHa (Friedl *et al.*, 1979) and CaSki (Pattillo, 1977), which contain no, one and 500 HPV16 genome copies per cell respectively (Yee *et al.*, 1985; Baker *et al.*, 1987), were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 120 mg/l each penicillin and streptomycin and 100 mg/l Ciprobay (Bayer, Leverkusen). The cells were transfected at 70–80% confluency by the calcium precipitation method as described previously (Stubenrauch *et al.*, 1992). 20 μg of *cat* gene reporter plasmids were co-transfected with 1 μg of the luciferase expression vector pRSV-LUC (DeWet *et al.*, 1987) used as an internal control of transfection efficiency. In some experiments 5 μg of the expression vector for the BPV1 or HPV16 E2 proteins (pC59, Yang *et al.*, 1985; pC859, Phelps and Howley, 1987) or the equivalent amounts of sonicated salmon sperm DNA were co-precipitated. Forty-eight hours after transfection the cells were harvested, washed in phosphate-buffered saline and lysed by four freeze–thaw cycles in 100 mM potassium phosphate pH 7.8, 1 mM DTT. The protein concentration in the lysates was determined by the method of Bradford (1976). The amount of CAT protein was measured by an ELISA system (Boehringer, Mannheim, Germany) according to the manufacturer's protocol. The assayed amounts of CAT were standardized according to the luciferase activity of the corresponding extracts.

Primer extension analysis

RNA was isolated from HT3 cells 48 h after transfection by the guanidinium lysis method of Chomczynski and Sacchi (1987). For primer extension analysis 40 μg of total cellular RNA was mixed with an excess of a ^{32}P-labeled oligonucleotide complementary to sequences at the 5′ end of the *cat* ORF (pBLCAT6, nt 156–185) in a total volume of 10 μl containing 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$. The hybridization mix was heated to 90°C for 3 min, to 55°C for 10 min and finally allowed to cool down slowly to room temperature. The volume was then increased to 20 μl with the addition of RNasin (BTS, St Leon-Rot) to 1 U/μl, dNTPs to 0.5 mM, 1 μl (200 U) of MMLV reverse transcriptase (BRL, Eggenstein)

and 2 μl of 5 × MRT buffer (250 mM Tris−HCl, pH 8.3, 375 mM KCl, 50 mM DTT, 15 mM $MgCl_2$). After incubation at 42°C for 1 h, EDTA (final concentration 25 mM) and RNase A (final concentration 200 μg/ml) was added and the reaction was further incubated for 30 min at 37°C. The reaction was phenol-extracted and ethanol-precipitated after addition of 20 μg of glycogen (Boehringer, Mannheim, Germany). Extension products were separated on a 5% denaturing polyacrylamide−urea gel. The gel was dried and exposed overnight to Kodak X-Omat at −80°C with an intensifying screen.

Expression and purification of YY1 protein

The YY1−6 × HIS fusion protein was expressed in the bacterial strain RR (Shi *et al.*, 1991). The bacteria were grown at 30°C to an OD_{600} of 0.7 and the expression plasmid was induced by addition of isopropyl-β-D-thiogalactoside to the end concentration of 1 mM. After a further 4 h the bacteria were harvested and lysed overnight in 6 M guanidine−HCl, pH 8.0. The YY1−HIS fusion protein was purified from the lysate by affinity chromatography on Ni-NTA agarose (Qiagen, Chatsworth) according to the protocol recommended by the manufacturer. The protein was dialysed against buffer as described by Dignam *et al.* (1983) and stored in aliquots at −80°C.

Gel retardation assays

The electrophoretic mobility shift assay has been described earlier (May *et al.*, 1991). The reactions were performed with aliquots of end-labeled ds oligonucleotides or PCR amplification products equivalent to 20 000 Cerenkov c.p.m. (corresponding to ~40 pg) in the presence of 0.5−1 μg poly(dI−dC)·poly(dI−dC) and 500−1000 ng YY1 protein. For competition experiments the labeled HPV16 sequences were incubated together with different amounts of homologous or heterologous unlabeled oligonucleotides. PCR amplification products G, H and I were obtained with primers 3, 4 and 5 and the lower strand of oligonucleotide F. The DNA−protein complexes were resolved on 4% native polyacrylamide gels in 0.5 × TBE (50 mM Tris, 42.5 mM boric acid, 1 mM EDTA).

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