

# Regulatory interactions of transcription factor YY1 with control sequences of the E6 promoter of human papillomavirus type 8

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Human papillomavirus type 8 (HPV-8) is a strictly cutaneous oncogenic virus known to induce malignant skin lesions in *epidermodysplasia verruciformis* patients. Our study shows that sequences surrounding transcription start sites of the HPV-8 oncogene E6 (nt 175–179) and comprising the presumable CCAAC and TATA boxes of the E6 promoter P<sub>175</sub> contain a cluster of four motifs similar to the DNA recognition site of the multifunctional cellular transcription factor *yin-yang 1* (YY1). Using DNase I footprinting and gel retardation tests it could be demonstrated that three of these motifs indeed act as YY1 binding sites. To test their functional relevance for P<sub>175</sub> activity, engineered YY1 binding site mutants were analysed in the

context of a P<sub>175</sub> test vector using transient expression assays with human keratinocytes. YY1 turned out to exert both positive and negative effects upon the activity of the HPV-8 E6 promoter; binding of YY1 to a site upstream of the promoter's cap-position (BS1) activated transcription, whereas the downstream site (BS2) mediated repression. The second downstream YY1 binding site (BS3) seemed to play an auxiliary role, enhancing the negative effect of YY1 BS2. These observations define YY1 as an important cellular protein involved in the control of E6 oncogene expression of the skin-specific 'high risk' HPV-8 and emphasize the potential regulatory role of sequences located downstream of the transcription start site.

## Introduction

Human papillomaviruses (HPV) induce squamous tumours of skin and mucosa. Most of these lesions are benign, persist only temporarily and eventually undergo spontaneous regression (Shah & Howley, 1996). However, persistent infections with specific HPV types may critically contribute to the development of human cancers. This is firmly established for HPV-16 and HPV-18 infection and cervical carcinomas as well as HPV-5 and HPV-8 infection and non-melanoma skin cancer in *epidermodysplasia verruciformis* (EV) patients (Fuchs & Pfister, 1997).

EV is a rare skin pathology characterized by a life-long occurrence of multiple flat warts and macular lesions (Jablonska *et al.*, 1972; Orth, 1987). EV lesions are induced by a group of at least 20 HPV types (Fuchs & Pfister, 1996). Up to 60% of EV patients have been reported to develop squamous cell carcinomas within their primary lesions 10–30 years after the onset of verrucosis. In contrast to the plurality of EV-associated HPVs in benign tumours, only a few virus types predominate

in EV cancers, HPV-5 and HPV-8 being the most frequent. In all EV lesions, the viral DNA persists as episomes, typically in high copy number per cell.

The cell-transforming potential of HPV-8 and several other EV-associated HPVs could clearly be assigned to the viral E6 protein (Iftner *et al.*, 1988; Kiyono *et al.*, 1989, 1992). Interestingly, no such properties were found for HPV-8 E7, a protein which constitutes a major oncogenic factor of 'high risk' genital HPV types involved in cervical carcinogenesis.

Transcription of the HPV oncogenes E6 and E7 is mediated by early promoters, invariably located in the ORF E6-proximal part of the non-coding regulatory region (NCR) of the viral genomes (Fuchs & Pfister, 1997). Also in EV-associated HPV-8 and HPV-47, slightly heterogeneous 5' ends of the E6-coding mRNAs map to the NCR–ORF E6 boundary (Kiyono *et al.*, 1989; Iftner *et al.*, 1990; Stubenrauch *et al.*, 1992). In the case of HPV-8, the cap sites of the E6 promoter are located between nt 175 and 179. However, in contrast to genital HPV types, very little is known about NCR sequences contributing to the function of the E6 promoters in EV-specific HPVs. The NCRs of EV-associated virus types clearly differ from those of other papillomaviruses; in EV-associated types, they are much shorter and the majority of them display a number of

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characteristic, highly conserved sequences (M33/API, CCAAC, M29 and A/T elements) and a unique distribution of binding sites of the viral *trans*-regulator protein E2 (Krubke *et al.*, 1987; Ensser & Pfister, 1990).

The activity of early HPV promoters in benign tumours is tightly regulated and correlates with the differentiation programme of keratinocytes, e.g. the oncogene transcripts of HPV-5 and HPV-16 are detectable by *in situ* hybridization primarily in superficial layers of the stratified epithelium (Dürst *et al.*, 1992; Stoler *et al.*, 1992; Higgins *et al.*, 1992; Böhm *et al.*, 1993; Haller *et al.*, 1995). On the other hand, poorly differentiated cells of malignant genital lesions uniformly express the oncogene messages.

In high-risk genital HPV infections, deregulation of expression in cancer cells may result from destruction of the viral E2 gene as a consequence of the frequently observed integration of the virus into the genome of the host cell, since E2 represses E6/E7 promoters at higher concentrations (zur Hausen, 1994). We have recently reported that extrachromosomally persisting HPV-16 DNAs from cervical carcinomas often present deletions and point mutations within their genomic control regions which involve binding sites of the cellular transcription factor *yin-yang* 1 (YY1) (Dong *et al.*, 1994; May *et al.*, 1994a). Since all these mutations result in upregulation of the HPV-16 E6/E7 oncogene promoter, we proposed that mutations of the cognate sequences for YY1 binding may offer another way to overcome negative control, resulting in deregulated expression of the E6/E7 proteins.

YY1 is an ubiquitously expressed multifunctional factor from the family of DNA-binding GLI-Krüppel zinc finger proteins which exerts regulatory functions in a variety of cellular and viral promoters (Shrivastava & Calame, 1994). The most striking feature of YY1 is its ability to act either as a transcriptional activator or repressor, depending on the local sequence context or secondary interactions with other proteins. Moreover, it could be shown that in some TATA-less genes YY1 may play a role as transcription-initiating factor (Seto *et al.*, 1991; Shi *et al.*, 1991).

In view of the fact that the DNA of EV-associated HPVs persists in skin cancers exclusively as episomes, we became interested in the role of YY1 in the regulation of their oncogene promoters. In a first approach to this question, we examined whether the E6 promoter of the strictly cutaneous EV virus HPV-8 is subject to transcriptional control by YY1.

## Methods

**■ Plasmid constructs and oligonucleotides.** The HPV-8 P<sub>175</sub> promoter test plasmid (p175-WT-CAT) was generated by successive cloning of an HPV-8 PCR amplificate (nt 53–248) and a dimer of a double-stranded oligonucleotide corresponding to the HPV-8 M33/API enhancer (nt 7422–7474; Horn *et al.*, 1993) into the filled-in *Bam*HI and *Xba*I sites of the promoter test vector pBLCAT6 (Boshart *et al.*, 1992), respectively. The primers used to amplify the HPV-8 promoter fragment were P1 (5' AAGTTGTTATTGCCAACCAACCATCGTCTA 3'; nt 53–

81) and P2 (5' GGTAGCTCGTCCTTATTAGTGTCT 3'; nt 248–225). The PCR reaction was run with a high fidelity Vent-polymerase (Biolabs).

The YY1 binding site mutants MT2 (p175-MT2-CAT), MT3 (p175-MT3-CAT) and MT2/3 (p175-MT2/3-CAT) were constructed using a PCR site-directed mutagenesis protocol (Ausubel *et al.*, 1995). Briefly, two separate PCR reactions were performed, each using either a 5' or a 3' flanking primer (P3, 5' AGGTTCGACGGATCCCCGGGAATTCATC 3'; P4, 5' GCGAGCTCGAGATCTAGATATCTCCA 3') and one of the central complementary primers carrying the desired mutations. Primers P3 and P4 were located over the boundaries of the HPV-8 insert (nt 7078–555) of plasmid pPF43 (Reh & Pfister, 1990) used as a PCR template. Both amplified DNA fragments were gel-purified, mixed and subjected to the second PCR with primers P3 and P4. The PCR products were purified again, digested with *Bam*HI and *Bgl*II and cloned into the *Bam*HI site of the vector pCR-Script-Amp (Stratagene). The mutated promoter sequences were amplified with primers P1 and P2 and inserted into the filled-in *Bam*HI site of pM33/API-Di-CAT plasmid, which contains a dimer of the M33/API element, cloned into the *Xba*I site of pBLCAT6. The presence of mutations was confirmed by sequencing. Mutation of YY1 BS1 (p175-MT1-CAT) and the triple mutation of sites 1, 2 and 3 (p175-MT1/2/3-CAT) were obtained using the Chameleon double-stranded site-directed mutagenesis kit (Stratagene) with p175-WT-CAT and p175-MT2/3-CAT DNAs as templates. A pBLCAT6-specific oligonucleotide (nt 1691–1710; Boshart *et al.*, 1992) with a mutated *Sma*I site served as a selection primer. Mutations introduced into YY1 binding sites 1, 2 and 3 and the initially considered site 4 affected five nucleotides each (MT1, nt 119–123; MT2, nt 195–199; MT3, nt 218–222; MT4, nt 233–237; see also Fig. 2a).

For DNase I footprint analysis, a pMM1-CAT plasmid with an HPV-8 fragment (nt 7626–301) cloned into the *Bam*HI site of pBLCAT6 has been used (M. May, unpublished work).

For band shift analyses, we used as target DNAs the double-stranded oligonucleotides A and B (nt 110–134) containing wild-type or mutated BS1, and PCR amplicates C–H (nt 168–248), containing wild-type or mutated YY1 binding motifs 2 through 4, respectively (Fig. 2a). PCR amplicates C–H were generated with primers complementary to nt 168–204 and nt 213–248, which contained wild-type or mutated HPV-8 sequences, respectively. The following double-stranded oligonucleotides served as competitor DNAs: P5 + 1 [nt –10 to +13 of adeno-associated virus (AAV) type 2 P5 promoter containing a well-characterized YY1 binding site; Shi *et al.*, 1991]; NRE-MT2 with no YY1 binding site (HPV-8 nt 7384–7421; May *et al.*, 1994b); and O/BS2 (HPV-8 nt 187–210), containing the YY1 binding motif 2.

All synthetic oligonucleotides were purchased from Eurogentec. Nucleotides of the HPV-8 sequence are numbered according to Fuchs *et al.* (1986).

**■ Cell culture and functional assays.** The human skin carcinoma cell line RTS3b (Purdie *et al.*, 1993) was grown in a keratinocyte medium as described previously (Stubenrauch *et al.*, 1996). Cells were transfected with test DNAs using Lipofectamine (Gibco/BRL) according to the manufacturer's protocol. Briefly, 24 h prior to transfection  $5.5 \times 10^5$  cells were plated into 6 cm culture dishes. Lipofectamine (12  $\mu$ l) was mixed with 3.5  $\mu$ g DNA in a total of 600  $\mu$ l OptiMEM medium (Gibco/BRL), left at room temperature for 30 min, mixed with 2.4 ml medium and added to cells that had been previously washed with PBS. After 6 h, the Lipofectamine-containing medium was replaced with 4 ml fresh keratinocyte medium containing 10% (v/v) FCS and the cells were incubated further for 42 h. Within one experiment, each test DNA was transfected onto three separate plates. To determine transfection efficiency, the fourth plate was transfected with 1.5  $\mu$ g of the expression vector pCMV-

$\beta$ -gal (Dong *et al.*, 1994) and stained *in situ* for  $\beta$ -galactosidase activity. No cotransfection approach has been chosen for this purpose since the  $\beta$ -gal vector inhibited the activity of the HPV-8 test constructs. Chloramphenicol acetyltransferase (CAT) activity was determined according to a standard protocol (Ausubel *et al.*, 1995). Reaction products were separated by thin-layer chromatography and quantified with a phosphorimager (Molecular Dynamics). All results presented are the average values of at least ten independent experiments.

**Gel retardation analysis.** YY1 protein was bacterially expressed as a 6  $\times$  His-tagged polypeptide and affinity-purified as described by Shi *et al.* (1991). A standard binding reaction was performed with  $^{32}$ P-end-labelled target DNAs (20000 Cerenkov c.p.m.) and 500 ng YY1 protein in 20  $\mu$ l buffer containing 10 mM HEPES pH 7.9, 5 mM KCl, 2 mM MgCl<sub>2</sub>, 0.01 mM dithiothreitol, 0.1 mM spermidine, 5  $\mu$ g BSA, and 1  $\mu$ g poly(dI-dC)·(dI-dC). In competition experiments, a 100-fold molar excess of unlabelled heterologous oligonucleotides with or without a YY1 binding site was included. The reaction proceeded for 20 min at room temperature and finally the DNA-protein complexes were resolved in 4% native polyacrylamide gels and exposed to X-ray film.

**DNase I footprints.** For DNase I footprint analysis, HindIII-linearized DNA of pMM1-CAT was labelled using [ $\alpha$ - $^{32}$ P]dATP and Klenow polymerase and digested with XhoI. After gel purification, the asymmetrically labelled NCR fragment was subjected to a binding reaction with 2  $\mu$ g purified YY1 protein under conditions described for band shift analysis. The DNA-YY1 complexes were partially digested with pancreatic DNase I (Worthington). The reaction with a predetermined amount of DNase I took place in a volume of 20  $\mu$ l for 90 s and was terminated by addition of 100  $\mu$ l of 0.2 M NaCl, 0.03 M EDTA, 100  $\mu$ g/ml yeast tRNA, 1% (v/v) SDS and 200  $\mu$ l phenol. The ethanol-precipitated digestion products were separated in 5% urea gels and analysed by autoradiography.

**Primer extension analysis.** Total cellular RNA from transiently transfected keratinocytes was isolated according to the guanidinium isothiocyanate method of Chomczynski & Sacchi (1987). RNA (55  $\mu$ g) was mixed with an excess of the  $^{32}$ P-end-labelled primer complementary to the 5' proximal *cat* gene sequence (pBLCAT6, nt 115-142; Boshart *et al.*, 1992) in 10  $\mu$ l of 225 mM KCl. Primer annealing, reverse transcription and purification steps were performed as described by Steger & Corbach (1997). Extension products were separated on 6% denaturing polyacrylamide-urea gels. The gels were dried and exposed at -80 °C for 2 weeks with an intensifying screen.

## Results

### Identification of YY1 binding sites around the cap sites of E6 transcripts

Inspection of sequences around the start sites of transcripts of the HPV-8 oncogene E6 showed the presence of four sequence motifs resembling the recognition site of the YY1 factor as defined by Lee *et al.* (1992). As shown in Fig. 1(a), one of the motifs matched the recognition site perfectly whereas the other three differed from the consensus sequence at one nucleotide position. These local sites have been designated BS1 to BS4.

In order to test the actual YY1 binding capacity of these sites, we initially used DNase I footprint analysis with bacterially expressed, affinity-purified YY1 protein (Fig. 1b). Clear protection could be demonstrated for sequences coinciding with sites 1 and 2. In contrast, no footprint could be

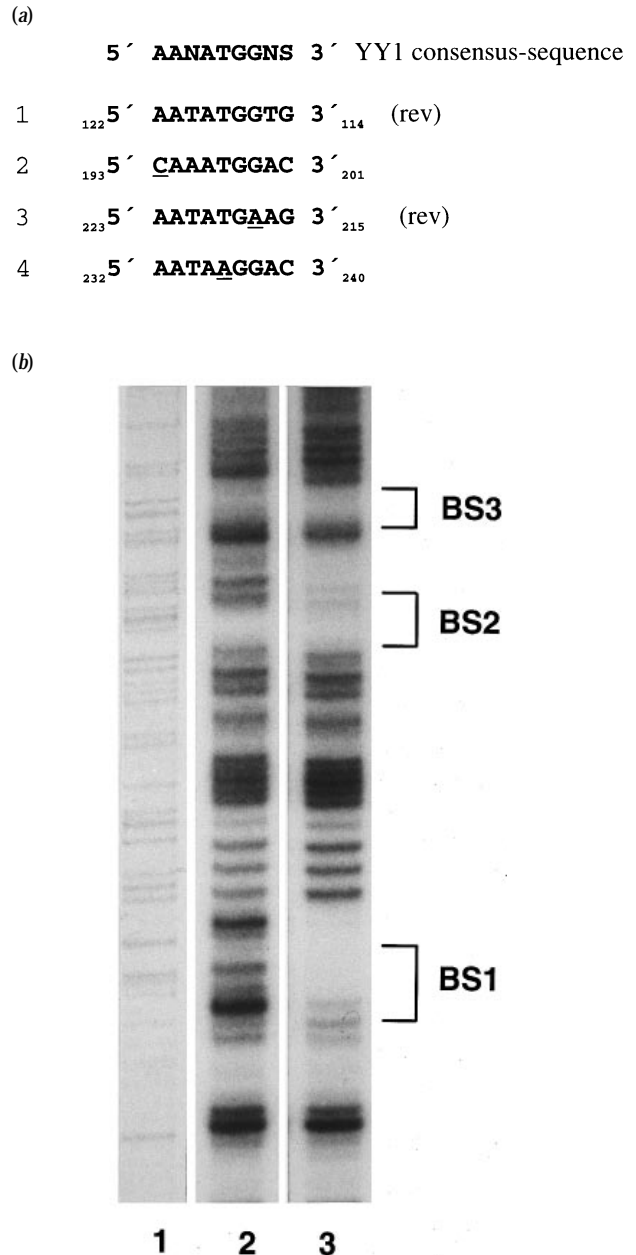


Fig. 1. DNase I footprint analysis of YY1 binding sites. (a) Sequence comparison of potential YY1 binding sites within the tested promoter fragment. The mismatches with a consensus YY1 site proposed by Lee *et al.* (1992) are underlined; rev, reverse orientation. In the YY1 consensus sequence, S represents C or G. (b) YY1 protein protects sites 1 and 2 against DNase I. An HPV-8 fragment (nt 7625-301) was end-labelled at the coding strand, incubated with purified YY1 protein (lane 3) or with the binding buffer only (lane 2), partially digested with DNase I and resolved in 6% urea-PAGE. The sequencing ladder (lane 1) was run in parallel as a DNA length marker. YY1 binding sites BS1, BS2 and BS3 are marked with brackets.

observed at site 4. Analysis of site 3 was not possible, since this region of the HPV-8 sequence seemed not to be attacked by DNase I. There were generally no indications of YY1 binding at other locations within the promoter fragment.

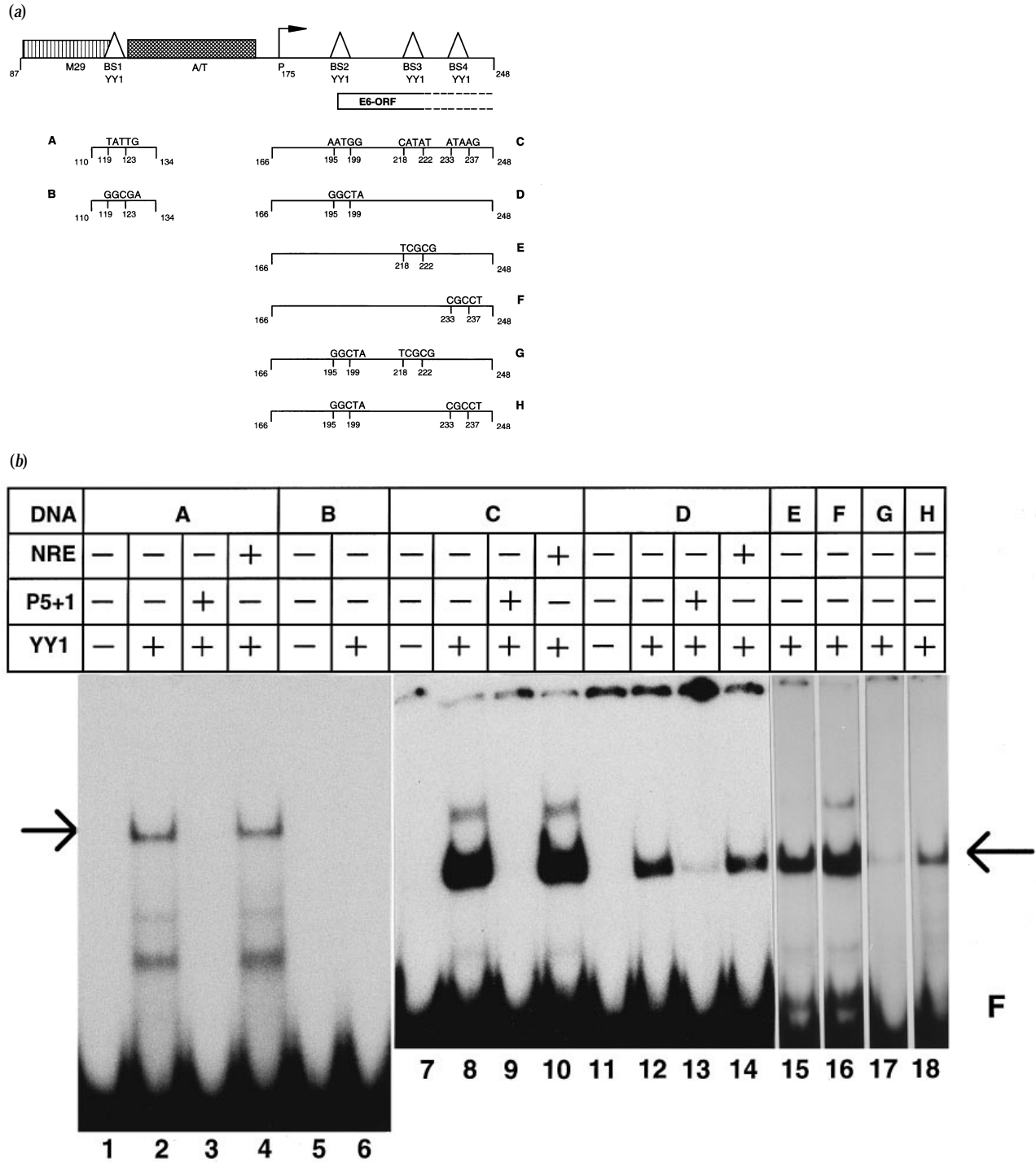
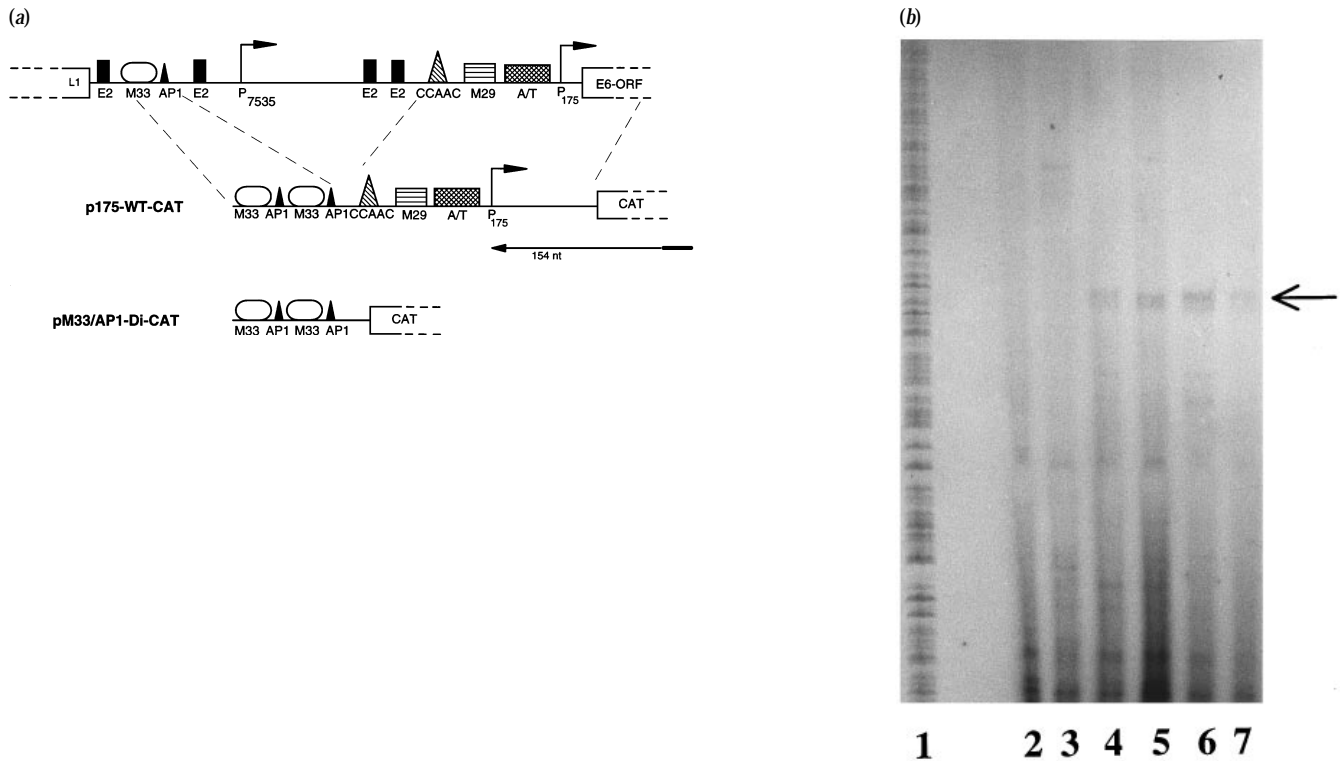


Fig. 2. (a) Localization of four potential YY1 binding sites ( $\Delta$ ) within the HPV-8 E6 promoter fragment and oligonucleotides (A and B) and PCR amplicates (C–H) used for gel retardation analyses. Sequences above individual target DNAs are wild-type (A and C) or mutated (B, D–H) parts of the potential YY1 binding sites. Nucleotide positions of the HPV-8 sequence are given. (b) Band shift analysis of promoter subfragments with purified YY1 protein. The table above the autoradiograms gives compositions of the binding reactions analysed in lanes 1–18. NRE, heterologous oligonucleotide containing no YY1 binding site; P5 + 1, oligonucleotide corresponding to a part of the AAV P5 promoter with an authentic YY1 binding site; A–H, target DNAs as defined in (a). The arrows mark the position of the main DNA–YY1 complex. F represents free DNA.



**Fig. 3.** Primer extension analysis of CAT RNA isolated from keratinocytes transfected with wild-type and mutated promoter constructs. (a) Structure of the HPV-8 NCR and its elements cloned into pBLCAT6. Different graphical symbols mark the localization of characteristic sequence motifs (M33, CCAAC, M29 and A/T) and binding sites for the transcription factors AP1 and E2. The arrows give the start sites of transcripts driven by the promoters P<sub>7535</sub> and P<sub>175</sub> as defined by Stubenrauch *et al.* (1992). The arrow below p175-WT-CAT represents an expected primer extension product. (b) Autoradiography of the cDNAs resolved in a denaturing polyacrylamide gel. Individual lanes show extension products obtained with RNA from cells transfected with pBLCAT6 (lane 2), pM33/AP1-Di-CAT (lane 3), p175-WT-CAT (lane 4), p175-MT2-CAT (lane 5), p175-MT3-CAT (lane 6) and p175-MT2/3-CAT (lane 7). For mutated promoter constructs see Methods. Technical problems due to very low RNA concentrations do not allow quantitative evaluation of the data. Lane 1, A+T sequencing ladder used as a length marker. The arrow points to the position of the correctly primed extension product(s).

To check for the sequence-specificity of YY1 interactions with the promoter region, we further analysed binding of YY1 protein in gel retardation experiments. As shown in Fig. 2(b), incubation of oligonucleotide A, which contains the potential BS1, with YY1 resulted in three DNA–protein complexes (lane 2). The same complexes could be seen when the binding reaction was performed in the presence of an excess of the unlabelled, heterologous oligonucleotide NRE-MT2 (lane 4). However, they disappeared entirely upon competition with either oligonucleotide P5 + 1, which contains a genuine YY1 binding site (lane 3), or oligonucleotide O/BS2, which corresponds to the sequence surrounding the HPV-8 YY1 binding motif 2 (data not shown). No complex formation was found with oligonucleotide B, which has mutations in five nucleotides of the BS1 sequence (Fig. 2a, b, lane 6). Moreover, unlabelled oligonucleotide A could compete for formation of a complex between YY1 and O/BS2. These results confirm that BS1 is a specific sequence target of the YY1 factor. We believe the multiple complexes observed in these experiments were due to degradation products present in the YY1 preparation used.

YY1 BS2 to 4 were tested by analysing a PCR amplificate containing all these motifs. In band shift experiments, two prominent complexes were observed for this DNA fragment (lane 8). Again, the competition tests (lanes 9 and 10) suggested a sequence-specific nature of these interactions. In order to examine the involvement of the individual potential YY1 binding motifs, we introduced mutations within each site as well as in sites 2 plus 3 and sites 2 plus 4 (Fig. 2a). Gel retardation analysis revealed that simultaneous disruption of sites 2 and 3 severely affected YY1 binding; as shown in Fig. 2(b) (lane 17), there were only trace amounts of the faster migrating complex left. Mutations introduced individually into sites 2 and 3 both resulted in the disappearance of the upper, slower migrating complex only (lanes 12 and 15, respectively). This was also the case for amplificate H, which carried mutations in sites 2 and 4. In contrast, a mutation of BS4 alone did not affect the pattern of complexes that were characteristic for the wild-type amplificate (lane 16). These data indicate that BS2 and BS3, but not BS4, are bona fide YY1 binding sites. They also suggest that the fast and slow migrating complexes observed with the wild-type amplificate are due to target

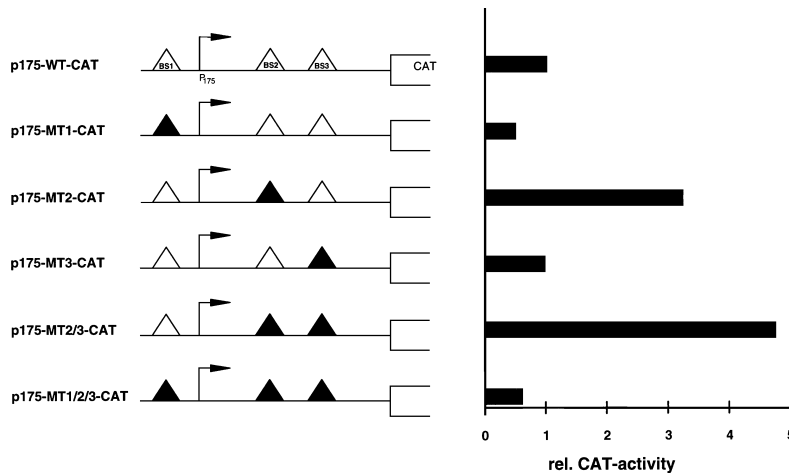


Fig. 4. Effects of mutations introduced into YY1 binding sites on activity of the E6 promoter construct. An overview of YY1 binding sites mutated in the individual test constructs is shown to the left.  $\Delta$ , wild-type YY1 binding sites;  $\blacktriangle$ , mutated YY1 binding sites. CAT activity in extracts from RTS3b keratinocytes transfected with different mutant constructs is given relative to that of p175-WT-CAT (wild-type promoter), which is taken as 1.

DNAs loaded with one or two YY1 molecules occupying sites 2, 3 or both.

#### Role of individual YY1 binding sites in $P_{175}$ activity

HPV-8 NCR contains in its 5' part the strong late promoter  $P_{7535}$  which, in transient assays, dominates the very weak E6 promoter (Stubenrauch *et al.*, 1992). To analyse the functional relevance of the interactions of the YY1 factor with its three binding sites identified in the vicinity of  $P_{175}$ , it was therefore necessary to construct an E6 promoter-specific test plasmid. Therefore, we cloned a 196 bp HPV-8 fragment (nt 53–248) into the promoterless vector pBLCAT6. The fragment chosen for this purpose encompasses the cap site at position 175 (Stubenrauch *et al.*, 1992) and contains a number of sequence elements conserved among many EV-specific HPV types in their E6-proximal NCR parts (CCAAC, M29 and A/T motifs). No promoter activity was observed for this construct in transient CAT assays with RTS3b keratinocytes. In order to identify sequence elements within the HPV-8 NCR which may activate the  $P_{175}$  promoter, we cloned a dimer of the previously characterized transcriptional activator element M33/AP1 (Horn *et al.*, 1993) in front of the candidate promoter fragment. In the case of this construct (p175-WT-CAT), expression of the reporter gene was observed in transient CAT assays. Primer extension analysis of RNA isolated from transfected cells (Fig. 3*b*) revealed the specific cap sites of the *cat* gene transcripts at HPV-8 positions 177 and 179, which suggests they are most probably directed by the E6 promoter  $P_{175}$ .

The above described mutations of YY1 BS1 to 3 were introduced into the context of the promoter test construct p175-WT-CAT and tested in transient CAT expression assays. As shown in Fig. 3*b* (lanes 5 and 6), mutations of BS2 and 3 did not influence the location of the transcription initiation points. Disabling of YY1 binding either to BS1 (p175-MT1-CAT) or to all three recognition sites (p175-MT1/2/3-CAT) resulted in a roughly 50% reduction of the wild-type promoter activity. In contrast, a mutation in BS2 alone stimulated  $P_{175}$

activity 3.2-fold. No significant effect was observed for the mutation in BS3. However, both these mutations in concert mediated a 4.8-fold enhancement of the  $P_{175}$  promoter activity (Fig. 4).

These results indicate that binding of YY1 to sites 1 and 2 results in opposite regulatory effects; BS1 activates whereas BS2 represses activity of the HPV-8-E6 promoter. Interestingly, BS3, although it has no effect on its own, seems to support the negative influence of BS2. Nevertheless, keeping in mind the reduced activity of the p175-MT1/2/3-CAT construct, the overall influence of YY1 interactions with its targets within the  $P_{175}$  must be regarded as an activating one.

#### Discussion

During the last few years it has become evident that the multifunctional transcription factor YY1 can control gene expression in numerous viruses. YY1-regulated promoters have been reported for AAV (Shi *et al.*, 1991), adenovirus type 12 (Zock *et al.*, 1993), Epstein-Barr virus (Montalvo *et al.*, 1995), herpes simplex virus type 1 (Chen *et al.*, 1992), human cytomegalovirus (Liu *et al.*, 1994), human immunodeficiency virus type 1 (Margolis *et al.*, 1994), parvovirus B19 (Momoeda *et al.*, 1994), polyomavirus (Martelli *et al.*, 1996), Moloney murine leukaemia virus (Flanagan *et al.*, 1992), and HPV-16 and HPV-18 (Bauknecht *et al.*, 1992; May *et al.*, 1994*a*). Since in the majority of these cases repression of transcription was observed as a net result of YY1 interactions with the viral regulatory sequences, it has been postulated that YY1 may contribute to the establishment of persistent or latent virus infections.

In this regard, the role of YY1 in the regulation of human oncogenic papillomaviruses deserves special attention. The genital mucosa-specific, high risk HPV-16 and HPV-18 contain YY1-controlled promoters (May *et al.*, 1994*a*; Bauknecht *et al.*, 1995; O'Connor *et al.*, 1996), which mediate expression of the viral oncoproteins E6 and E7 that are actively involved in the promotion of cervical cancer, and overexpression of the

oncogenes due to impaired YY1 control seems to contribute to tumour progression (May *et al.*, 1994a; Dong *et al.*, 1994).

We focused in this work on the function of YY1 in the regulation of the E6 oncogene promoter of HPV-8, a virus clearly different from genital HPVs in its epidemiology, tissue tropism and in several genomic features. Prior to our study, we demonstrated by Western blot analysis with an anti-YY1 antibody that RTS3b keratinocytes do contain substantial amounts of the YY1 protein (data not shown). The HPV-8 E6 promoter fragment (nt 53–248) could be shown to contain three binding sites for the YY1 factor. All these sequence motifs correspond within a one nucleotide-mismatch limit to the YY1 consensus recognition site proposed by Lee *et al.* (1992) and Shrivastava & Calame (1994) and determined empirically by Hyde-DeRuyscher *et al.* (1995), respectively. They share a common trinucleotide core, 5' CAT 3', which seems to be critical for the YY1–DNA interaction. The failure of the potential site 4 to bind YY1 protein (CAT vs CTT misfit) supports this emerging rule. However, the CAT motif alone is not indicative of a YY1 binding site, which has doubtlessly been proven by the study of O'Connor *et al.* (1996) on potential YY1 recognition sites in the HPV-16 NCR.

Mutational analysis of the identified YY1 binding sites demonstrated that all three of them were involved in the control of P<sub>175</sub> activity. However, it could also be shown that individual sites exert fairly different effects upon promoter function. Whereas a mutation of BS1 reduced P<sub>175</sub> activity by 50%, about 3-fold stimulation was observed for the BS2 mutant. The BS3 mutation, although being neutral on its own, could clearly enhance the effect of the BS2 inactivation. Such a cooperative effect between YY1 binding sites has not been reported so far. Furthermore, it is noteworthy that functionally relevant YY1 recognition sites are located both upstream and downstream of the HPV-8 transcription initiation site, which represents an unusual arrangement. In papillomaviruses, downstream YY1 binding sites have not been previously described.

The sequence of BS2 was found to be highly conserved among EV-specific HPVs. Comparison of 17 sequenced EV-viruses revealed potential YY1 binding sites in the same genomic position. Interestingly, this location corresponds to the translational start codon of E6. The obvious similarity between the YY1 binding site core (C)CAT and the preferred translational initiator motif ATG(G) (Kozak, 1984) suggests that the surroundings of translation initiation codons in many genes may represent targets of the YY1 factor.

A picture seemed to emerge several years ago that upstream YY1 sites generally mediate transcriptional repression, whereas downstream sites mostly exert positive effects on promoter activity (reviewed by Shrivastava & Calame, 1994). However, upstream stimulatory YY1 binding sites [e.g. in the promoters of herpes simplex virus type 1 (Chen *et al.*, 1992), parvovirus B19 (Momoeda *et al.*, 1994) and intracisternal A particles (Satyamoorthy *et al.*, 1993)] and downstream negatively regulating sites [e.g. in the adenovirus type 12 (Zock *et al.*,

1993) and embryonic  $\beta$ -type globin (Wandersee *et al.*, 1996) promoters] have been identified, which is in line with the function of the HPV-8 BS1 and 2. It should be noted that BS2 and 3 show only one mismatch each when compared to the consensus repressor YY1 binding site proposed by Shrivastava & Calame (1994). In contrast, BS1 presents only a rather poor homology (five mismatches; 60%) with the consensus of a transcription-activating YY1 binding site.

Interestingly and in contrast to other HPVs, the repression mediated by downstream sites is counterbalanced by the activating, upstream site BS1. At least in the tested context, BS1 plays the role of a 'master site', which is decisive for promoter activity. In HPV-18, YY1 mediated activation in HeLa cells because of the presence of C/EBP $\beta$  (Bauknecht, *et al.*, 1996) whereas repression was observed in SiHa, 444 and GM1604 cells (Jundt *et al.*, 1995). HPV-8 appears to be distinguished by balanced positive and negative YY1 effects via different binding sites in the same cell line under identical cellular conditions, thus allowing particular fine tuning of HPV-8 promoter activity. The involvement of similar mechanisms in other papillomaviruses is unclear since no attention has so far been paid to the control functions of a region downstream of their promoter's cap sites.

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