Human papillomavirus type 8 contains cis-active positive and negative transcriptional control sequences

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Human papillomavirus type 8 (HPV-8) is one aetiological agent of macular and flat wart-like lesions in patients with epidermodysplasia verruciformis and appears to be closely linked to skin carcinogenesis. A 1-2 kb region of the genome, which was previously shown to contain a viral E2-dependent enhancer, was progressively shortened from both ends with Bal 31. The resulting fragments were tested for their ability to stimulate chloramphenicol acetyltransferase (CAT) expression from the simian virus 40 (SV40) promoter. This analysis showed a complex interaction between cis-active, positive and negative control elements located throughout the non-coding region and the flanking reading frames. Two separate positively acting sequences significantly stimulated expression only in cooperation with a third region, which led to 12-fold, E2-dependent enhancement on its own. A major negative element was not only active in the context of HPV-8 sequences, but also down-regulated SV40 enhancer–promoter-driven CAT expression when cloned downstream of the transcription unit. It acted at the transcriptional level as shown by RNase protection assays and can therefore be regarded as a cis-acting silencer of transcription.

Human papillomaviruses (HPV) induce benign tumours of the skin and mucosa, some of which may progress to carcinomas. HPV DNA can also frequently be detected in healthy epithelium, which indicates that many HPV infections remain clinically inapparent (Pfister, 1988). The establishment of a latent persistent infection will depend on sophisticated regulatory mechanisms as exemplified by herpesviruses or human immunodeficiency virus.

Most cis-active control sequences of HPVs were mapped within non-coding regions, so-called long control regions (LCRs) between the late gene L1 encoding the major capsid protein and the early transforming gene E6 (Iftner, 1990). Nothing is known so far about the origins of HPV DNA replication, but both constitutive (Cripe et al., 1987; Swift et al., 1987; Gius et al., 1988; Hirochika et al., 1988; Chin et al., 1989; Steinberg et al., 1989) and inducible (Gloss et al., 1987; Hirochika et al., 1987, 1988; Phelps & Howley, 1987; Seeberger et al., 1987) enhancers have been identified. The major trans-activating protein is the viral E2 gene product which binds to the palindrome ACCN₆GGT which occurs several times within the LCRs of all HPVs (Iftner, 1990). When present in two or more copies, the E2-binding palindrome is sufficient to constitute an E2-dependent enhancer (Hawley-Nelson et al., 1988). A negative control of transcription was described for amino-terminally truncated E2 proteins, which compete with the activating full-length E2 for the common palindromic binding site (Giri & Yaniv, 1988). In genital HPVs, even the full-length E2 protein can function as a transcriptional repressor, presumably by sterically hindering initiation when binding to palindromes between the viral CAAT and TATA elements (Thierry & Yaniv, 1987; Chin et al., 1988). A cis-active negative regulatory element was identified in the LCR of an HPV-6 subtype (Wu & Mounts, 1988). HPV-8 induces flat warts and macular lesions in patients with the hereditary disease epidermodysplasia verruciformis (EV) and persists in skin cancers of these patients (Pfister et al., 1981; Orth, 1987). In the normal population there are no clinical manifestations of HPV-8 infection, although antibody screening suggests a prevalence of at least 10% (Pfister et al., 1981). HPV-8 (Fuchs et al., 1986) and related EV-associated HPVs (Krubke et al., 1987) are distinguished from all other HPVs by their short control regions (400 bp compared to 1000 bp). The HPV-8 LCR contains four ACCN₆GGT palindromes and constitutes an E2-dependent enhancer (Seeberger et al., 1987). No constitutive enhancer activity could be detected in C127 mouse fibroblasts or human HeLa cells.
In view of the carcinogenic activity of HPV-8 in the context of a specific genetic background of the host, it is important to understand the control of viral transcription in more detail. To dissect the HPV-8 enhancer region, two series of Bal 31 deletion mutants were generated. The plasmid pPF43 contains the HPV-8 EcoRI–EcoRV fragment spanning the entire non-coding region (NCR) and parts of the L1 and E6 open reading frames (ORFs) cloned into the corresponding cleavage sites of pIC19H (Marsh et al., 1984). We linearized pPF43 with EcoRI or EcoRV and then digested with Bal 31. The shortened HPV-8 fragments were cut free using BglII or BamHI, and ligated to the enhancer-dependent chloramphenicol acetyltransferase (CAT) vector pRZ2, which was cleaved within the polylinker upstream of the simian virus 40 (SV40) promoter (Fig. 1). The vector pRZ2 is a derivative of pSV2cat (Gorman et al., 1982) containing the SV40 early promoter, the bacterial CAT gene, the SV40 small t intron and the SV40 polyadenylation site cloned into pIC20H (Marsh et al., 1984). Recombinant plasmids with HPV-8 inserts of different length were selected and the exact map positions of the deletions (Fig. 2) were confirmed by sequencing by the method of Maxam & Gilbert (1980). The deletions from the EcoRI

Fig. 2. CAT expression under the control of HPV-8 DNA fragments. A partial physical map of the HPV-8 genome is presented on top, showing the position of the ORFs L1 (3' end) and E6 (5' end) and the non-coding sequences between. Arrows point to the E2-binding sites and grey boxes indicate 33 bp and 29 bp motifs conserved among EV-associated HPVs. The position and size of the fragments tested is given beneath. Nucleotides are numbered according to Fuchs et al. (1986). For transient expression assays, cells were plated 24 h prior to transfection (3 x 10⁶ cells per 10 cm dish) and incubated in Dulbecco's modified Eagle's medium (Gibco) supplemented with 5% foetal calf serum (Gibco), penicillin (120 µg/ml) and streptomycin (120 µg/ml). Calcium phosphate DNA precipitates were prepared according to the method of Graham & van der Eb (1973). A glycerol shock was performed 4 h after adding the precipitate using 15% glycerol in phosphate-buffered saline. Cells were harvested 48 h after transfection. Protein extracts were prepared and assayed for CAT activity according to the method of Gorman et al. (1982). Extracts were adjusted for different protein concentrations using a commercial protein assay (Bio-Rad). Chloramphenicol and its acetylated forms were separated by thin-layer chromatography, visualized by autoradiography and measured by liquid scintillation. The relative enzyme activity induced by the various constructs is given to the right.
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(a)  

AluI HpaI  

insert in pHR31  

(c) 1 2  

(b) 1 2 3 4  

:ki:k  

i  

Fig. 3. CAT expression from pHR31 and pRR52 (a) determined by enzyme assay (b) and RNase protection (c). Riboprobes were synthesized from 1 μg linearized plasmid DNA under standard conditions (Green et al., 1983) in the presence of 5 units RAgaurd (Pharmacia), 50 μCi [α-32P]UTP (400 Ci per mmol) and 10 units T7 or SP6 polymerase. The template was removed by digestion with 40 units of RNase-free DNase (Boehringer Mannheim). After phenol treatment and ethanol precipitation the pellet was dissolved in a hybridization buffer containing 80% formamide, 40 mM-PIPES pH 6.4, 400 mM-NaCl and 1 mM-EDTA (sp. act. 250000 c.p.m. per 10 μl). Before use in RNase protection assays (Zinn et al., 1983), an aliquot was run on a 7 M-urea, 6% polyacrylamide gel to ascertain that the probes contained at least 90% full-length transcripts. (b). pRR52, lanes 1 and 2; pHR31, lanes 3 and 4. Cellular RNA (10 μg) was dissolved in 10 μl of the adjusted riboprobe. After denaturing at 70 °C for 10 min, the probes were incubated in a water bath at 37 °C for at least 12 h. Then 300 μl of freshly prepared, ice-cold RNase mixture was added, containing RNase A at 6 μg per ml and RNase T1 at 12 units per ml in 300 mM-NaCl, 5 mM-EDTA, 10 mM-Tris-HCl pH 7.5. After a second incubation at 37 °C for 1 h, SDS and proteinase K were added to denature RNases, which were finally removed by the following phenol treatment. After precipitation, probes were run on a 6% polyacrylamide urea gel and the protected RNAs were visualized by autoradiography (c). The upper arrow points to bands corresponding to

and EcoRV sites have been cloned in the opposite orientation relative to the promoter. Only deletions with the same orientation will be directly compared in the present report. The entire EcoRI–EcoRV fragment was cut from the multiple cloning site of pPF43 and inserted in both sense and antisense orientations into pRZ2 to create pH5+ and pH5−.

To test for transient expression of the CAT gene, the DNAs were transfected into C82 cells, which are derivatives of C127 mouse fibroblasts expressing the HPV-8 E2 gene under control of a retroviral large terminal repeat (Iftner et al., 1988). The CAT activities shown in Fig. 2 are mean values of seven independently performed transfections normalized to the baseline activity of pRZ2. Care was taken that the chloramphenicol conversion was in the range of 1% to 60% to guarantee a linear correlation between chloramphenicol acetylation and CAT enzyme activity. The entire EcoRI–EcoRV HPV-8 DNA fragment present in pH5 enhanced CAT expression about 20-fold when transactivated by the viral E2 protein. The deletion of the E2-binding site at position 7327 (pHR15) had almost no effect, but the loss of two more palindromes (pHR11) led to a dramatic decrease in enhancer activity. The low activity of pHR11 was surprising in view of the presence of two E2-binding sites. The palindromes at positions 7649 and 22, present in pHR11, showed no enhancer activity at all when tested in the Sau3A fragment from position 7604 to position 108, which is in contrast to the findings of Hawley-Nelson et al. (1988), who demonstrated that two palindromes are sufficient to create an E2-dependent enhancer. This difference may be due to a low E2 affinity of the palindromes at positions 7649 and 22 or to surrounding repressor sequences. A deletion of nucleotides 243 to 555 (compare pH5 to pH26) and 5 to 163 (compare pH22 to pH23) led to a marked decrease of enhancer activity, which indicates that additional positive regulatory elements exist in these regions.

There are three pieces of evidence for an increase of CAT expression due to the deletion of HPV-8 sequences, which is consistent with the existence of cis-active negative control elements. These are defined by the slightly lower activity of pH24 in comparison to pH25 (70%) and the lower activities of pH13 compared to pH14 (60%) and pH26 compared to pH22 (42%), which point to a silencer around the 5' terminus of ORF E6. This is adjacent to the Sau3A fragment which is supposed to contain sequences responsible for the repression of its two E2-binding

CAT mRNA fragments initiated at the SV40 promoter and the lower arrow to the band corresponding to β-globin mRNA transcribed from the cotransfected SVOVEC vector. Lane 1, pRR52; lane 2, pHR31.
palindromes. To obtain further experimental evidence for silencer activity in this region of the HPV-8 genome, we cloned the Hpal–AluI fragment (positions 1 to 243) downstream of a SV40 promoter–enhancer-driven CAT gene (Fig. 3). Restriction endonuclease digests and Southern blot analysis confirmed that the fragment was inserted in the sense orientation. This plasmid was compared in transfection experiments with the parent vector, pRR52, which expresses the CAT gene under the control of the SV40 promoter–enhancer. Extracts from pHR31-transfected cells showed only 30% CAT activity in comparison to pRR52.

RNase protection experiments were performed to assess whether the reduction in CAT expression is due to reduced levels of correctly initiated CAT mRNAs. For this purpose, a fragment composed of the entire SV40 early promoter and the 5’ part of the cat gene was cloned into pGEM1. Using the T7 RNA polymerase promoter, a 570 bp antisense riboprobe could be transcribed in vitro after appropriate linearization of the plasmid.

The length of the protected RNA fragments proved the correct initiation of the CAT messengers at the SV40 early start sites and the different intensities of the signals obtained with RNA from pHR31- and pRR52-transfected cells, respectively, were in agreement with the data from the CAT assays. The cells were cotransfected in these experiments with SVOVEC (Westin et al., 1987) as an internal control of transfection efficiency. SVOVEC is an SV40 enhancer-driven expression vector for the rabbit β-globin gene and its transcript could be detected in the protection reactions together with CAT mRNA using an SP6 riboprobe transcribed from SP6βTTS (Westin et al., 1987). The analysis of β-globin expression revealed almost identical transfection efficiencies in all assays.

The data on CAT gene expression from pHR31 in comparison to pRR52 confirmed the existence of a transcriptional silencer within the Hpal–AluI fragment of HPV-8. A cis-active, negative control element in papillomaviruses has been so far described for only HPV-6 (Wu & Mounts, 1988). The 413 bp fragment with silencer activity differed from the homologous non-silencing DNA of a closely related subtype by a 3 bp deletion and the exchange of one nucleotide. The ACTGTT core defined by these mutations was not detectable in the HPV-8 silencer. This HPV-8 silencer did have, however, some similarity to other silencer elements (Brand et al., 1985, 1987; Laimins et al., 1986; Wu & Mounts, 1988; Burt et al., 1989; Cao et al., 1989; Pech et al., 1989) as summarized in Fig. 4. A highly similar AT-rich region was also observed in the silencer of the yeast silent mating type locus HMR (Brand et al., 1985, 1987) and was shown to represent an origin of yeast DNA replication (Brand et al., 1987). This could indicate an interdependence of transcription control and replication. The similarity with other silencers was found throughout the whole Hpal–AluI fragment of HPV-8, including the 29 bp motif which appears to be highly conserved among HPV-8-related, EV-associated papillomaviruses (Krubke et al., 1987).

In conclusion, there are both positive and negative transcriptional control elements in HPV-8 which appear to be connected and interact in a complex way. One enhancer resides within the L1-proximal non-coding sequences and extends into ORF L1. This genome segment contains three E2-binding palindromes (positions 7327, 7401 and 7491) and a sequence of 33 nucleotides (positions 7425 to 7457), which was shown to be highly conserved among HPV-8-related, EV-associated papillomaviruses (Krubke et al., 1987).

Fig. 4. Sequence similarity between the HPV-8 Hpal–AluI fragment (positions 1 to 243) and silencer elements of different genes (Grindlay et al., 1984; Brand et al., 1985, 1987; Lakshmikumaran et al., 1985; Laimins et al., 1986; Wu & Mounts, 1988; Burt et al., 1989; Cao et al., 1989; Pech et al., 1989). Upcase letters designate identical nucleotides. REV indicates reverse orientation relative to the direction of mRNA transcription. The line above the nucleotides marks the conserved 29 bp (see text). a stands for the upstream sequences of the human renin gene (Burt et al., 1989); β for rat insulin 1 gene upstream sequences (Lakshmikumaran et al., 1985; Laimins et al., 1986); t for the PDGF-2 promoter silencer element (Pech et al., 1989); e for the human e-globin upstream sequences (Grindlay et al., 1984; Cao et al., 1989) and μ for the yeast HMR silencer (Brand et al., 1985, 1987).
into ORFs L1 and E6 and are not confined to the NCR, which should be considered in the context of its small size relative to HPV5s not associated with EV.

The arrangement of enhancers and silencers in HPV-8 will allow a fine adjustment of gene expression, which may be relevant to its usually inapparent persistence in the normal population in contrast to its pathogenicity in EV patients. To achieve a better understanding of the role of these elements it will be essential to evaluate their effects on authentic HPV-8 transcription and their activity in different host cells.

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References


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