Early promoters of genital and cutaneous human papillomaviruses are differentially regulated by the bovine papillomavirus type 1 E2 gene product

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The physical state of the human papillomavirus (HPV) genome is usually different in malignant lesions of the skin, in which it is generally found in episomal form, and genital mucosa, in which it is frequently integrated with disruption of the E2 gene. Using chimeric or natural HPV promoters in the presence of the bovine papillomavirus type 1 E2 gene product, we observed transcription activation or repression, depending on the distance of E2-binding motifs from the start site. We found a clear difference in the positions of E2-binding motifs in cutaneous and genital HPVs that may partly explain the selective pressure for genome integration of genital HPV types in malignant lesions.

Introduction

Human papillomaviruses (HPVs) are aetiological agents of benign and malignant epithelial lesions (Pfister, 1987; zur Hausen & Schneider, 1987). Sixty different types of HPV have been described and are traditionally grouped into those predominantly found in cutaneous and those in mucosal lesions (de Villiers, 1989).

Some of the HPVs commonly found in lesions of mucosal origin, like HPV-6 and -11, are usually present in benign lesions of the genitals, i.e. condylomas and intraepithelial neoplasias, mainly in an episomal state (Dürst et al., 1985; Pfister, 1987; zur Hausen & Schneider, 1987). By contrast, HPV-16, -18, -31, -33 and -35 are frequently found associated with malignant neoplasias. In a high percentage of cervical carcinomas and cell lines derived from them, HPV-16, -18 and -33 DNA has been found integrated into the cellular genome (Baker et al., 1987; Dürst et al., 1985; Matsukura et al., 1986; Schwarz et al., 1985; Yee et al., 1985). When it has been possible to analyse the integration pattern of the viral DNA, the E1–E2 gene region is usually found to be disrupted (Baker et al., 1987; Matsukura et al., 1986; Schwarz et al., 1985). However, in premalignant dysplastic lesions the same HPV types are usually found in an extrachromosomal state (Crum et al., 1985; Dürst et al., 1985). The majority of HPVs associated with skin neoplasias have been isolated from lesions of patients with epidermodysplasia verruciformis (EV). Some of them, like HPV-5 and -8, have been associated with squamous cell carcinomas, in which viral DNA has been found mainly in an extrachromosomal state (Orth, 1987).

The molecular organization of the genomes of different HPV types is very similar; they have a number of early genes (E1 to E7) and two late genes (L1 and L2). Genes E6 and E7 have been found to be necessary and sufficient for immortalization of primary human keratinocytes (Münger et al., 1989). In addition, the long control region (LCR) harbours the early promoter and the replication origin. This region is the target for the interaction of viral and cellular factors which presumably control most transcriptional events in the HPV genome (Chin et al., 1988; García-Carrancá et al., 1988; Hirochika et al., 1988).

The E2 gene is highly conserved in all sequenced papillomaviruses. The E2 protein is a sequence-specific DNA-binding protein that recognizes and binds to a 12 bp sequence, ACCGNNCGGT, the E2-binding site (E2BS), which is repeated several times in the LCR of all papillomaviruses (Androphy et al., 1987; Dartman et al., 1986).

The E2 protein is functionally conserved, especially the C-terminal third which is involved in dimerization
and DNA binding (Dostatni et al., 1988; McBride et al., 1989). The N-terminal third, harbouring two acidic amphipathic α-helices, which seem to be necessary for transcriptional activation (McBride et al., 1989), and the internal hinge domain, are much less conserved in sequence and in length.

Originally described as a very potent trans-activator (Spalholz et al., 1985), full-length E2 protein from bovine papillomavirus type 1 (BPV-1) was later shown to repress the genuine HPV-18 early promoter (Thierry et al., 1987; Thierry & Yaniv, 1987). The BPV-1 E2 protein has also been found to repress the HPV-11 and -16 early promoters (Chin et al., 1988; Romanczuck et al., 1990), and the full-length E2 proteins from HPV-16 and -18 act similarly (Bernard et al., 1989; Romanczuck et al., 1990; Thierry & Howley, 1991). However, there is published experimental evidence suggesting that repression/trans-activation by the full-length E2 protein may be a rather complex phenomenon. For instance, an increase in HPV-16 P97 transcription has been found in the presence of the BPV-1 E2 protein (Cripe et al., 1987; Gloss & Bernard, 1990). Whereas the HPV-11 E2 protein has been found to stimulate the HPV-11 enhancer–E6 promoter, repression of the same construct by the BPV-1 E2 protein has been observed (Chin et al., 1988).

In the case of HPV-18, it has been shown that full-length BPV-1 E2 protein binds to a perfect double palindrome that is located just upstream from the E6/E7 TATA box (Garcia-Carrancá et al., 1988). This palindrome is perfectly conserved in all the genital HPVs sequenced (García-Carrancá et al., 1988). In contrast, cutaneous HPVs contain an E2BS further upstream from the TATA box (Ensser & Pfister, 1990).

In this study we analysed the effect of the full-length BPV-1 E2 trans-regulator protein on the transcription of chimeric promoter elements containing E2BS at different positions relative to the start site. In addition, we compared cutaneous and genital promoter trans-regulation by the BPV-1 E2 protein.

**Methods**

**DNA constructs.** The basic construct used in this study (pGCo) contains a modified simian virus 40 (SV40) early promoter linked to the chloramphenicol acetyltransferase (CAT) reporter gene. This modified promoter contains five base changes that create two new restriction sites: a BamHI site between the 72 bp repeats and the 21 bp repeats, and a SalI site between these repeats and the TATA box (Takahashi et al., 1986). The fragment containing this SV40 early promoter was obtained from plasmid p80 (Takahashi et al., 1986; kindly provided by Dr H. Barret-Saldana) by EcoRI restriction, the ends were filled using the Klenow fragment of Escherichia coli DNA polymerase I, and the promoter was isolated by HindIII restriction and electrophoresis on an agarose gel. This promoter fragment was ligated to a CAT construct without a promoter (pS2), a derivative of pSB1 (Herbomel et al., 1984) (kindly provided by Dr M. Yaniv), from which we removed a unique BamHI site by cutting with the enzyme and filling using the Klenow fragment. pS2 was digested with SalI and the ends were filled using the Klenow fragment, after which it was HindIII-digested and ligated to the SV40 promoter fragment.

To introduce E2BS in a position distal to the SV40 early promoter, we cloned an oligonucleotide containing a single E2BS by digesting pGCo with KpnI, treating with T4 DNA polymerase and ligating it to the blunt-ended double-stranded oligonucleotide 5' GATCGTACCG- AAAAAAGGTTCGCA 3' producing pE2 Kpn (Fig. 1b). To generate chimeric constructs containing E2BS located proximal to the start site, we substituted the 21 bp motifs from the SV40 promoter with different numbers of oligonucleotides containing the E2BS. pGCo was digested with SalI and BamHI, and ligated to small DNA fragments from a group of pGEM-4Z recombinant plasmids containing E2BS flanked by SalI and BamHI sites. Two different double-stranded oligonucleotides were used: 5' CTATGAGAAGCTTTCTGAGGGTGCTGCTATTGTTGTCATTTGAGGTCAGATT 3' and 5' CTATGAGAAGCTTTCTGAGGGTGCTGCTATTGTTGTCATTTGAGGTCAGATT 3'. Positive clones were selected and partially sequenced by chemical modification of the DNA (Maxam & Gilbert, 1980) to confirm the orientation and number of oligonucleotides inserted. Thus, plasmids pSEK143, pSEK254 and pSEK453, containing one, two and four copies of the first oligonucleotide, and pSEA21, containing two copies of the second, were obtained (Fig. 1b, Table 1).

**Cell lines and DNA transfection.** HeLa and SW13 cells were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% foetal bovine serum in a 5% CO2 atmosphere. For calcium phosphate-mediated DNA transfection experiments, SW13 cells were grown in 60 mm tissue culture dishes to a density of 1 x 106 cells/dish. Each transfection was performed at least twice with a total of 10 μg of DNA per dish. Usually, in cotransfection experiments 2.5 μg of CAT reporter construct was mixed with 2.5 μg of a Rous sarcoma virus–β-galactosidase plasmid (kindly provided by Dr M. Thierry) and, when indicated, 2.5 μg of a full-length BPV-1 E2 protein-expressing vector (pC59; kindly provided by Dr P. M. Howley) in the presence of carrier DNA (pBR322 or pGEM-4Z). Cells were incubated with the precipitate for 18 h, washed and refed with fresh medium for another 22 h. Electroporation was performed using 12 x 106 HeLa cells grown under the conditions described. After mixing the DNA in HeBs (137 mM-NaCl, 5 mM-KCl, 0.7 mM-Na2HPO4, 6 mM-dextrose, 20 mM-HEPES), it was incubated for 10 min on ice with cells and then subjected to a pulse of 260 V and 960 μF using a Bio-Rad apparatus. After another 10 min incubation on ice, cells were placed in a dish containing medium and incubated for 16 h before changing of the medium.

CAT and β-galactosidase assays. Extracts were prepared and assayed for CAT and β-galactosidase activity essentially as described previously (Herbomel et al., 1984; Thierry et al., 1987).

**Results and Discussion**

Fig. 1 shows a representative CAT assay with different constructs tested in the absence or presence of the full-length E2 gene product from BPV-1. As can be seen, in some instances E2 protein reduces (panels ii and iii), whereas in others it enhances (panel i) the expression of the CAT gene (see also Table 1). Control experiments with the natural HPV-18 enhancer/promoter driving the CAT gene (p18/42) or the HPV-16 enhancer/promoter fused upstream to the SV40 early promoter (p863.1) gave
the expected results; the HPV-18 construct was repressed, whereas the HPV-16 construct (having 21 bp boxes separating E2BS and the start site by 155 bp) was activated, as reported previously (Thierry et al., 1987; Thierry & Yaniv, 1987; Phelps & Howley, 1987).

We compared the positions of the E2S relative to the start site in these and other constructs (Table 1) and found that constructs in which the E2BS is proximal to the start site were repressed, whereas those with a more distant E2BS were activated. In agreement with published data, the number of E2BSs was not found to be important for repression by the BPV-1 E2 protein; a single E2BS was sufficient for repression, as described recently for the repression of the HPV-18 P105 promoter by the BPV-1 E2 protein (Thierry & Howley, 1991). In all the constructs tested that were repressed, the distance between the most proximal E2BS and the cap site was less than 70 bp. In contrast, there were more than 150 bp between the cap site and the most proximal E2BS in the two constructs that were stimulated.

To explore the idea that the distinct response to the E2 trans-regulator protein (depending on whether the binding sites are close to or distant from start sites) could have a biological significance for the life cycle of HPV, we analysed the natural position of E2BS in HPVs from cutaneous and mucosal lesions (Fig. 2). We found that the positions differ between these two groups; HPVs usually found in lesions of the genital mucosa have two E2BSs very close to the E6/E7 start site (Fig. 2b), whereas those usually found in lesions of the skin either have their most proximal E2BS more than 100 bp from the E6/E7 start site or possess an incomplete single E2BS palindrome near the E6/E7 TATA box, as observed in HPV-9 and HPV-17 (see Fig. 2a). To confirm that genital and cutaneous HPV early promoters are differentially regulated by the E2 protein, we cotransfected cells with a construct containing the HPV-20 LCR (kindly provided by Dr P. Fuchs) linked to the CAT gene and pC59 or carrier DNA. Fig. 3 shows that, as expected, the cutaneous HPV-20 construct is activated whereas the HPV-18 construct is repressed.

It is well established that HPV DNA present in malignant lesions from the genitals or in cell lines derived from them is often integrated into the cellular genome (Dürst et al., 1985; Schwarz et al., 1985; Pfister, 1987; zur Hausen & Schneider, 1987). In all cases in which virus integration has been studied, viral molecules are interrupted in the E1 or E2 open reading frames (Baker et al., 1987; Lehn et al., 1985; Pfister, 1985; Matsukura et al., 1986; Schneider-Maunoury et al., 1987; Schwarz et al., 1985). In contrast, in malignant lesions from the skin (like those found in patients with EV) viral DNA is found in an episomal state (Orth, 1987). These facts and the finding that in all sequenced genital papillomaviruses there are two proximal E2BSs located just upstream from the E6/E7 TATA box could mean that in genital carcinomas continuous expression of the E6 and E7 genes is incompatible with the presence of E2 protein; in cutaneous lesions, continuous expression of the E6 and E7 genes seems to be compatible with the presence of the E2 protein. This fact will impose great selection pressure for genital mucosa cells in which viral DNA integration
Fig. 2. Localization of E2BS in the control region of HPVs. DNA sequences upstream from the E6 start site were used to localize perfect E2BS (ACCGN4CGGT; □), or related (○ or △) palindromes containing only a perfect half site. References: HPV-1a (Danos et al., 1982), HPV-5 (Zachow et al., 1987), HPV-6b (Schwarz et al., 1983), HPV-8 (Fuchs et al., 1986), HPV-9, HPV-17, HPV-20 and HPV-36 (Ensser & Pfister, 1990), HPV-11 (Dartmann et al., 1986), HPV-16 (Seedorf et al., 1985), HPV-18 (Cole & Danos, 1987), HPV-8, HPV-19 and HPV-25 (Krubke et al., 1987), HPV-33 (Cole & Streeck, 1986), HPV-39 (Volpers & Streeck, 1991), HPV-47 (Kiyono et al., 1990) and HPV-58 (Kirri et al., 1992). (a) Cutaneous HPVs; (b) genital HPVs. The broken vertical lines are 100 bp upstream from known or putative E6 gene start sites.

Fig. 3. Differential response of genital HPV-18 (p18/42, lanes 3 and 4) and cutaneous HPV-20 (lanes 1 and 2) (a) promoter regions to the BPV-1 E2 gene product. Lanes 1 and 3, no E2 gene product; lanes 2 and 4, E2 gene product. HeLa cells were electroporated and a CAT assay was performed as described in Methods. The putative start site of transcription in HPV-20 is indicated by the arrow. (b) A schematic representation of the constructs.

has resulted in the interruption of the E2 gene leading to continuous expression of the immortalizing genes (Schneider-Maunoury et al., 1987).

E2 protein repression of promoter activity has been demonstrated in HeLa cells, in an adrenocarcinoma cell line (SW13) and in primary human keratinocytes (Thierry & Yaniv, 1987; Bernard et al., 1989; Romanczuck et al., 1990). The results described here have been obtained by using the heterologous BPV-1 E2 gene product. Although different results have been reported for HPV and BPV E2 proteins (Chin et al., 1988), there is experimental evidence suggesting that full-length HPV-16 and HPV-18 E2 proteins perform similarly to that of BPV-1 (Bernard et al., 1989; Romanczuck et al., 1990; Thierry & Howley, 1991).
Although we have not explored the molecular mechanism by which a full-length BPV-1 E2 protein represses CAT expression, the possibility that the E2 protein imposes a particular bend to the DNA that impedes it from looping out to form an active transcription complex has to be considered. Indeed, evidence exists for very strong bending of DNA upon binding of the E2 protein (Bedrosian & Bastia, 1990). The possibility that the binding of E2 protein to a site near the TATA box could physically hinder the binding of transcription factor IID (Bedrosian & Bastia, 1990). The possibility that the interference were the sole explanation for repression, the construct in which the distance between the proximal E2BS and the TATA box is 37 nucleotides might be expected to exhibit some decrease in repression compared to those constructs in which E2BS are closer. We are currently exploring these possibilities.

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