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Differences in transcriptional activity of cutaneous human papillomaviruses

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ABSTRACT

The interaction between UV-B irradiation and cutaneous human papillomaviruses (HPV) has been suggested to be of relevance for the development of non-melanoma skin cancers. We investigated the activity within the upstream regulatory region (URR) of the HPV types 8, 38, 92, 93 and 96, as well as their responsiveness to UV-B irradiation and cellular differentiation. Promoter activities were higher in HaCaT than in SiHa cells, corresponding to the HPV tissue tropism. Transcriptional start sites were mapped at P_{92} (HPV-38), P_{45} (HPV-92), P_{7439} (HPV-93) and P_{256} (HPV-96). Transcription from HPV-8, 93 and 96 URR was up-regulated by cellular differentiation, linking the activity of these HPVs to the cellular state. UV-B irradiation activated HPV-8 but inhibited HPV-38 and HPV-93 whereas HPV-92 and 96 were not affected. As there are variable UV-B responses among the HPV types, further studies of interactions between UV-B and HPV need to consider the HPV type.

1. Introduction

Human papillomaviruses are small double-stranded DNA viruses that infect epithelial cells of the skin or mucosa and cause various degrees of epithelial proliferation (zur Hausen, 1996). To date, there are over 100 identified HPV types (de Villiers et al., 2004) of which approximately 15 are known as mucosal high-risk types and are important carcinogens in the development of anogenital and oral cancers (Woodman et al., 2007). Among the high-risk types, HPV-16 is the type most commonly detected in cervical cancer (Munoz et al., 2003). A role for cutaneous HPV types in development of non-melanoma skin cancer (NMSC) has been suggested (Karagas et al., 2006). NMSC, comprising the two main histological diagnoses: basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), is the most prevalent cancer in the Caucasian population (Kiviat, 1999) and UV-radiation is known to be the main risk factor. Other risk factors are fair skin, immune status of the host and certain inherited conditions such as Epidermodysplasia Verruciformis (EV) (Alam and Ratner, 2001). It was among the EV patients that an association between HPV infection and development of NMSC was first described (Majewski and Jablonska, 1995; Orth et al., 1978). The EV patients have numerous lesions and commonly develop SCC at sun-exposed sites. HPV types belonging to the genus *Betapapillomavirus* can be detected in nearly all of these SCC lesions, with the most prominent types being HPV-5 and 8 (Pfister, 2003; Pfister and Ter Schegget, 1997). *Betapapillomaviruses* are also common on healthy skin of immunocompetent patients (Antonsson et al., 2000). As cutaneous HPV infection is more prevalent at sun-exposed sites, it has been suggested to be a co-factor to UV-irradiation in development of NMSC (Forslund et al., 2007; Jackson et al., 2000).

The life cycle of HPV is tightly linked to the differentiation program of the keratinocytes (zur Hausen, 2002). In part, this is due to the interplay of differentiation specific cellular and viral transcriptional factors affecting the regulatory sequences located mainly in upstream regulatory region (URR) (Sailaja et al., 1999). The URR is a non-coding regulatory region located between the L1 and E6 ORFs and it contains both viral enhancer and promoter sequences with binding sites for numerous transcription factors. Location, frequency and characteristics of these binding sites are to a large extent conserved within each HPV genus but differ between the genera (Garcia-Vallve et al., 2006). Most HPV types have a promoter in the 3′-end of the URR, and a transcription start site immediately upstream of the E6 ORF. In HPV-8 this transcription start site is at P_{175} (Stubenrauch et al., 1992) whereas in HPV-16 it is at P_{97} (Smotkin and Wettstein, 1986). For the expression of the late genes, the position of the promoters differs between the mucosal and cutaneous types. In HPV-8, a strong late promoter, P_{7535}, initiates a late transcript (Stubenrauch et al., 1992). HPV-16 has several late promoters both within and outside the URR (Geisen and Kahn, 1996; Rohlfs et al., 1991). The knowledge of promoters and transcriptional regulation of mucosal HPV types is substantially more

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explored than that of cutaneous HPVs. We show in this study the location and the activity of the putative promoters from HPV types within different species of *Betapapillomavirus*: HPV 38 (species 2) (Scheurlen et al., 1986), HPV 92 (species 4) (Forslund et al., 2003), HPV-93 (species 1) and HPV-96 (species 5) (Vasiljevic et al., 2007). Furthermore, we investigated the responses of the viral promoters to UV-B irradiation and cellular differentiation.

2. Materials and methods

2.1. Plasmid constructs

The URRs of HPV-8, 38, 92, 93 and 96 were PCR amplified with AmpliTaq Gold™ polymerase (Applied Biosystems, Foster City, CA) and specific primers containing restriction enzyme (RE) sites for KpnI or NheI (Table 1). The primers were constructed so that the PCR product included the first base after the L1 stop codon and the last base before the E6 start codon. Cleaved PCR products were ligated into unmodified pGL3-enhancer (Promega, Mannheim Germany) vector and into unmodified pGL3-Basic vector (Promega) so that the first start codon after the URR sequence was the ATG of the luciferase gene. A 103 bp HPV-16 fragment (nt 2428–2530) was cloned upstream of the URR (Braunstein et al., 1999) in order to eliminate the possibility of translation of the luciferase gene from the vector. The correct insertion of the URR in all constructs was verified by DNA sequencing.

2.2. Cell culture and transfection

HaCaT and SiHa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 200 mM l-glutamine and 1% Penicillin–Streptomycin (Sigma–Aldrich, Steinheim, Germany). For luciferase assays, 2×10^5 cells were plated in each well of six-well plates and transfected 24 h later with 1.67 μg vector-DNA using Lipofectamine plus (Invitrogen, Carlsbad, CA) according to the manufacturers manual. Forty eight hours post-transfection luciferase activity was measured as previously described (Braunstein et al., 1999).

2.3. Luciferase assay

In brief, cells were washed and lysed, whereupon 20 μl of lysate was mixed with 50 μl substrate (Promega) and the activity immediately measured with a TD-20/20 luminometer (Turner Designs). Each construct was tested in triplicate in at least 2–4 independent experiments, representing a total of at least 6 transfections. In every experiment an empty vector was used as a negative control and the vector 97LCR, containing the early promoter of HPV 16, was used as a positive control (Braunstein et al., 1999). Internal control of a dual luciferase reported assay was not used because the CMV promoter in the Renilla construct has in our hands demonstrated interference with promoters of HPV and also decreased activity in response to UV-treatment (data not shown).

Table 1
Sequences and coordinates of primers used for amplification of URRs of HPV-8, 38, 92, 93 and 96 types

Name	URR sequence	Primer sequence (5′ → 3′)
Forward (sense) primers		
HPV8URR	7381–7410	AAACGGAAAA*GGTACC*TTGTACCGTTTTCG[a]
HPV38URR	7181–7210	AAGGACCCA*GGTACC*GTTTTCGGTCGCCCA
HPV92URR	7161–7190	AAGAAAAA*GGTACC*AAAACGGTCAGTATTC
HPV93URR	7161–7190	AAAAGAAAAA*GGTACC*AATAATTTTACCAC
HPV96URR	7381–7410	GAAAACGACG*GGTACC*GCTATCGGTTCGGG
Reverse (antisense) primers		
HPV8URR	181–210	GTCCTGCCCC*GCTAGC*TTGCTTAGGAAAATT
HPV38URR	191–220	TTGAGGTTTTGGTAG*GCTAGC*GAATACATC
HPV92URR	91–120	AGGAGGTTTT*GCTAGC*TGTGATGGGAACACT
HPV93URR	116–142	CTTACTGC*GCTAGC*TAAGATGTCTGCC
HPV96URR	339–368	TCTTTAAA*GCTAGC*TCAACAATGAACAGAG

[a] The restriction enzyme sites are underlined in each primer.

2.4. UV-light irradiation

HaCaT cells were irradiated with UV-B light using a UV-B-Bulb (TL 20W/12 RS SLV, Philips). In order to calculate time exposure equivalent to 15 mJ/cm^2 of irradiation, the bulb was placed 11.5 cm above a measurement instrument, Solatell Sola-Hazard (4D Controls LTD, United Kingdom). Twenty-four hours post-transfection cells were washed with PBS whereupon 300 μl of PBS was added to each well and the plates, with the lids off, were placed on ice and irradiated for 28 s. Luciferase activity was measured at 4 and 24 h after UV-irradiation. The experiment was set up in duplicate, where one set of cells was irradiated and the other was not in order to be able to calculate the relative effect by comparing the two sets of cells. At all times pGL3-Basic vector was used as the reference and its activity was set to 1.

2.5. Differentiation study

After the transfection of HaCaT cells, the cells were harvested for luciferase assay or cytoplasmic RNA isolation at 13, 45 and 117 (5 days) hours. The media of the cells were changed to high Ca^{2+}-containing DMEM (5.8 mM) after 13 h, in order to induce cellular differentiation (Gniadecki et al., 2001). To assess the level of differentiation by transfection itself, a triplicate of cells were transfected with empty pGL3-Basic vector and RNA isolation was performed using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manual. Contaminating DNA was removed with both the RNase-Free DNase Set (Qiagen) and afterwards DNase treatment with Deoxyribonuclease I, Amplification Grade (Invitrogen). The QuantiTech SYBR Green RT-PCR kit (Qiagen) allowed both reverse transcription and PCR in a single reaction. The reaction mix to detect the differentiation markers consisted of 12.5 pmol forward primer (K10 5′-AGCATGGCAACTCACATCAG-3′ or involucrin 5′-TGCCTGAGCAAGAATTGAG-3′), 12.5 pmol reverse primer (K10 5′-TGTCGATCTGAAGCAGGATG-3′ or involucrin 5′-TGCTCTGGGTTTTCTGCTTT-3′), 12.5 μl 2 × QuantiTect SYBR Green RT-PCR Master Mix, 0.25 μl Quantitect RT Mix, 500 ng of RNA and MilliQ water in a final volume of 25 μl. The PCRs were run on the Engine Opticon Continuous Fluorescence Detector (MJ Research) with the following parameters: 30 min at 50 °C, 15 min at 95 °C and then 40 cycles of 95 °C for 15 s, 57 °C for 30 s and 72 °C for 1 min. The C_t-values were converted to linear values by the formula $2C_t$ and shown as mRNA equivalents.

The protein level of involucrin was analysed at 13, 45 and 117 (5 days) hours in HaCaT cells, transfected with the HPV93 URR construct, by western blot with anti-involucrin antibody (1:100; Sigma–Aldrich) as previously described (Vasiljevic et al., 2007). GAPDH was used as the loading control and visualised with anti-GAPDH antibody (1:2000; Abcam. Cambridge, UK). Secondary antibody was rabbit-anti-mouse biotinylated antibody (1:1000; DakoCytomation A/S, Glostrup, Denmark) and streptavidin horse-raddish peroxidase conjugate (Amersham Biosciences, Buckinhamshire, England). The protein bands were visualised using the enhanced chemiluminescence system (Amersham Biosciences).