Cell-type-dependent activities of regulatory regions and E2 proteins derived from carcinogenic and non-carcinogenic human alphapapillomaviruses

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INTRODUCTION

A large number of studies have revealed that persistent infections with certain human papillomavirus (HPV) types are necessary for the development of invasive cancer of the cervix. Recent studies have shown that not only do the major carcinogenic HPV types 16 and 18 encode E6 and E7 oncoproteins with immortalizing activity but also the very weakly or non-carcinogenic types 53, 66, 70 and 82. Currently, it is unknown whether transcriptional differences exist between these viruses that account for carcinogenicity in vivo. Therefore, we compared for the first time the activities of the upstream regulatory regions (URRs) that drive E6 and E7 expression derived from HPV16, -18, -31, -53, -66, -70 and -82 in the absence and presence of the viral E2 transcriptional regulator. URR activities in the absence of E2 varied widely and were further modulated by the cellular background. The co-expression of homologous E2 proteins resulted in repression of the URRs of only some HPV types and this varied with cell type. Activation by E2 proteins was less cell-type dependent but differed in an HPV-type-dependent manner. However, basal URR activity, repression of the URR by E2 and transcriptional activation by E2 did not correlate with HPV carcinogenicity in vivo. In summary, our data do not support the model that the transcriptional activity of human alphapapillomavirus types correlates with epidemiological risk classification.
immortalizing activity in vitro. The oncogenic activities of the HPV16/18 E6 and E7 proteins are due to binding of the viral oncoproteins to key regulatory host-cell proteins such as p53 and members of the retinoblastoma family that control cell-cycle progression and apoptosis (Howie et al., 2009; McLaughlin-Drubin & Münger, 2009; Moody & Laimins, 2010).

However, recent comparative analyses of carcinogenic, non-carcinogenic and weakly carcinogenic HPV types from the a5, a6, a7 and a9 genera revealed surprisingly that HPV53, -66, -70 and -82 are also able to immortalize human keratinocytes in vitro, as well as HPV16 and -18 (Hiller et al., 2006; Muench et al., 2009). Biochemical analyses have confirmed that the E6 proteins of HPV53, -66, -70 and -82 degrade p53, as well as HPV16 and -18 E6 (Burk et al., 2009; Hiller et al., 2006; Mespède et al., 2012). Furthermore, the binding of E6 proteins to cellular PDZ-domain proteins such as hDlg, MAGI1 and scribble, which has also been suggested to be a distinctive feature of carcinogenic HPVs, did not disclose significant differences between highly carcinogenic HPV16 and -18 and non-carcinogenic HPV70 and -82 (Muench et al., 2009).

It has been postulated that the malignant progression of cervical lesions induced by HPV16 and -18 is favoured by enhanced expression of the E6/E7 oncogenes (Zur Hausen, 2002). Along these lines, it has been demonstrated that the HPV upstream regulatory region (URR) that controls expression of E6 and E7 contributes to the differential immortalization capacities of HPV16 and -18 (Romanczuk et al., 1991). Thus, the differences between carcinogenic and weakly/non-carcinogenic HPVs that encode immortalizing E6/E7 proteins could result from differences in the transcriptional control of the E6/E7 promoter. The viral URR consists of several binding sites for cellular transcription factors, which can have a positive or negative influence on E6/E7 transcription. In addition, viral proteins derived from the E2 gene bind to conserved motifs (5'-ACCNC3GGT-3') in the URR and activate or repress the E6/E7 promoter (McBride et al., 1991). The full-length form of E2 can be both an activator and repressor, whereas the E8E2C protein, which is generated by alternative splicing from the E2 gene, acts only as a repressor of E6/E7 transcription (Fertey et al., 2011). Both proteins bind via their C terminus to E2-binding sites and recruit cellular proteins to modulate transcription (Ammermann et al., 2008; Fertey et al., 2010; McPhillips et al., 2006; Powell et al., 2010; Schweiger et al., 2006; Smith et al., 2010; Wu et al., 2006). The E2 gene is highly conserved among papillomaviruses, but E8E2C transcripts have so far only been described for alphapapillomaviruses HPV16, -18, -31 and -33 and several non-alphapapillomaviruses. It is currently not established whether other a5, a6, a7 and a9 HPV types express E8E2C proteins. Interestingly, the binding of E2 proteins to the cellular Brd4 protein, which has been implicated in mediating both activation and repression by E2, varies among papillomaviruses (McPhillips et al., 2006; Schweiger et al., 2006; Smith et al., 2010; Wu et al., 2006). A large number of studies have investigated the influence of E2 on its corresponding E6/E7 promoter URR using different HPV types and different cell types, making it difficult to decide whether differences in HPVs exist that may contribute to carcinogenicity in vivo. A recent study compared the URR activities of HPV11, -16 and -18 in the presence and absence of the respective E2 proteins in HPV-positive and -negative immortalized human keratinocytes and other cell lines and revealed an unexpected complexity of transcriptional regulation among HPVs (Ottinger et al., 2009). Consistent with other observations, differences between HPV16 and -18 were observed (Ottinger et al., 2009; Romanczuk et al., 1991).

Therefore, we wanted to investigate whether transcriptional differences exist between the highly carcinogenic HPV16 and -18, intermediarily carcinogenic HPV31 and weakly/non-carcinogenic HPV53, -66, -70 and -82. We compared the activities of the respective HPV URR–reporter constructs alone and in the presence of E2 proteins in NHKs, the cervical cancer cell line HeLa (which is HPV18 positive) and the HPV-negative RTS3b keratinocyte cell line. This revealed unexpected cell- and HPV-type-dependent differences in transcriptional activities but no correlation between transcriptional activities and epidemiological risk classification.

**RESULTS**

**HPV16, -18, -31, -53, -66, -70 and -82 URRs display virus- and cell-type-dependent promoter activities**

For comparative analysis of the activities of the different URRs, all URRs were cloned into the promoterless luciferase plasmid pGL3 in such a way that the luciferase start codon corresponded to the E6 start codon and all URRs started with the stop codon of the L1 gene. Exceptions were HPV16 in which the second ATG of the E6 gene (nt 104) was used to include the major transcription start site at nt 97 (Smotkin & Wettstein, 1986) and HPV66 in which a naturally occurring restriction site in nt 6954 was used. NHKs, HPV18-positive HeLa cervical cancer cells and HPV-negative RTS3b cells were transfected with the respective URR plasmids and luciferase activities were determined at 48 h post-transfection (Fig. 1). In NHK cells, the activities of the HPV31, -53 and -66 URRs were three- to fivefold higher than that of HPV16. In contrast, the activities of HPV18 and -70 were threefold lower than HPV16. The HPV82 URR displayed the highest activity and was 20-fold more active than that of HPV16. In RTS3b cells, the HPV82 URR also displayed the highest activity and was 30-fold more active than that of HPV16. The HPV31 and -53 URRs were four- and tenfold more active than that of HPV16, respectively, which was similar to the results in NHKs. In
contrast to NHKs, the HPV18 and -70 URRs were approximately threefold more active and the HPV66 URR was threefold less active than the HPV16 URR. In HeLa cells, the highest URR activity was observed with HPV18, followed by HPV82, which was fivefold more active than that of HPV16. Similar to in NHK and RTS3b cells, the HPV31 and -53 URRs were more active than that of HPV16. Similar to in RTS3b cells and NHK cells, the HPV66 and -70 URRs were, respectively, threefold less active than that of HPV16. In summary, these experiments revealed unexpected type-dependent and cell-type-specific activities of the different HPV URRs.

**Activation by E2 proteins is not cell-type dependent**

We next investigated the influence of the different E2 proteins on viral transcription. All E2 genes were cloned into the pSG5 expression vector. To identify the optimal amount of E2 expression vector, we performed titration experiments with a synthetic promoter construct (pC18-Sp1-luc), which is strongly activated by HPV31 E2 (Stubenrauch et al., 2001) and expression vectors for HPV18 and -70 E2 in RTS3b and HeLa cells (Fig. S1, available in JGV Online). In addition, a reference plasmid encoding Gaussia luciferase (pCMV-Gluc) was included to control for transfection efficiency. This revealed that activation started to plateau at 3 ng of the two E2 expression vectors in both RTS3b and HeLa cells. As activation slightly decreased with HPV18 E2 in HeLa cells at 30 ng expression vector, 10 ng expression vector was chosen for subsequent experiments. All E2 proteins were able to stimulate transcription from the reporter plasmid but to different extents (Fig. 2). Generally, it was observed that the activities of the different E2 proteins relative to each other were similar in the different cells (Fig. 2). The greatest stimulation of the reporter was achieved by the HPV70 E2 protein and the lowest activation by HPV53 E2 (Fig. 2). The HPV82 E2 protein also only weakly activated the reporter but to slightly higher levels than HPV53 E2 in RTS3b and HeLa cells (Fig. 2). HPV16, -18, -31 and -66 E2 had similar activities in all three cell types, with the exception of HPV18 E2, which had a slightly reduced activity in RTS3b cells. To test whether the reduced activities of HPV53 and -82 E2 were due to different protein expression levels, haemagglutinin (HA) epitope tags were added to the different E2 proteins. The activities of the tagged E2 proteins were compared with untagged version in co-transfections with the pC18-Sp1-luc plasmid. This revealed that the addition of the epitope tag did not change the activities of HPV18, -31, -53 and -66 and only slightly impacted the activities of HPV16 (2.4-fold), -70 and -82 (twofold) E2. Immunoblot analyses of transfected RTS3b cells revealed similar expression levels of the HPV16, -18, -31, -70 and -82 E2 proteins and lower levels of the HPV53 and -66 E2 proteins (Fig. 3). This suggested that there was no correlation between E2 protein levels and the ability to stimulate the E2–reporter plasmid, indicating that the HPV53 and -82 E2 proteins are weaker activators of transcription.

**Effects of E2 proteins on URR promoter activity**

As shown in Fig. S1, co-transfection experiments of pGL-18URR-luc or pGL-70URR-luc and different amounts of the respective E2 expression vectors revealed that repression of both URR constructs was observed at 10 ng expression vector in both RTS3b and HeLa cells and could only be slightly enhanced by adding more expression vector. Therefore, to address how E2 proteins modulate the

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**Fig. 1.** Activities of HPV URRs in different keratinocyte cells. NHK, RTS3b and HeLa cells were transfected with the indicated HPV URR–luciferase plasmids (50 ng) and luciferase activities were determined at 48 h after transfection. Data represent the means of six (NHK), five (RTS3b) and five to seven (HeLa) independent transfections performed in duplicate. Luciferase activities are presented as relative light units (RLU) (mean ± SEM).
activity of the respective E6/E7 promoters, co-transfection experiments with URR reporter plasmids and 10 ng untagged E2 expression constructs were carried out in the different cell lines (Fig. 4). The HPV18 URR was repressed by E2 in all cell lines tested, with the strongest repression observed in HeLa cells. These levels of repression were similar to or better than has been reported in previous studies using human keratinocyte cell lines for the highest amount of E2 expression vectors (Ottinger et al., 2009; Steger & Corbach, 1997), indicating that pSG5-derived vectors express E2 proteins very efficiently. The HPV53 URR was also repressed more than twofold by E2 in all cell lines. In contrast, the HPV70 URR was only 20–30% inhibited by E2 in all cell types. The HPV16 and -82 URRs were inhibited more than twofold in NHK and RTS3b cells but only weakly in HeLa cells. The HPV66 URR was repressed in NHK and HeLa cells but activated in RTS3b cells. The HPV31 URR was repressed in HeLa cells and activated in NHK and RTS3b cells.

DISCUSSION
A large number of epidemiological studies have demonstrated that HPV16 and -18 are more potent inducers of cervical cancer by far in humans than other HPV types. Recent tissue-culture studies have challenged the idea that the ability to immortalize human keratinocytes is unique to carcinogenic HPV (Hiller et al., 2006; Muench et al., 2009). Therefore, we investigated whether differences in transcriptional regulation correlated with the carcinogenic behaviour of HPVs in vivo. Our studies revealed an unexpected cell-type-dependent complexity in transcriptional control among HPVs.

Transcriptional activities of α-HPVs do not correlate with epidemiological risk classification

Neither URR activity in a given cell type nor the activities of E2 proteins correlated strongly with the epidemiological risk of an HPV type for cervical cancer development. In all cell types investigated, the URR activities of the non-carcinogenic/weakly carcinogenic HPV types were higher...
URR activities of HPV16 and -18 were lower in the presence of E2 than those of less- and non-carcinogenic HPV types (Fig. 4). However, this trend could not be observed for RTS3b and HeLa cells, as the activity of the HPV66 URR in the presence of E2 was lower than that of HPV16 and -18. In addition, in HeLa cells, the activity of the HPV70 URR in the presence of E2 was in between the activity levels of that of HPV16 and -18. The investigation of the URR activities in the presence of the respective E2 proteins from all 25, 56, 76, and 99 HPV types may help to resolve this issue in the future. Furthermore, in this study only the reference sequence of a given HPV type was analysed. It has been shown for HPV16 that naturally occurring URR variants differ in their transcriptional activity (Hubert, 2005; Kämmer et al., 2000); it is therefore possible that the transcriptional properties of non-/weakly carcinogenic HPV types are influenced by variants.

**Differences in transcription among α-HPVs**

We observed differences in the basal activity of HPV URRs that were modulated by the cellular background, unravelling an unexpected complexity in transcriptional regulation that confirms and extends the findings by Ottinger et al. (2009). The basal activity of the HPV11, -16, -18 and -31 URRs has been shown to be critically dependent on the cellular transcription factors AP-1 and Sp1 (Chong et al., 1990; Cripe et al., 1990; Gloss & Bernard, 1990; Hoppe-Seyler & Butz, 1992; Hubert et al., 1999; Kyo et al., 1995; Mack & Laimins, 1991; Thierry et al., 1992; Zhao et al., 1997). Furthermore, several other transcription factors such as YY1, NF1, Oct1 and TEF1 have been implicated in a virus-type- and/or cell-type-dependent manner to contribute to HPV URR activities (Bauknecht et al., 1992; Butz & Hoppe-Seyler, 1993; Chong et al., 1990, 1991; Hoppe-Seyler et al., 1991; Ishijii et al., 1992; Kanaya et al., 1997; May et al., 1994). Sequence alignments have revealed that the Sp1-binding site immediately upstream of E2-binding site 3 is present in HPV16, -18 and -31, as well as in HPV53, -66, -70 and -82, indicating that it is not responsible for the different activities of the URRs. HPVs use both canonical and non-canonical AP-1-binding sites to control URR activity (Wang et al., 2011). We identified putative AP-1-binding sites using sequences described by Wang et al. (2011) in HPV URRs but did not observe a correlation between URR activity and the number of putative AP-1 sites. Both HPV16 and -31 contain six AP-1-binding sites but differed greatly in URR activity in the three cell lines investigated (Fig. 1). Similarly, both HPV53 and -82 URRs harbour two putative AP-1 sites, but HPV82 always displayed a much higher activity (Fig. 1). Furthermore, the URR activities of HPV18 and -70, both of which have three AP-1 sites, were similar in NHK and RTS3b cells but differed greatly in HeLa cells. Thus, future studies combining mutational analyses with transcription factor-binding studies of different HPV URRs are required to resolve these issues.
Repression by E2 is mainly HPV-type dependent

Repression of HPV URRs from α-HPVs by E2 proteins requires a conserved promoter region consisting of an Sp1-binding site, two E2-binding sites and the TATA box, and occurs mainly with higher levels of E2 protein, as judged by titration experiments (Ottinger et al., 2009; Steger & Corbach, 1997; Stubenrauch et al., 2000). Surprisingly, the HPV18 and -70 URRs differed markedly in their ability to be repressed by the respective E2 proteins, despite having identical Sp1- and E2-binding sites and TATA box elements. Furthermore, repression was not due to differences in E2 protein levels (Fig. 3). Repression of the HPV URR by E2 has been recently linked to interaction of E2 with the cellular Brd4 protein (Smith et al., 2010; Wu et al., 2006). The E2–Brd4 interaction, which is highly conserved among papillomaviruses, is also required for the activation of E2-dependent reporter plasmids such as the pC18-Sp1-luc plasmid used in this study (McPhillips et al., 2006; Schweiger et al., 2006). HPV18 and -70 E2 were both equally able to stimulate transcription from the reporter, indicating that both proteins engage in functional Brd4 interactions to a similar extent. This also held true for the HPV16 and -31 E2 proteins, which weakly or did not repress their URRs but were expressed at similar levels as HPV18 E2 and stimulated pC18-Sp1-luc transcription comparably to HPV18 E2. This may indicate that E2 proteins bind differentially to additional factors that contribute to repression, as suggested by a recent genome-wide small interfering RNA screen (Smith et al., 2010). It is also possible that URR sequences outside the conserved core promoter contribute to repression by E2.

A recent comparative study by Ottinger et al. (2009) demonstrated that repression of the HPV11, -16 or -18 URR by the homologous E2 proteins is cell-type dependent, as none of the URR/E2 combinations led to repression in SiHa or BJ cells. In contrast, we mainly observed differences between HPV types with regard to repression but saw little influence of the cell type used. Thus, for example, HPV16 was always weakly, HPV18 always strongly and HPV31 never repressed by the respective E2 proteins. The only exception was HPV66, which could be repressed in NHK and HeLa cells but was activated in RTS3b cells. Our data suggest that repression is determined mainly by the virus type and not by the cell type.

METHODS

Plasmid constructions. The luciferase reporter plasmids pC18-Sp1-luc, pGL-16URR-luc, pGL-18URR-luc and pGL-31URR-luc have been described previously (Fertey et al., 2011). URRs were amplified by PCR using cloned HPV genomes as templates and primers that added restriction sites. The amplicons were digested after cloning into pGL3-Basic (Promega). The HPV66 URR was released with EcoRI, BamHI and NcoI from the cloned HPV66 genome construct and then cloned in a three-component ligation into pBRL/NcoI-digested pGL3. Expression vectors for HPV16, -18 and -31 E2 have been described previously (Fertey et al., 2011). E2-coding sequences were amplified by PCR using cloned HPV genomes as templates and primers that added restriction sites. The amplicons were cloned after digestion with NheI and BamHI and cloned into pBRL/NheI-digested pIRESpuro3 (Clontech). The E2 genes were then subcloned into a pSG5 expression vector with a modified polylinker of pSG5. An oligonucleotide encoding a HA tag was introduced into pIREs3 constructs between the EcoRV and NheI sites and HA–E2 fusions were then subcloned into pSG5 plasmids. Expression vectors encoding internally HA-tagged E2 proteins have been described previously (Fertey et al., 2011). An internal HA tag was added to HPV70 E2 by inserting an oligonucleotide into the SfoI site. All cloned sequences were verified by DNA sequencing (GATC Biotech).

Cell culture. NHK cells were derived from neonatal foreskins and cultivated in complete keratinocyte serum-free medium (Invitrogen) supplemented with gentamicin (0.5 mg ml⁻¹). The cervical carcinoma HPV18-positive HeLa cell line was maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with gentamicin and 10% FBS. RTS3b cells were expanded in E-medium supplemented with penicillin, streptomycin and 10% FBS (Stubenrauch et al., 2001).

Luciferase-based reporter assays. Approximately 3 × 10⁴ HeLa, NHK or RTS3b cells were seeded into 24-well dishes 1 day before transfection. Cells were transfected with 50 ng reporter plasmid alone or together with 10 ng pSG5 or a pSG E2 expression construct using Fugene HD (Roche) and OptiMEM (Invitrogen). Luciferase assays were carried out 48 h after transfection as described previously (Fertey et al., 2011). For the titration experiments shown in Fig. S1, pCMV-Gluc (New England Biolabs) was added as an internal control.

Immunoblot analyses. To detect E2 proteins by immunoblotting, 3 × 10⁶ RTS3b cells were transfected with 2 μg pSG expression plasmid. Transfected cells were lysed 48 h after transfection in SDS-PAGE loading buffer, heated to 95 °C and then briefly sonicated. Aliquots were separated in SDS-PAGE (10% acrylamide) and transferred to a nitrocellulose membrane. Membranes were incubated with 1:1000-diluted primary antibodies (α-HA, Covance; α-HSP90, Santa Cruz). Bound antibodies were detected with fluorescently labelled antibodies (IRDye 680RD goat anti-mouse IgG or IRDye 800CW goat anti-rabbit IgG; LI-COR Biosciences) and an Odyssey Fc Infrared Imaging System (LI-COR Biosciences).

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