Infectious human papillomavirus type 31b: purification and infection of an immortalized human keratinocyte cell line

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Human papillomaviruses (HPVs) are aetiological agents of human malignancies, most notably cervical cancers. The life-cycles of HPVs are dependent on epithelial differentiation, and this has impeded many basic studies of HPV biology. The organotypic (raft) culture system supports epithelial differentiation such that infectious virions are synthesized in raft tissues from epithelial cells that replicate extrachromosomal HPV genomes. The CIN-612 9E cell line maintains episomal copies of HPV type 31b (HPV31b), an HPV type associated with cervical cancers. Many previous studies, including our own, have focused on characterizing the later stages of the HPV31b life-cycle in CIN-612 9E raft tissues. In this study, we have used the raft system to generate large numbers of HPV31b viral DNA (vDNA)-containing particles. We found a biologically contained homogenization system to be efficient at virion extraction from raft epithelial tissues. We also determined that vDNA-containing particles could be directly quantified from density-gradient fractions. Using an RT–PCR assay, the presence of newly synthesized, spliced HPV31b transcripts was detected following HPV31b infection of the immortalized HaCaT epithelial cell line. Spliced E6 and E1'E4 RNAs were detected using a single round of RT–PCR from cells infected with a dose as low as 1.0 vDNA-containing particle per cell. Spliced E1*I,E2 transcripts were found in cells infected with an HPV31b dose as low as 10 vDNA-containing particles per cell. Infectivity was blocked by HPV31 antiserum, but was not affected by DNase I. This work lays a foundation for a detailed analysis of the early events in HPV infection.

Introduction

Papillomaviruses (PVs) are small DNA viruses that cause benign and malignant tumours in their natural hosts (Lancaster & Olson, 1982; Lowy & Howley, 2001). Humans are the only known hosts for human papillomaviruses (HPVs); attempts to transfer HPVs to other species have failed (Rowson & Mahy, 1967). Over 85 types of HPV have been defined to date, but more than 130 additional types have been partially characterized based on PCR amplification of genomic regions (de Villiers, 1999). Only certain HPV types are associated with human cancers (de Villiers, 1989; zur Hausen, 1996). For example, HPV1 and HPV2, types typically found in common and plantar warts, are not generally associated with carcinomas. HPV6 and HPV11, associated with laryngeal papillomatosis and anogenital lesions, rarely lead to carcinomas. The latter are designated low-risk viruses. HPVs commonly associated with malignant conversion include those involved in epidermodysplasia verruciformis (e.g. HPV5 and HPV8) and the majority of the types that infect the anogenital region. Examples of high-risk anogenital viruses include HPV types 16, 18, 31, 33 and 45 (reviewed in Lowy et al., 1994; zur Hausen, 1996). In fact, high-risk HPV infections are involved in greater than 99% of all cervical malignancies (Walboomers et al., 1999).

According to current models, HPVs infect the mitotically active basal cell layer in vivo through a micro-abrasion or wound in the epithelium. The ability of HPVs to undergo a complete replication cycle resulting in the production of virions (i.e. infectious progeny) is tightly linked to the differentiation state of the infected cells (Laimins, 1996; Lowy & Howley, 2001; Meyers et al., 1992). Epidermal cells are not fully permissive for PVs at the onset of their cellular differentiation process, but become permissive with increasing differentiation. Viral genomes are replicated in three stages. In stage I, the autonomously replicating episomal viral DNA
(vDNA) is established at low (10–200) copy number per cell in the basal stem cells. This event is necessary for establishing virus persistence. Stage II occurs randomly during the cell cycle and provides daughter cells with an approximately equal copy number of the viral genome (Gilbert & Cohen, 1987; Ravnan et al., 1992). Stage III yields amplified copies of vDNA in differentiating cells (Bedell et al., 1991; Ozbun & Meyers, 1998a; Stoler et al., 1990). Epithelial differentiation also results in the induction of late gene synthesis (Frattini et al., 1996; Meyers & Meyers, 1998; Meyers et al., 1992; Pray & Laimins, 1995), regulation and structural characterization of viral transcripts (Hummel et al., 1992, 1995; Klumpp & Laimins, 1999; Ozbun & Meyers, 1997, 1998a, b, 1999b; Stubenrauch et al., 2000; Terhune et al., 1999, 2001) and the replication of vDNA (Bedell et al., 1991; Ozbun & Meyers, 1998a). Yet, the early events in HPV infection of host cells remain to be characterized. In addition, a detailed analysis of infection of keratinocytes in vitro by virions synthesized in organotypic cultures has not been described thus far.

In the present study, we have purified high-risk HPV31b virions from raft tissue cultures grown from the latently infected CIN-612 9E cell line and used these virions to begin to characterize the process of HPV infection. Methods for the safe isolation of large quantities of HPV virus were identified as well as techniques to quantify virus stocks. Furthermore, we used infection of the HaCaT cell line to characterize the HPV31b transcripts expressed following infection.

**Methods**

### Cell and tissue culture.

The CIN-612 cell line was established from a cervical intraepithelial neoplasia (CIN) grade I biopsy (Bedell et al., 1991). The CIN-612 clonal derivative 9E maintains the HPV31b genome episomally at an average of 50 copies per cell (Hummel et al., 1992). CIN-612 9E cells were maintained in monolayer culture using E medium containing 5% foetal bovine serum (FBS; Summit Biotechnology) in the presence of mitomycin c-treated J2 3T3 feeder cells, as previously reported (McCance et al., 1988; Meyers, 1996). Epithelial organotypic (raft) tissue cultures for in vitro differentiation were maintained as previously described (McCance et al., 1988; Meyers, 1996; Meyers et al., 1992). Raft tissue cultures were treated every other day with 10 µM 1,2-dioctanoyl-sn-glycerol (C8:0; Sigma) in E medium containing 5% FBS. Epithelial tissues were allowed to stratify and differentiate at the air–liquid interface for 14 days. The HaCaT cell line (a generous gift of N. Fusenig, DKFZ, Heidelberg) is a spontaneously immortalized epithelial cell line established from normal adult skin (Boukamp et al., 1988). HaCaT cells were maintained in DMEM/F12-Ham’s Nutrient mixture containing 10% FBS, 4 mM amino acids, 2 mM l-glutamine, 100 U/ml penicillin and 1 µg/ml streptomycin (Sigma).

### Virion purification and quantification.

CIN-612 9E raft tissues were extracted using a modified protocol of Favre et al. (Favre et al., 1975; Meyers et al., 1997). Briefly, 50–75 raft tissues were ground with sea sand (Fisher Scientific) in a mortar using 25 ml of buffer A (1 M NaCl, 0.05 M sodium phosphate buffer, pH 8.0). The debris was pelleted at 8000 g for 10 min at 4 °C. The supernatants were kept on ice while the pellet was re-extracted with 25 ml of buffer A and pelleted again under the same conditions. The efficacy of virus extraction was also tested by grinding the tissues in buffer A using a sealed BeadBeater (BioSpec Products) homogenizer and 1-0 mm glass beads. Both extractions proceeded as described below. Supernatants from the first set of extractions were
Table 1. Oligonucleotide primers used to characterize HPV31b infections

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5' → 3')*</th>
<th>Sense or antisense</th>
<th>Nucleotide position*</th>
<th>ORF†</th>
</tr>
</thead>
<tbody>
<tr>
<td>E6A</td>
<td>CCTGCAGAAAAGCCTAGCCG</td>
<td>Sense</td>
<td>120–137</td>
<td>E6</td>
</tr>
<tr>
<td>E6.2A</td>
<td>GCATGAACTAAGCTGCAGC</td>
<td>Sense</td>
<td>142–160</td>
<td>E6</td>
</tr>
<tr>
<td>E7.3A</td>
<td>GGAGAAGACCTCCTGAC</td>
<td>Sense</td>
<td>526–541</td>
<td>E7</td>
</tr>
<tr>
<td>E7.4A</td>
<td>GGCTCTTGGGAAATGCTGTGC</td>
<td>Sense</td>
<td>812–832</td>
<td>E7</td>
</tr>
<tr>
<td>E7.2B</td>
<td>GACACGATTCACAAATGACCC</td>
<td>Antisense</td>
<td>812–832</td>
<td>E7</td>
</tr>
<tr>
<td>742A</td>
<td>CTCACAAGGCTATGCAG</td>
<td>Sense</td>
<td>857–874</td>
<td>E7</td>
</tr>
<tr>
<td>742B</td>
<td>CTGAGTCGTCATGAC</td>
<td>Antisense</td>
<td>857–874</td>
<td>E7</td>
</tr>
<tr>
<td>E1.4A</td>
<td>GACAGAAGACAGACGACAGCAGC</td>
<td>Sense</td>
<td>1042–1064</td>
<td>E1</td>
</tr>
<tr>
<td>E1A</td>
<td>CATGCAAGAGAACGAGAG</td>
<td>Sense</td>
<td>1072–1099</td>
<td>E1</td>
</tr>
<tr>
<td>E1B</td>
<td>TGTTCTCTTTCTCTCGTGCC</td>
<td>Antisense</td>
<td>2067–2083</td>
<td>E1–E2</td>
</tr>
<tr>
<td>E2.2B</td>
<td>GTTCTCAATATGTATATGACAC</td>
<td>Antisense</td>
<td>2772–2795</td>
<td>E2</td>
</tr>
<tr>
<td>E2.4B</td>
<td>GTGCACTGTGTCGTAGTTTG</td>
<td>Antisense</td>
<td>3327–3440</td>
<td>E4</td>
</tr>
<tr>
<td>E4B</td>
<td>CGGACTGTGCGCCAGAG</td>
<td>Antisense</td>
<td>3395–3378</td>
<td>E4</td>
</tr>
<tr>
<td>E4.3B</td>
<td>GCTCTTCTCTTGTCG</td>
<td>Antisense</td>
<td>3425–3440</td>
<td>E4</td>
</tr>
<tr>
<td>β-actin OA</td>
<td>GATGACCCAGATCATGGTTT</td>
<td>Sense</td>
<td>1578–1587/2029–2039</td>
<td>β-Actin</td>
</tr>
<tr>
<td>β-actin OB</td>
<td>GAGAATGATCTTGTACTCTC</td>
<td>Antisense</td>
<td>2735–2744/2857–2867</td>
<td>β-Actin</td>
</tr>
</tbody>
</table>

* Corresponding to the sequence and numbering of HPV31 (Goldsborough et al., 1989) or human β-actin (Smith et al., 1995).
† Open reading frame or region of specified gene.

pooled and the vDNA-containing particles were pelleted based on a sedimentation coefficient of 29S-30S for ‘full’ vDNA-containing particles (Crawford & Crawford, 1963) for 1 h at 4 °C in a swinging bucket rotor at 130000 g. The supernatants were discarded and the virus particles were suspended in 2 ml of buffer B (0.05 M NaCl, 0.01 M EDTA, 0.05 M sodium phosphate buffer, pH 7.4) using a disposable homogenizer. The debris was pelleted at 80000 g for 10 min at 4 °C. The supernatants were kept on ice while the pellet was re-extracted with 2 ml of buffer B and then pelleted again under the same conditions. Caesium chloride was added to the pooled supernatants and the refractive index was used to verify the density at 1.3 g/ml. A gradient was formed by centrifugation at 135000 g for 24 h at 4 °C. The tubes were punctured and 0.5 ml fractions were collected from the bottom of the gradient. Refractive indices of the fractions were measured. Each fraction was placed in a bucket rotor at 135000 g for 24 h at 4 °C. The fractions were collected from the bottom of the gradient. The dispersion of the fractions was measured. Each fraction was placed in a bucket rotor at 135000 g for 24 h at 4 °C. The particles were collected based on a cut-off and the fractions were collected from the bottom of the gradient. The dispersion of the fractions was measured. Each fraction was placed in a bucket rotor at 135000 g for 24 h at 4 °C.

**HPV31b infections.** Cells were seeded at 3 × 10^5 cells per well in 4 cm^2 wells or at 5 × 10^5 cells per well in 9 cm^2 wells and allowed to attach overnight. The cells were washed and re-fed as described above. Virion stocks were incubated with dilutions of anti-PV monoclonal antibodies (a generous gift of Neil Christensen, Penn State College of Medicine) at 37 °C for 1 h. Virion stocks were treated with 0.4 units of RQ DNase I (Promega) in 10 mM MgCl₂ and Tris buffer for 15 min at 37 °C. Normal medium was added and cells were exposed to the suspensions for 1 h at 4 °C with rocking. The inoculum was removed, and the cells were washed with an excess of normal medium. The cells were re-fed with normal medium and moved to 37 °C. The medium was changed every other day, and cells were expanded when they reached confluence.

**HPV31b treatment.** Virion stocks were incubated with dilutions of anti-PV monoclonal antibodies (a generous gift of Neil Christensen, Penn State College of Medicine) at 37 °C for 1 h. Virion stocks were treated with 0.4 units of RQ DNase I (Promega) in 10 mM MgCl₂ and Tris buffer for 15 min at 37 °C. Normal medium was added and cells were exposed to the suspensions for 1 h at 4 °C. Normal medium was added and cells were exposed to the suspensions for 1 h at 4 °C. Virion stocks were treated with 0.4 units of RQ DNase I (Promega) in 10 mM MgCl₂ and Tris buffer for 15 min at 37 °C. Normal medium was added and cells were exposed to the suspensions for 1 h at 4 °C. Virion stocks were treated with 0.4 units of RQ DNase I (Promega) in 10 mM MgCl₂ and Tris buffer for 15 min at 37 °C. Normal medium was added and cells were exposed to the suspensions for 1 h at 4 °C. Virion stocks were treated with 0.4 units of RQ DNase I (Promega) in 10 mM MgCl₂ and Tris buffer for 15 min at 37 °C. Normal medium was added and cells were exposed to the suspensions for 1 h at 4 °C.

**Viral neutralization assays and DNase I treatment.** Virion stocks were incubated with dilutions of anti-PV monoclonal antibodies (a generous gift of Neil Christensen, Penn State College of Medicine) at 37 °C for 1 h. Virion stocks were treated with 0.4 units of RQ DNase I (Promega) in 10 mM MgCl₂ and Tris buffer for 15 min at 37 °C. Normal medium was added and cells were exposed to the suspensions for 1 h at 4 °C. Virion stocks were treated with 0.4 units of RQ DNase I (Promega) in 10 mM MgCl₂ and Tris buffer for 15 min at 37 °C. Normal medium was added and cells were exposed to the suspensions for 1 h at 4 °C. Virion stocks were treated with 0.4 units of RQ DNase I (Promega) in 10 mM MgCl₂ and Tris buffer for 15 min at 37 °C. Normal medium was added and cells were exposed to the suspensions for 1 h at 4 °C.
M. A. Ozbun

Results

HPV31b virion purification

In order to synthesize large quantities of HPV virions in culture, we used our previous observations that CIN-612 9E rafts treated with PKC inducers reach open reading frames and viral late gene production near days 12–14 after lifting to the air–liquid interface (Ozbun & Meyers, 1997). Therefore, we harvested raft tissue for virus extraction at 14 days after lifting to the air–liquid interface. Our previous method of harvesting virus involved a modified protocol of Favre and co-workers (Favre et al., 1975; Meyers et al., 1997) in which a mortar and pestle are used to grind the tissues with sea sand to help release the virions from keratinized epithelium. This procedure is prone to aerosolization of virus and may not be the optimal technique for isolating large quantites of virus. HPVs are potential oncogenic pathogens and genital HPVs have been detected in laryngeal papillomas and carcinomas (Atula et al., 1999; Dickens et al., 1991; Moore et al., 1999; Sakakura et al., 1996). Therefore, we sought to develop an efficient approach for extracting HPV virions that was more biologically contained and that reduced aerosolization of this human pathogen. We investigated the use of the BeadBeater device, which employs glass beads in a closed mechanical Teflon homogenization apparatus. For these studies, we directly compared the sand, mortar and pestle extraction technique with the BeadBeater. A total of 150 raft tissues grown simultaneously were equally divided and virus particles were extracted side-by-side using each technique. HPV particles were then isolated by first pelleting at 130,000 g, which leaves only vDNA-containing particles in the pellet (Crawford & Crawford, 1963). The pellet was resuspended, and virus particles were centrifuged to equilibrium in a CsCl gradient. Fractions were collected from the bottom of the gradient, dialysed against PBS, extracted for DNA and analysed by Southern blot hybridization (an example is shown in Fig. 1). In comparison with copy number standards, we found that fractions 8 and 9 contained 1.7 × 10^7 vDNA-containing particles/μl (Fig. 1). We also determined that the number of vDNA-containing particles could be accurately and directly quantified from the fractions before dialysis. This was achieved using a direct particle lysis and denaturation with NaOH followed by dot-blot hybridization. Dot-blot hybridization indicated that the BeadBeater technique yielded similar, if not slightly more, vDNA-containing particles compared with the sand extraction method (data not shown). From five separate extractions of 25–150 CIN-612 9E rafts each, we recovered an average of 7.7 × 10^7 vDNA-containing particles per raft tissue.

HPV31b infection of HaCaT monolayer cells with various doses of vDNA-containing particles per cell

HaCaT cells are an immortalized human epithelial cell line that display normal cell differentiation as raft tissues (Schoop et al., 1999). Cells express high levels of αv integrin (Klein et al., 1990; Yoon et al., 2001), a proposed PV receptor, and have been shown to bind HPV types 6b, 11 and 16 virus-like particles (VLPs) (Evander et al., 1997; Joyce et al., 1999; Yoon et al., 2001). Previous studies using virus isolated from mouse xenografts have shown that HPV11 and HPV16 are able to infect HaCaT cells, using RT and nested PCR to detect newly synthesized, spliced viral RNAs (Smith et al., 1995; White et al., 1998). We therefore designed an array of PCR primers to detect and characterize spliced HPV31b early transcripts in single-round and nested PCR assays (Table 1). These primers

![Fig. 1. Southern blot hybridization for vDNA presence in dialysed fractions from the isopycnic gradient purification of HPV31b virions. Each of the ten fractions from the bottom to top of the CsCl gradient is shown in lanes 1–10, respectively. DNA was extracted and purified from each fraction. The DNA preparations were restricted with XbaI to linearize the 7912 bp HPV31b genomes. Cloned HPV31a genome copy number controls (10^5, 10^6 total copies of vDNA) were restricted with EcoRI to release the cloned genome from the vector. The positions of DNA molecular size markers, lambda phage DNA restricted with HindIII, are shown at the right.](image-url)
were systematically optimized for annealing temperatures and magnesium concentration. Serial tenfold dilutions of cloned HPV31b cDNA template copy number controls were used with these primer pairs in single-round PCR assays to compare the relative efficiency of the primer pairs. These results are summarized in Table 2.

We next used RT–PCR to examine the spectrum of HPV31b transcripts expressed following infection of HaCaT cells with our virus preparation. Subconfluent HaCaT monolayers were incubated with doses of DNA-containing virus particles equivalent to 0, 2, 20, 200, and 2000 genomes per cell. We defined the dose of virus inoculation based on the number of vDNA-containing virus particles determined by Southern or dot-blot hybridization as described above, subconfluent HaCaT monolayers were incubated with serial tenfold dilutions of the HPV31b virion stock in normal media at 4 °C, then washing to remove the unbound particles. As described above, subconfluent HaCaT monolayers were incubated with serial tenfold dilutions of the HPV31b virion stock in normal media at 4 °C for 1 h. In one set of infections, the inocula were aspirated and the cells were washed, re-fed and returned to 37 °C. In a second set of infections, the cultures were shifted to 37 °C for 1 h before removing the inoculum, and returning to 37 °C. The cells were harvested for 4 days post-infection (p.i.). RNA samples were DNase I-treated and subjected to RT. The known spliced HPV31b RNAs targeted for detection are summarized in Table 2.

Table 2. A comparison of the relative efficiencies of HPV31b primer pairs in 45 cycles of PCR

<table>
<thead>
<tr>
<th>Left primer</th>
<th>Right primer</th>
<th>cDNA template</th>
<th>Product size (bp)</th>
<th>Sensitivity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>E6A</td>
<td>742B</td>
<td>E6<em>E7,E1</em>E2 or</td>
<td>553</td>
<td>10^7</td>
</tr>
<tr>
<td>E6A</td>
<td>E2.2B</td>
<td>E6<em>E7,E1</em>E2</td>
<td>706</td>
<td>10^7</td>
</tr>
<tr>
<td>E6.2A</td>
<td>E1B</td>
<td>E6<em>E7,E1</em>E2</td>
<td>571</td>
<td>10^7</td>
</tr>
<tr>
<td>E7.3A</td>
<td>E2.2B</td>
<td>E6<em>E7,E1</em>E2</td>
<td>502</td>
<td>10^7</td>
</tr>
<tr>
<td>E7.4A</td>
<td>E2.2B</td>
<td>E6<em>E7,E1</em>E2</td>
<td>216</td>
<td>10^7</td>
</tr>
<tr>
<td>742A</td>
<td>E1B</td>
<td>E6<em>E7,E1</em>E2</td>
<td>59</td>
<td>10^7</td>
</tr>
<tr>
<td>E6A</td>
<td>E4.3B</td>
<td>E6<em>E7,E1</em>E4.L1</td>
<td>702</td>
<td>10^7</td>
</tr>
<tr>
<td>E6.2A</td>
<td>E4B</td>
<td>E6<em>E7,E1</em>E4.L1</td>
<td>633</td>
<td>10^7</td>
</tr>
<tr>
<td>E7.3A</td>
<td>E4.3B</td>
<td>E6<em>E7,E1</em>E4.L1</td>
<td>498</td>
<td>10^7</td>
</tr>
<tr>
<td>E7.4A</td>
<td>E4.3B</td>
<td>E6<em>E7,E1</em>E4.L1</td>
<td>212</td>
<td>10^7</td>
</tr>
<tr>
<td>742A</td>
<td>E4B</td>
<td>E6<em>E7,E1</em>E4.L1</td>
<td>122</td>
<td>10^7</td>
</tr>
<tr>
<td>E1.4A</td>
<td>E4B</td>
<td>E6*E2C</td>
<td>356</td>
<td>10^4</td>
</tr>
<tr>
<td>E1A</td>
<td>E4B</td>
<td>E8*E2C</td>
<td>326</td>
<td>10^4</td>
</tr>
<tr>
<td>E1A</td>
<td>E4.4B</td>
<td>E8*E2C</td>
<td>277</td>
<td>10^4</td>
</tr>
</tbody>
</table>

* Copies of cDNA template per PCR reaction.

were transferred to 37 °C for 1 h before aspirating the inocula, washing, refeeding, and returning to 37 °C. The cells were harvested for 4 days post-infection (p.i.). RNA samples were DNase I-treated and subjected to RT. RT–PCR amplification was used to detect the spliced transcripts of cellular β-actin (Fig. 3c), or the HPV31b splice junctions E6*E7.1210*113 (Fig. 3d), E1*E2.477*1246 (Fig. 3e), E1*E4.77*3295 (Fig. 3f), and E8*E2C.1296*3295 (Fig. 3g). RNAs were analysed from mock-infected HaCaT cells, from HaCaT cells incubated with vDNA or from contaminating vDNA. An intermediate-sized product, which has been refractory to cloning, was also observed.

A previous study demonstrated that BPV1 virions attached but did not penetrate the cells at 4 °C (Zhou et al., 1995). With the goal of analysing virus infection in a more synchronized fashion, we determined the sensitivity of detecting new spliced HPV31b RNAs following a 1 h attachment of particles at 4 °C, then washing to remove the unbound particles. As described above, subconfluent HaCaT monolayers were incubated with serial tenfold dilutions of the HPV31b virion stock in normal media at 4 °C for 1 h. In one set of infections, the inocula were aspirated and the cells were washed, re-fed and returned to 37 °C. In a second set of infections, the cultures were shifted to 37 °C for 1 h before aspirating the inocula, washing, refeeding, and returning to 37 °C. The cells were harvested for total RNAs at 4 days post-infection (p.i.). RNA samples were DNase I-treated and subjected to RT. RT–PCR amplification was used to detect the spliced transcripts of cellular β-actin (Fig. 3c), or the HPV31b splice junctions E6*E7.1210*113 (Fig. 3d), E1*E2.477*1246 (Fig. 3e), E1*E4.77*3295 (Fig. 3f), and E8*E2C.1296*3295 (Fig. 3g). RNAs were analysed from mock-infected HaCaT cells, from HaCaT cells incubated with a dose of DNA-containing virus particles equivalent to 0, 1, 10, 100 and 1000 genomes per cell, and from CIN-612 9E cells. The results of HPV infection were indistinguishable between the set of infections where virus was bound at 4 °C for 1 h, then transferred to 37 °C for 1 h before removing the inoculum, and...
Fig. 2. Genomic organization of HPV31b and summary of the known early spliced polycistronic transcripts targeted by RT–PCR. The circular HPV31b genome of 7912 base pairs is depicted linearized at the late polyadenylation (pA) signal for the purpose of illustrating the ORFs and the characterized transcripts, and the nucleotide numbering of the viral genome is given below (Goldsborough et al., 1989). The long control region (LCR) is indicated and the open boxes indicate viral ORFs in all three reading frames of the genome. The viral promoters are shown by bent arrows with their initiation sites indicated (Hummel et al., 1992; Ozbun & Meyers, 1998b, 1999b). Placement and orientation of the PCR primers used in Fig. 3 to amplify specific spliced segments are illustrated with arrows below the ORFs. The basic RNA organizations were characterized by sequencing cloned cDNAs obtained from CIN-612 9E cells and raft tissues (Hummel et al., 1992; Ozbun & Meyers, 1998a, b; Stubenrauch et al., 2000). Grey boxes illustrate the ORFs contained within the polycistronic transcripts; thick lines represent noncoding sequences. Thin lines show regions spliced out of transcripts (introns); the nucleotide positions of splice donors and acceptors are indicated below the transcripts. The regions and ORFs contained in each mRNA are indicated to the right side of each.

To investigate the expression of the four major spliced HPV31b transcripts following infection, single RT reactions for each RNA sample were divided into PCR amplifications targeting E6*I (Fig. 3d, lanes 3–5, 8–10). Amplimers derived from spliced E1*I,E2 RNAs were detected at a viral dose as low as 10 vDNA-containing particles per cell (Fig. 3e, lanes 4–5, 9–10), whereas new E8*E2C transcripts were not detected following HPV31b infection of HaCaT cells under the described conditions (Fig. 3g). Targeting the E1*E4 RNAs by RT–PCR was the most sensitive means of detecting HPV31b infection at 4 days p.i. The use of serial tenfold dilutions of cloned cDNA template copy number controls demonstrated that the primer pairs used for detecting E6*I and E1*I,E2 were each able to detect targets present at \( \geq 10^2 \) copies per PCR.
Analysis of infectious HPV31b

Fig. 3. RT–PCR analysis of HPV31b transcripts following infection of HaCaT cells at various viral doses. (a, b) Subconfluent HaCaT monolayers were incubated with serial tenfold dilutions of an HPV31b stock as indicated in the text. The virus inoculum was left on the cells, and the cells were harvested at 4 days p.i. DNase I-treated total RNAs (3 µg) were subjected to RT. RNAs were analysed from CIN-612 9E monolayers (9E), mock-infected HaCaT cells (M) and HaCaT cells infected with viral doses corresponding to 0–2, 2–0, 20, 200 and 2000 vDNA-containing particles per cell. No RNA input (W RNA) served as a negative amplification control. The RT reactions were divided equally into PCR amplifications of 40 cycles. (a) Primers β-actin OA → β-actin OB were used to detect a 641 bp amplimer derived from spliced β-actin RNA. (b) Primers E6.2A → E7.2B were used to target a 499 bp amplimer arising from spliced E6*I RNA. The input RNA corresponded to 1–5 µg for each PCR reaction. Molecular size standards (X174 digested with Hae III) are shown to the left of each panel. (c–g) Subconfluent HaCaT monolayers were incubated with serial tenfold dilutions of an HPV31b stock in normal media at 4 °C for 1 h. As indicated, the inocula were aspirated, washed and the cultures were re-fed (4 °C) or were shifted to 37 °C for 1 h before aspirating the inocula, washing and re-feeding (4 °C, 37 °C). The cells were harvested at 4 days p.i. DNase I-treated total RNAs (8 µg) were subjected to RT. RNAs were analysed from mock-infected HaCaT cells (M), HaCaT cells infected with doses corresponding to 0.1, 1.0, 10 and 100 vDNA-containing particles per cell, and CIN-612 9E monolayers (9E). No RNA input (W RNA) served as a negative control. Each RT reaction was divided into PCR amplifications of 45 cycles targeting the spliced transcripts of cellular β-actin or HPV31b. (c) Primers β-actin OA → β-actin OB detected a 641 bp amplimer derived from spliced β-actin RNA. The input RNA corresponded to 380 ng. (d) Primers E6A → E742B targeted a 553 bp amplimer arising from spliced E6*I RNA. (e) Primers E7.3A → E2.2B targeted a 502 bp amplimer resulting from spliced E1*I,E2 RNA. (f) Primers E7.3A → E4.3B targeted a 498 bp amplimer derived from spliced E1gE4 RNA. (g) Primers E1A → E4B targeted a 326 bp amplimer derived from spliced E8gE2C RNA. For (d)–(g), the input RNA corresponded to 1–2 µg for each PCR reaction. Molecular size standards (100 bp ladder, New England Biolabs) are shown to the left of each panel.

Antibody-mediated neutralization assays and DNase I treatment of virions were performed to confirm the specificity of infection. Monoclonal antibodies raised to HPV31, HPV16 and CRPV VLPs (Christensen & Kreider, 1991; Christensen et al., 1996) were tested for neutralization activity against HPV31b virion preparations as described in Methods. As expected, antibodies against CRPV and HPV16 had no effect on HVP31b infectivity (Fig. 4a, lanes 1–6 and 13–18, respectively). The monoclonal antibody H31.A6 raised to HPV31 VLPs completely neutralized HPV31b infection of HaCaT cells (Fig. 4a, lanes 7–12). Additionally, pretreatment of virion stocks with DNase I did not affect their infectivity (Fig. 4a, lanes 19–21), verifying that unpackaged vDNA was not contributing to our observations. These data indicate that detection of spliced viral transcripts in infected cells is a result of bona fide HPV31b infection.
**Discussion**

We used the organotypic, or raft, tissue culture system to cultivate large amounts of HPV31b, a viral type that induces cervical lesions with a high risk of progressing to malignancy. PV life-cycles are intimately linked to the differentiation state of infected epithelial cells and the raft tissue culture system is the only in vitro system shown to mimic epithelial differentiation to the extent that infectious HPVs can be recovered (Meyers et al., 1992, 1997). Traditionally, we utilized a mortar and pestle to grind the tissues with sand and release the virions from keratinized epithelium. Although this procedure is effective, the preparations are not contained and there is potential for spillage or aerosolization of infectious virus particles. As HPVs are human oncogenic viruses, we felt it important to devise a means of increased biological containment on purification of concentrated viral stocks. Thus, we compared the sand extraction procedure to the use of the beadbeater, which utilizes glass beads in a sealed mechanical Teflon homogenization device. Quantification of HPV31b vDNA-containing particles by DNA hybridization demonstrated that the beadbeater was as efficient as the sand grinding procedure; furthermore, the beadbeater provided increased biological containment of the infectious HPV virions. A direct dot-blot analysis obviated the need to dialyse each of the CsCl gradient fractions against PBS before quantifying the number of vDNA-containing particles in the virus preparations. This further streamlined the HPV virion purification and quantification process. Reproducible purification of HPV types from the organotypic culture system will permit investigations of the earliest stages of infection, from virion attachment to the establishment of persistent infections. Such investigations have not been possible for HPVs capable of causing malignancies.

As there is currently no way to quantitatively titre HPVs based on infectivity, we have defined the dose of viral infection based on the number of vDNA-containing particles. Newly synthesized, spliced viral RNAs were targeted by RT–PCR as a qualitative indication of infection. We have found the detection of HPV infection to be inconsistent among various isolates of low passage human foreskin keratinocytes (Meyers et al., 1997; Ozbun, 2002). Comparing HPV31b infections among a number of human keratinocyte cell lines, we found infection of the HaCaT cell line to be the most efficient and reproducible (Ozbun, 2002). Spliced viral RNAs were detected by RT and a single round of PCR in a population of HaCaT cells infected with a dose as low as 1 × 10⁶ viral genome per cell. Furthermore, we showed that HPV31b-infected HaCaT cells synthesize late gene transcripts on epithelial raft tissue differentiation (Ozbun, 2002). This suggests that early infection events in HaCaT cells reflect an accurate view of HPV31b infection biology.

On comparing the efficiency of infection between cells where the virus inoculum remained on the cells and cells in which the unbound virions were washed away after a 1 h binding at 4 °C, we found the minimal detectible doses were similar. This suggests that viral particles capable of binding to the cells were able to bind in 1 h at 4 °C, and is in agreement with the findings of Volpers et al. (1995), showing that approximately 70% of HPV33 VLPs were bound to cells under these conditions. However, Christensen et al. (1995) found that maximum foci production in C127 cells by BPV1 was not obtained unless the virus inoculum remained on the cells for 8 h, suggesting that BPV1 binding leading to infection was relatively slow. The focus assay is a quantitative assessment of infection, whereas our RT–PCR detection is a qualitative assay. Therefore, we are probably unable to detect a quantitative change in infectivity by basic RT–PCR. Nevertheless, the sensitivity of RT–PCR permitted us to qualitatively assay viral transcripts resulting from infection following the binding of virions to the cells for 1 h at 4 °C.

Our RT–PCR amplification from spliced viral RNAs is a sensitive assay for HPV31b infection. Using optimized PCR
primer pairs targeting the known early spliced HPV31b RNAs, we found that the primers specific to spliced E1’E4 transcripts were able to reproducibly detect targets with the greatest sensitivity at 4 days post-infection. Targeting spliced E6’E1 and E1’E4 transcripts, we detected infection in HaCaT cells inoculated with a dose as low as 10^2 vDNA-containing particles per cell. We have used the primer pairs listed in Table 2 to characterize the temporal initiation of viral transcripts following HPV31b infection (Ozbun et al., 2002). Others found that detection of newly spliced viral transcripts in HPV11- and HPV16-infected HaCaT cells required the use of RT and nested PCR (Smith et al., 1995; White et al., 1998, 1999). Our ability to detect PV infection of cells at an apparently lower viral dose and using RT with a single round of PCR could be due to a number of experimental variables. RT–PCR for PV transcripts is not a standardized technique. Differences in primer sensitivities, the amount of input RNA, the efficiency of reverse transcription, reaction conditions like magnesium concentration and primer annealing temperatures, type of polymerase and the number of PCR cycles are each expected to affect the sensitivity of the assay. Infectivity also could vary among HPV and animal PV types. We are in the process of devising standardized techniques to quantitatively compare the infectivities of various PV types. Although we were unable to assess the number of infectious units for HPVs, if we assume the particle-to-infectious-unit ratio is ~10^3, as reported for BPV1 (Roden et al., 1996), then we calculate that our RT–PCR system is capable of detecting ~50 infectious events in a background of 5 × 10^5 cells.

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