In Vitro HPV-11 Infection of Human Foreskin

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Study of the infectious process of human papillomavirus type 11 (HPV-11) has been facilitated by the discovery that HPV-11-infected neonatal human foreskin epithelium can proliferate as xenografts into condyloma-like growths within athymic nude mice. Here we describe detection of HPV-11 infection of neonatal human foreskin-derived keratinocytes, infected and cultured entirely in vitro, by use of the polymerase chain reaction and primers straddling the splice donor/acceptor site of the most prevalent early gene (E1/E4). Expression of the E1/E4 mRNA is abrogated by 60°C heat inactivation of the inoculum. HPV-11-infected foreskin explants continue to produce the E1/E4 mRNA for up to 5 weeks in culture, and second-passage keratinocytes derived from infected explant outgrowths continue to produce the E1/E4 mRNA. The in vitro system described here provides a new way to study HPV-11 infection and may be useful in evaluating early events of infection. Key Words: human papillomavirus type-11/in vitro infection/human epithelium/PCR. J Invest Dermatol 101:292–295, 1993

Human papillomaviruses (HPV) comprise a large family of genetically related types that have distinct tissue tropism and are associated with clinically important epithelial proliferations (condylomata, intraepithelial neoplasia, carcinoma) [1]. The discovery of the association between certain genital HPV types and cervical carcinoma has been followed by a recognition of the key role of early HPV gene function in initiating keratinocyte transformation. Yet many gaps exist in the understanding of HPV infection partly because there exists no in vitro system in which the process can be studied in detail from its earliest events.

HPV-11 is a common genital-type HPV associated with genital condylomata and juvenile laryngeal papillomatosis. Detailed study of the HPV-11 infectious cycle has been made possible by the development of the athymic mouse xenograft system described by Kreider [2,3]. In this system, HPV-11-infected neonatal human foreskin epithelia implanted beneath the renal capsule of nude mice grow into condyloma-like proliferations, and produce viral particles sufficient for purification and further propagation. The xenografted condyloma tissue exhibits an mRNA transcription pattern similar to that seen in HPV-11-associated genital condyloma from patients [4,5] with the E1/E4 transcript most prevalent [5]. Further refinement in detecting HPV-11 transcription has been made possible by detection of spliced mRNAs using the polymerase chain reaction [6]. Recently, Bonnez et al [7] have used PCR detection of the HPV-11 E1/E4 transcript to reliably demonstrate HPV-11 infection of human foreskin epithelium grown as xenografts in nude mice at 6 weeks post-infection. In addition, HPV-11 neutralizing antiserum abrogated the appearance of the E1/E4 transcript in the xenografted tissues, suggesting that the E1/E4 mRNA can be used as a marker of infection. In this report, using similar methods to detect the HPV-11 E1/E4 mRNA, we demonstrate HPV-11 infection of neonatal human foreskin explants cultured entirely in vitro.

In addition, analysis of second-passage keratinocytes derived from the explant cultures confirmed the presence of HPV-11 E1/E4 mRNA within these cells.

MATERIALS AND METHODS

HPV-11 Virus Production The Hershey strain of HPV-11 was generously supplied by Drs. John Kreider and Neil Christensen. Xenografted condyloma tissue (produced using the HPV-11 Hershey inoculum and methods previously described by Kreider et al [8]) was the source of HPV-11 virus used to perform the experiments described here. Approximately 5 g of tissue was pulverized under liquid nitrogen in a stainless steel mortar and suspended in phosphate-buffered saline, pH 7.4, at 4°C. Debris was removed by low-speed centrifugation and virus was pelleted from the resulting supernatant at high speed as described by Favre et al [9]. The high-speed pellet was resuspended in 2 ml phosphate-buffered saline, aliquoted, and stored at –70°C.

Western Blot Analysis For Western blot analysis [10], 5 µl of the HPV-11 virus preparation was subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis [11], transferred to nitrocellulose by semi-dry blotting, blocked with 5% nonfat milk in phosphate-buffered saline, and exposed to rabbit anti-bovine papillomavirus (BPV) (1:500; Dako, Torrance, CA) followed by alkaline phosphatase-labeled goat anti-rabbit IgG (1:1000; Sigma Chemical Co., St. Louis, MO). Color development was with nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate chromogen (Fisher Chemical Co., Pittsburgh, PA). Control BPV was purified from bovine fibropapillomas (obtained from Grand Laboratories, Larchwood, IA) using methods previously described [12].

Neonatal Human Foreskin Explant Cultivation and HPV-11 Infection Neonatal foreskins were obtained after elective circumcision and used within 24 h. After transport to the laboratory, dermal tissues were excised as completely as possible and the remaining epidermal tissue dissected into 2 X 2-mm fragments. For HPV-11 infection, 10–20 foreskin fragments were mixed with 100 µl of the virus preparation and incubated at 37°C (5% CO2, 100% humidity) for 1 h. Control foreskin fragments were treated with phosphate-buffered saline instead of virus preparation. The fragments were then either implanted beneath athymic mouse renal capsule or allowed to adhere to sterile culture dishes for establishment of explant culture at 37°C (5% CO2, 100% humidity). Culture media consisted of Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum, epidermal growth factor (10 µg/ml), cholera toxin (10 µg/ml), and hydrocortisone (1 mg/ml). Keratinocyte outgrowths from the edges of the explants were generally noted at 2 weeks of culture, and continued to
increase in size with continued time in culture. For establishment of secondary keratinocyte cultures, the explant was removed from the culture dish after 4 weeks, the remaining outgrowth of keratinocytes was trypanpurified, replated in fresh culture dishes, and allowed to grow to confluence. Fore skin implants, explants, and secondary cultures were subsequently harvested and RNA extracted [13].

cDNA and PCR For generation of cDNA, 0.5–1 µg RNA was used as a template for DNA synthesis. The first-strand DNA synthesis was with SuperScript reverse transcriptase (RT; Gibco-BRL, Gaithersburg, MD) and was carried out according to manufacturers recommendations with one exception: the downstream primer was used instead of an oligo dT. Amplification of cDNA for detection of the E1/E4 spliced mRNA utilized primers that straddled the HPV-11 donor/acceptor site (847/3325) as follows: upstream primer nucleotides 794–814, downstream primer nucleotides 3880–3900 (GenBank). Thirty rounds of amplification were performed on an Ericomp thermocycler with a temperature profile of 94°C/30 seconds, 55°C/30 seconds, 72°C/35 seconds, followed by a final 10-min extension at 72°C [14]. The resulting products were electrophoresed on a 2% agarose gel, visualized by ethidium bromide fluorescence, and transferred to a nylon membrane for Southern blot analysis.

Southern Blot Analysis Southern blot analysis was performed as previously described [15] but with modifications to a nonradioactive detection system including the use of a horseradish peroxidase– conjugated oligonucleotide probe (Research Genetics, Huntsville, AL) complementary to nucleotides 3561–3586 of the sense strand. Briefly, the nylon membrane was blocked in Tris-Blotto for 1 h at room temperature and rinsed in 5 M sodium citrate, pH 7.0) without urea. Chemiluminescent detection was then performed using the Amersham enhanced chemiluminescence detection system (Arlington Heights, IL). The filter was exposed to Kodak X-OMAT AR scientific imaging film (Kodak, Rochester, NY) for 2 h.

Nucleotide Sequence Analysis Aliquots of polymerase chain reaction (PCR) mixtures were directly cloned into the pCR II vector using the TA Cloning protocol (Invitrogen Corp., San Diego, CA) according to manufacturers specifications. Colonies were screened for the presence of an insert by digestion with Eco RI restriction enzyme, followed by ethidium bromide–agarose gel electrophoresis (not shown).

RESULTS

Visual observation of explant cultures of uninfected and HPV-11–infected foreskins revealed no gross differences through 5 weeks. The primary explants were also examined histologically after formalin fixation and no differences between uninfected and infected tissues were apparent after 5 weeks of culture (not shown). Consistent with previously published observations of organ cultures of neonatal human foreskin [16] the original spinous and granular layers demonstrated pyknotic nuclei and separated from the explant, changes consistent with epidermolysis and loss of viability of the original outer differentiated epidermal layers. However, basal keratinocytes remained viable, forming a three to four–cell–thick layer above the basal lamina, and keratinocytes migrated to cover the sides of the explant and adjacent culture dish, phenomena also described previously [16]. Hallmarks of HPV infection such as multinucleated cells or koilocytes were not observed in the viable basal layers from HPV-11–infected cultured explants. Despite the appearance of the outer layers of the cultured explants, cellular RNA extracted from 5-week foreskin explant cultures and outgrown keratinocytes appeared intact with clear ribosomal and high molecular weight components on ethidium–agarose electrophoresis (not shown).

The HPV-11 virus preparation used for these experiments contained infectious HPV-11 by the following criteria: 1) the preparation was successful in producing xenografted condyloma after inoculated neonatal human foreskin fragments were placed under the renal capsule of nude mice; 2) the virus preparation, when subjected to Western blot analysis, yielded a strong band in the position expected for the HPV-11 major capsid protein (Fig 1); and 3) infection of human foreskin fragments in vitro was associated with the appearance of the HPV-11 E1/E4 mRNA, a phenomenon abrogated by preincubation of the HPV-11 inoculum for 1 h at 60°C, but not at 37°C (Fig 2).

We used PCR amplification to study the appearance of the HPV-11 E1/E4 mRNA, as demonstrated by the presence of the characteristic 628-bp band, in HPV-11–infected neonatal human foreskin

![Figure 1](image1.png) **Figure 1.** Western blot analysis of HPV-11 Hershey inoculum (gift of N. Christensen and J. Kreider) and virus prepared from xenograft condyloma (generated in our laboratory). Rabbit anti-BPV antiserum (Dako) was reacted to the following antigens: lane 1, 5 µl purified BPV; lane 2, 5 µl original HPV-11 virus inoculum (Hershey inoculum); and lane 3, 5 µl HPV-11 preparation (virus stock for experiments described here). Note: repeat Western blot analysis with 50 µl of Hershey HPV-11 inoculum showed a band coincident with the major capsid protein of HPV-11, identical to that shown in lane 3 (not shown).

![Figure 2](image2.png) **Figure 2.** Effect of heat on in vitro infectivity of HPV-11. The HPV-11 inoculum (described in Materials and Methods) was incubated for 1 h at either 60°C or 37°C. Each inoculum was used to infect 20 foreskin fragments, which, after culture for 5 weeks, were extracted together as a 37°C specimen or 60°C specimen. After PCR amplification of cDNA, ethidium-agarose gel electrophoresis was performed. Lane 1, 6X174 DNA marker digested with HaeIII, molecular weight standards; lane 2, 37°C specimen; lane 3, 60°C specimen; lane 4, PCR products of cDNA from HPV-11 condyloma (positive control).
Figure 3. Ethidium bromide-agarose electrophoresis of polymerase chain reaction from *in vivo* implant and *in vitro* explant analysis. Lane 1, uninfected foreskin control; lane 2, week 1, infected implanted foreskin cDNA; lane 3, week 2, infected implanted foreskin cDNA; lane 4, week 3, infected implanted foreskin cDNA; lane 5, week 4, infected implanted foreskin cDNA; lane 6, week 5, infected implanted foreskin cDNA; lane 7, φX174 DNA marker digested with Hae III; lane 8, patient condyloma cDNA; lane 9, uninfected second-passage keratinocyte RNA, no reverse transcriptase (RT); lane 10, infected second-passage keratinocyte RNA, no RT; lane 11, week 4 infected explant foreskin RNA, no RT; lane 12, uninfected second-passage keratinocyte cDNA; lane 13, infected second-passage keratinocyte cDNA; lane 14, week 4 infected explant cDNA; lane 15, no sample; and lane 16, φX174 DNA marker digested with Hae III.

Fragments grown both as explant cultures and implants in nude mice (Fig 3). In HPV-11-infected foreskin fragments grown as xenografted implants in *vitro*, the E1^E4* mRNA was detectable by 3 weeks and sometimes earlier. HPV-11-infected foreskin explant cultures also expressed the E1^E4 mRNA as early as 3 weeks (not shown). HPV-11-infected foreskin fragments (derived from nine different foreskin specimens) cultured in *vitro* for 4 weeks or longer have been consistently positive for the E1^E4 mRNA. Figure 3 demonstrates the E1^E4 mRNA from a 4-week HPV-11-infected explant culture and also from second-passage keratinocytes derived from the explant outgrowth, which were regrown to confluence prior to extraction. Second-passage keratinocytes derived from HPV-11-infected explant cultures originating from three different foreskin specimens were all positive for the E1^E4 mRNA after 11 d, 14 d, and 42 d in secondary culture. Table I summarizes our experience to date on detection of the E1^E4 mRNA. Negative controls included ten different, uninfected specimens cultured for up to 5 weeks, and PCR reactions conducted using RNA that had not been reverse transcribed showed no evidence of the E1^E4 mRNA.

Southern blot analysis confirmed the sequence homology of the 628-bp cDNA bands by positive hybridization of the HPV-11 probe with samples generated from both infected xenografted implants and infected cultured explants (Fig 4).

Nucleotide sequence analysis was performed on cloned inserts recovered from PCR reaction mixtures of cDNA from HPV-11-infected foreskin explant cultures. The splice donor/acceptor sequence at nucleotides 847/3325 (Fig 5), as well as the nucleotide sequence of the entire insert region of the clones, confirmed the identity of the 628-bp PCR product as the cDNA of the HPV-11 E1^E4 spliced mRNA. Sequence analysis indicated the cDNA clones represented the HPV-11 sequence from nucleotides 794–3900, with a deletion spanning nucleotides 848–3324 (Los Alamos GenBank). A less intense band of 379 bp was also seen in some PCR reaction mixtures. Nucleotide sequence analysis indicated the origin of the 379-bp band to be from false priming by the downstream primer occasionally primed in the upstream-to-downstream direction from nucleotides 3521–3541.

**DISCUSSION**

The data presented here show that HPV-11 infection was detected in neonatal human foreskin explant cultures that had been infected and cultivated entirely *in vitro*. Although hallmarks of HPV infection were not seen histologically in the tissue cultured *in vitro*, HPV-11 infection under the conditions described involved sufficient numbers of cells so that infection was reliably detected by 4 weeks postinfection. The appearance of the HPV-11 E1^E4 mRNA in second-passage keratinocytes derived from infected explant cultures, suggests continued propagation or at least persistence of the virus in these cells. Infection is inferred by the appearance of the

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**Table I. Detection of HPV-11 infection by E1^E4 mRNA PCR**

<table>
<thead>
<tr>
<th>Time (weeks post-infection)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo implants</td>
<td>0/3*</td>
<td>1/3</td>
<td>1/3</td>
<td>2/3</td>
<td>3/3</td>
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<td>In vitro explants</td>
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<td>NT</td>
<td>3/4</td>
<td>13/13</td>
<td>2/2</td>
</tr>
</tbody>
</table>

* Number of experiments positive for E1^E4/total number of experiments.

* Not tested.

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**Figure 4. Chemiluminescent photograph of Southern blot analysis from *in vivo* implant and *in vitro* explant analysis of PCR reactants. Lanes as in Fig 3.**

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**Figure 5. Autoradiograph of Sanger dideoxysequence analysis of a cDNA clone derived from PCR products of HPV-11 infected foreskin explant cDNA at 3 weeks post-infection. The DNA sequence 3' to the upstream primer (794–814) demonstrates the splice donor (847)/acceptor (3325) site of the HPV-11 E1^E4 mRNA.**
The model described here has several important attributes including:
1. The earliest events of HPV-11 infection can be studied entirely in vitro.
2. There is no interference from host animal physiology.
3. A natural target tissue for HPV-11 infection is utilized.
4. The system has obvious applications for the study of virus-neutralizing antibodies, the mechanism of cellular entry, and the effects of HPV infection on keratinocyte biology.

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REFERENCES