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Cloning of E6/E7 Genes of Human Papillomavirus Type 16 DNA

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To obtain the specific probe which can detect human papillomavirus type 16 (HPV-16)-specific transcripts encoding for E6 and E7 genes in cells or cell lines containing HPV-16 DNA, we cloned 0.57-kbp fragment (nucleotides 198-767) of the HPV-16 DNA into pUC18 and named pHPV16-E6E7R. To test the specificity of the cloned probe compared with whole HPV-16 genome, we cultured normal human oral keratinocytes, and HOK-16B cells, human oral keratinocytes immortalized by transfection with recombinant HPV-16 DNA, in keratinocyte growth medium supplemented with pituitary extract, isolated poly(A$^+$)RNAs from the cells, and hybridized them with the cloned probe and whole HPV-16 genome. Northern blot analysis showed that multiple poly(A$^+$)RNAs hybridized to whole HPV-16 genome were expressed from the HPV-16-immortalized HOK-16B cells, while only two transcripts with sizes of 1.9-kb and 1.6-kb hybridized to HPV-16 E6/E7 gene fragment were detected from the cells. However, both probes did not detect the viral transcripts in normal human oral keratinocytes which are not infected with HPV-16. These data indicate that 0.57-kbp fragment representing HPV-16 E6/E7 genes can be used to detect HPV-16-specific transcripts encoding for E6 and E7 genes in cells or cell lines containing HPV-16 DNA.

Key words: *Human papillomavirus, HPV-16 E6/E7, pHPV16-E6E7R*

Introduction

Human papillomavirus (HPV) infection is closely related to benign and malignant oral lesions (Kellokoski *et al.*, 1992) as well as to female genital epithelial cancer (zur Hausen, 1986). Among the known HPV types, type 16 (HPV-16) and type 18 (HPV-18) are most frequently associated with oral squamous cell carcinomas (Woods *et al.*, 1993). Recent studies demonstrated that the role of HPV in carcinogenesis derived from its transforming capacity by means of E6 and E7 early gene products (von Knebel Doeberitz *et al.*, 1988; Dürst *et al.*, 1989). The E6 and E7 proteins of HPV-16 and HPV-18 were shown to bind *in vitro* to the tumor suppressor proteins p53 and $p105^{Rb}$, respectively (Dyson *et al.*, 1989; Münger *et al.*, 1989; Werness *et al.*, 1990). This protein-protein interaction may interfere with the regulation of normal cell growth and may offer one explanation of how HPV infection leads to abnormal cell proliferation (Rohlfs *et al.*, 1991).

The genome of HPV-16 in precancerous lesions exists generally in an episomal state, whereas in carcinoma integrated viral DNA is frequently observed (Dürst *et al.*, 1985; Lehn *et al.*, 1985, 1988; Cullen *et al.*, 1991). The break in the viral genome due to integration often occurs within the HPV-16 E1-E2 gene region and this event is presumed to result in de-regulation of the expression of the HPV-16 E6/E7 genes and promotion of neoplasia (zur Hausen, 1989). The transcription pattern was extensively studied in carcinomal cell lines, in some tumor biopsies, and in HPV-16-immortalized keratinocyte cell lines (Smotkin *et al.*, 1986; Baker *et al.*, 1987; Doorbar *et al.*, 1990; Sherman *et al.*, 1992). According to the alternative splicing patterns, various HPV-16-specific transcripts were detected. Four types of E6-E7 mRNA were identified (Smotkin *et al.*, 1989; Doorbar *et al.*, 1990); E6, E 6I, E6II, and E6III. The E6I transcript is presumed to encode the E7 protein which is abundantly expressed in carcinomas and their derived cell lines (Smotkin *et al.*, 1989).

We previously immortalized primary human oral keratinocytes by transfection with cloned HPV-16 DNA and established a cell line, human oral keratinocytes-16B (HOK-16B). This line contained intact HPV-16 DNA as an integrated form, expressed viral genes, and demonstrated indefinite life span. However, the cells proliferated only in keratinocyte growth medium (KGM) containing a

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low level of calcium, and were not tumorigenic in nude mice. In these study, we cloned 0.57-kbp fragment into pUC18 to obtain the specific probe which can detect the HPV-16-specific transcripts encoding for E6 and E7 open reading frames (ORFs) in cells or cell lines infected with HPV-16, and then tested the specificity of this probe compared with whole HPV-16 DNA by northern blot analysis.

Materials and Methods

Cells and Culture Condition

Excised gingival tissue from the oral cavity of a healthy volunteer was washed in calcium- and magnesium-free Hanks' balanced salt solution (CMF-HBSS, Gibco/BRL, Grand Island, NY). To separate the epithelium from the underlying submucosa, the tissue was incubated in CMF-HBSS containing collagenase (type II; 1.0 mg/ml, Sigma, St. Louis, MO) and dispase (grade II; 2.4 mg/ml, Boehringer-Mannheim, Indianapolis, IN) for 90 min at 37°C in an atmosphere of 95% air and 5% CO_2. Separated epithelial sheets were then dissociated into single cells by incubation in trypsin with agitation at 37°C for 8 min. The cells were washed with CMF-HBSS, resuspended with keratinocyte growth medium (KGM) supplemented with pituitary extract (Clonetics Corp., San Diego, CA), and plated at 2×10^5 cells per 60-mm Petri dish. The HOK-16B cells, human oral keratinocytes immortalized by transfection with recombinant HPV-16 DNA, were cultured in KGM supplemented with pituitary extract as described previously (Park *et al.*, 1991).

E. coli HB101, used as the recipient for transfection, was grown in LB broth or LB agar plate. *E. coli* HB101 transformed with recombinant DNA was cultured in LB broth containing 100 μg/ml of ampicillin.

Cloning of HPV E6/E7 subgenomic DNA

The 1.33-kbp DNA fragment containing HPV E6/E7 genes was excised by *Pst*I and *Eco*RI double digestion from HPV-16 DNA. The 0.57-kbp DNA fragment (nucleotides 198-767) containing HPV E 6/E7 genes was prepared from the 1.33-kbp DNA fragment cleaved by *Rsa*I. The blunt-ended 0.57-kbp DNA fragment purified by QIAEX II Gel Extraction Kit (QIAGEN Inc., Chatsworth, CA) was incubated with *Hin*dIII linker [d(pCAAGCTTG)] in the presence of bacteriophage T4 DNA ligase, and then cleaved with *Hin*dIII restriction enzyme. The resulting DNA fragment was cloned into pUC18 digested with *Hin*dIII restriction enzyme and dephosphorylated by bacterial alkaline phosphatase. The recombinant plasmid was then transformed into *E. coli* HB101. The resulting *E. coli* HB101 recombinants were grown in LB broth containing ampicillin for restriction analysis of small-scale preparation of plasmid DNA (Sambrook *et al.*, 1989). The plasmid DNAs were prepared by the alkaline lysis method and purified by precipitation with polyethylene glycol (Sambrook *et al.*, 1989).

Northern Analysis

To determine the transcription of HPV-16 DNA, HPV-16 E6/E7, and β-actin genes, cytoplasmic poly(A$^+$)RNAs were extracted from cells using standard procedures. Probes used for northern blot analysis were as follows: 7.9-kbp HPV-16 DNA, 0. 57-kbp fragment (nucleotides 198-767) representing the major early HPV-16 message including E6/E7 genes, and human β-actin cDNA (from Dr. L. Kedes, Stanford University, Palo Alto, CA). All were labeled with [^{32}P]dCTP (Amersham Corp., Arlington Heights, IL) by megaprime DNA labeling (Amersham Corp.). Specific radioactivities of labeled probes were always higher than 5×10^8 cpm/μg of DNA. Five μg of poly(A$^+$)RNAs were denatured and run on a 1.2% formaldehyde agarose gel with marker RNAs (9.5-, 6.2-, 3.9-, 2.8-, 1.9-, 0.9-, 0.6-, and 0.4-kb RNA marker, International Biotechnologies, Inc., New Haven, CT). The RNAs were transferred onto nylon filters (Amersham Corp.) and crosslinked with ultraviolet light for 5 min. The filters were hybridized to [^{32}P]-labeled probe at 42°C for 24 h in 50% formamide/10% dextran sulfate/5X SSPE (0.15 M NaCl, 0.01 M Na_2HPO_4, and 0.001 M EDTA)/5X Denhardt's solution/ denatured salmon sperm DNA (20 μg/ml). Filters were washed twice in 5×SSPE for 15 min at 42°C, then in 1X SSPE/0.1% SDS for 30 min at 42°C, and finally in 0.1×SSPE/0.1% SDS for 15 min at room temperature. Filters were then exposed to Hyperfilm-MP (Amersham Corp.) for 24 h at -70°C. After exposure, the probe was stripped off the filter for rehybridization to the next radiolabeled probe.

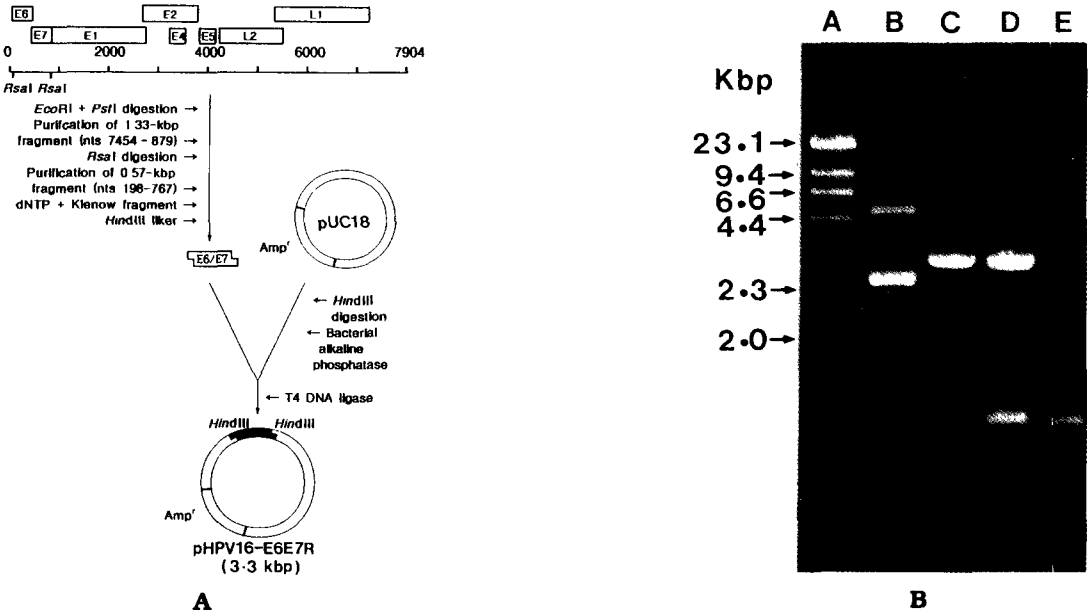

Fig. 1. Cloning of HPV-16 E6/E7 gene fragment. (A) Construction and restriction map of pHPV16-E6E7R. (B) Agarose gel electrophoresis of *Hin*dIII-digested pUC18 and pHPV16-E6E7R. Lane A; *Hin*dIII-digested λDNA size marker. Lane B; undigested pHPV16-E6E7R. Lane C and D; *Hin*dIII-digested pUC18 and pHPV16-E6E7R, respectively. Lane E; purified 0.57-kbp HPV-16 E6/E7 gene fragment.

Results

Cloning of HPV-16 E6/E7 genes

Ampicillin resistant transformants were screened for isolation of recombinant plasmid DNAs. Plasmid DNAs were purified from these transformants and digested with *Hin*dIII restriction enzyme. The fragmented plasmids were run in a 0.8% agarose gel. One recombinant plasmid was selected, and named pHPV16-E6/E7R according to the origin of viral DNA (Fig. 1). The plasmids were digested with *Hin*dIII restriction enzyme, run in a agarose gel, purified 0.57-kbp fragment (nucleotides 198-767) representing the major early HPV-16 message including E6/E7 genes, and used HPV-16 specific probe for northern blot analysis.

Viral RNA

Northern blot hybridization to [^{32}P]HPV-16 DNA showed that multiple poly(A$^+$)RNAs hybridized to HPV-16 DNA were expressed from the HPV-16-immortalized cell line, while no HPV-16 poly(A$^+$)RNAs were expressed from normal human oral keratinocytes (NHOK) (Fig. 2A). Northern blot analysis using the probe containing 0.57-kbp HPV-16 E6/E7 gene fragment showed that HOK-16B cells expressed two transcripts with sizes of 1.9-kb and 1.6-kb, but NHOK did not express the viral transcripts (Fig. 2B). The expression patterns of β-actin gene from NHOK and HOK-16B cells were active (Fig. 2B). Both cells expressed 2.0-kb mRNA in a similar manner, indicating that both cells were metabolically active.

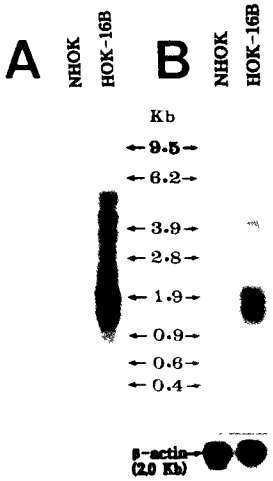

Fig. 2. Northern blot hybridization of cellular polyadenylated RNAs to [^{32}P]-labeled whole HPV-16 DNA (A) and to [^{32}P]-labeled 0.57-kbp HPV-16 E6/E7 gene fragment (B). The RNAs were prepared as described in "Materials and Methods". Kb, kilobase.

Discussion

These data show that the 0.57-kbp HPV-16 E6/E7 gene fragment (nucleotides 198-767) cloned into pUC18 can be used to detect HPV-16-specific transcripts encoding for HPV-16 E6/E7 genes in cells or cell lines containing HPV-16 DNA. There is much evidence to support the involvement of 'high risk' HPV in the induction of female genital epithelial and oral cancers. Among the known HPV types, type 16 (HPV-16) and type 18 (HPV-18) are most frequently associated with malignant oral lesions (Maitland *et al.*, 1987; Syrjanen, 1992). Recent studies show that up to 30-40% of oral cancer biopsies contain the viral DNA (Syrjanen, 1992), indicating the close association between oral cancer and HPV infection.

Recently, it was shown that 'high risk' HPV such as HPV-16 and HPV-18 can immortalize human oral keratinocytes in culture (Park *et al.*, 1991; Shin *et al.*, 1994). This immortalizing activity was mapped to the E6 and E7 ORFs of the viral genome (Barbosa *et al.*, 1989; Hawley-Nelson *et al.*, 1989). The viral E6 proteins make complex with wild-type p53 proteins (Werness *et al.*, 1990) and promote the degradation of the latter *in vitro* (Scheffner *et al.*, 1990) and *in vivo* (Hubbert *et al.*, 1992). Similarly, the viral E7 proteins bind to Rb proteins and inactivate tumor suppressor activity in cells infected with 'high risk' HPV (Dyson *et al.*, 1989; Stirdivant *et al.*, 1992). Previous report also demonstrates that Rb is often found to be inactivated in oral cancer cells, but the inactivation of Rb may be less frequently than that of p53 (Kim *et al.*, 1993). Because of the role of E6 and E7 as transforming proteins in virus-linked carcinogenesis, attention has mainly focussed on the expression of the HPV-16 and -18 E6/E7 ORFs in oral cancers and cancer derived cell lines. Therefore, detection of HPV-16-specific transcripts encoding for E6 and E7 ORFs in oral cancers and their derived cell lines may play an important role to understand the relationship between inactivation of tumor suppressor genes and oral carcinogenesis. However, the predominant probe used in the detection of the transcription of HPV-16 E6/E7 genes was 1.2-kbp fragment (nucleotides 24-880, 3357-3820) representing the major early HPV-16 message including E6/E7 genes, and part of noncoding region, E1, E2, and E4 genes (Woodworth *et al.*, 1989; Park *et al.*, 1995). These data indicate that 0.57-kbp HPV-16 E6/E7 gene fragment can be detect more specifically HPV-16-specific transcripts encoding for HPV-16 E6/E7 genes than the predominant 1.2-kbp HPV-16 probe.

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