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☐ Original Article ☐

Expression and Localization of Human Papillomavirus Type 16 E6 and E7 Open Reading Frame Proteins in Human Epidermal Keratinocyte

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Over 60 different types of human papillomavirus (HPV) have been identified, and they are classified into high and low risk groups based on the risk for malignant progression of HPV associated lesions. HPVs belonging to a high risk group have been shown to express two major transforming proteins, E6 and E7. With respect to the transforming activity of these proteins, many investigators have reported the location of these proteins in the cell, but their results are still controversial. In the present study, HPV type 16 E6 or E7 open reading frame (ORF) proteins were expressed and localized in human epidermal keratinocytes (RHEK-1) using the vaccinia virus as an expression vector. Immunofluorescence detection using monoclonal antibodies against E6 or E7 ORF proteins revealed that E6 or E7 proteins of HPV type 16 were located in the cytoplasm of RHEK-1 cells. These results suggest that E6 and E7 proteins bind to the tumor suppressor counterparts, thereby preventing transport of these proteins into the nucleus. These antioncogene products that fail to be rapidly transported out of the cytosol may be degraded by certain proteases such as the ubiquitin dependent system. In this way, the precise function of antioncogene products in the regulation of cell growth could be destroyed, and abnormal cell growth could occur.

Key Words: Human papillomavirus type 16 E6 and E7 ORF proteins, vaccinia virus expression system

Among the 60 different types of human papillomaviruses (HPVs) which have been isolated thus far from a variety of squamous epithelial lesions, eighteen types have been shown to be associated with anogenital tract lesions (deVilliers 1989). Some of these, such as HPV 6 and HPV 11, are associated with benign proliferative tumors (e.g., condyloma acuminatum) which have a low risk for malignant progression, whereas others, such as HPV16, HPV18, HPV31, HPV33, and HPV35 are associated with potentially precancerous genital tract lesions, and with a high percentage of anogenital cancers (zur Hausen and Schneider 1987). The cloned DNA of these HPV types which are associated with lesions that have a high risk for malignant progression encode cellular transformation properties in established rodent fibroblasts (Yasumoto *et al.* 1986), in primary rodent cells (Matlashewski *et al.* 1986; Phelps *et al.* 1988; Storey *et al.* 1988), and in primary human cells (Dürst *et al.* 1987; Pirisi *et al.* 1987; Schlegel *et al.* 1988). In cervical carcinomas and in cell lines derived from cervical carcinomas, the E6 and E7 open reading frames (ORFs) of the high risk HPVs are found to be intact and actively transcribed, implicating the involve-

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ment of E6 and E7 genes in the malignant phenotype (Backer 1988). It has been shown that E6 and E7 proteins can bind to p53 and p105RB tumor suppressor gene product, respectively (Dyson *et al.* 1989; Münger *et al.* 1989; Werness *et al.* 1990), although the role of these viral proteins in the oncogenesis at the molecular level is not clearly understood. Recently, it was suggested that inactivation of p53 and p105RB by viral proteins or by mutations plays a key role in the oncogenesis of cervical carcinoma (Scheffner *et al.* 1991).

In the present study, expression of E6 and E7 proteins of HPV in RHEK-1 human epidermal keratinocytes were carried out by the vaccinia virus expression system, and the localization of these proteins was determined in order to understand the possible intracellular interaction between these proteins and other cellular macromolecules such as p53 or p105 RB.

MATERIALS AND METHODS

Reagents

Analytical grade reagents and materials were obtained from the following sources: Mouse anti-E6 ORF protein monoclonal antibody (Ab) was purchased from Oncogene Science, Uniondale, NY, USA. Mouse anti-E7 ORF protein monoclonal Ab was obtained from Triton Diagnostics, Alameda, NY, USA; and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG Ab was obtained from Zymed Laboratories, Inc., San Francisco, CA, USA. Restriction enzymes, Taq DNA polymerase and other molecular biology grade reagents were purchased from Boehringer Mannheim GmbH Biochemica, Mannheim, Germany. Culture dishes and plates were purchased from Costar, Cambridge, MA, USA. Other chemicals were obtained from Sigma Chem. Co., St Louis, USA.

Cells and plasmid

CV-1, monkey kidney epithelial cells were grown in Dulbecco's modified Eagles media (DMEM) containing 10% fetal calf serum (FCS), 100units/mL penicillin and 100 μg/mL streptomycin. Hu-TK$^-$ 143B, human osteogenic sarcoma cells that have a thymidine kinase negative phenotype, were grown in a minimal essential media (MEM) containing 10 % FCS, 25 μg/mL bromodeoxyuridine (BUdR), 100 units/mL penicillin, and 100 μg/mL streptomycin. RHEK-1, human epidermal keratinocytes were grown in DMEM containing 10% FCS, 5 μg/mL hydrocortisone, 100units/mL penicillin, and 100 μg/mL streptomycin (Rhim *et al.* 1984).

The pSC11 plasmid (Chakrabarti *et al.* 1985) is a vaccinia virus transfer vector. It contains a unique SmaI cloning site which is immediately downstream from the vaccinia virus early/late p7.5 promoter. It is flanked by sequences from the vaccinia virus thymidine kinase which direct the insertion of the foreign gene into the non essential thymidine kinase gene of the vaccinia virus. It also expresses β-galactosidase, which can be used to select recombinant viruses.

Amplification of E6 and E7 ORF DNA fragments

To subclone the E6 and E7 ORF DNA fragments into the pSC11 vaccinia virus transfer vector, E6 ORF (HPV 16 nucleotide (nt) 83-563) and E7 ORF (HPV 16 nt 562-874) DNA fragments were obtained by the polymerase chain reaction (PCR) technique. The primers for PCR were as follows. The primers for E6 were 5′-CTC*GGATCC*ATG CACCAAAAGAG-3′ (nt 83-96) and 5′-CTC *GGATCCAAGCTT*ATGATTACAGCT-3′ (nt 552-563) and the primers for E7 were 5′-CTC *GGATCC*ATGCATGGAGATACA-3′ (nt 562-576) and 5′-CTC*GGATCCAAGCTT*GAT CAGCCA-3′ (nt 866-874), respectively. Each upstream and down stream primer contained a BamHI recognition site (*GGATCC*) at its 5′ ends, and the HindIII recognition site (*AAGCTT*) was additionally located at 3′ to the BamHI site in the downstream primer. Forty nanograms of cloned HPV type 16 DNA was used as a template. Typical optimal PCR conditions were: primers, 50 pmoles each; Taq DNA polymerase, 2.5 units; 10x PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 20 mM $MgCl_2$, 0.01% (w/v) gelatin) 5 μL; each dNTP, 200 μM, and H_2O in a total volume of 50 μL. Samples were overlaid with mineral oil. Each cycle of PCR consisted of 30 seconds of template denaturation at 94° C, 40 seconds of annealing at 60°C, and 40

seconds of extension at 72℃. The cycle was repeated for 30 times and final extension was kept for 10 minutes at 72℃.

Construction of recombinant plasmid

The pSC11 vector was cleaved at nt 6502 with SmaI, and the PCR amplified E6 or E7 ORF DNAs were inserted. For blunt end ligation, PCR amplified E6 or E7 ORF DNA fragments were digested with BamHI and then filled-in 3′-recessive ends with an *E.coli* Klenow fragment.

Selection of recombinant vaccinia virus

Recombinant viruses were selected for thymidine-kinase negative phenotype and for expression of β-galactosidase. CV-1 cells infected with the wild type vaccinia virus were transfected with 1 μg of each recombinant plasmid DNA by using calcium phosphate. TK^- recombinants were isolated by a plaque assay on Hu-TK^-143 B cells in the presence of BUdR (25 μg/mL) and 300 μg/mL X-gal in the agarose overlay. Plaques were examined after four to six hours incubation at 37℃. Plaques were then purified three times in succession.

Intracellular localization of E6 or E7 ORF protein

RHEK-1 cells were plated onto eight well culture slides, infected at a multiplicity of approximately four plaque forming unit (pfu) /cell with the E6 or E7 recombinant vaccinia virus or wild type vaccinia virus. Twelve hours post infection, cells were fixed for 30

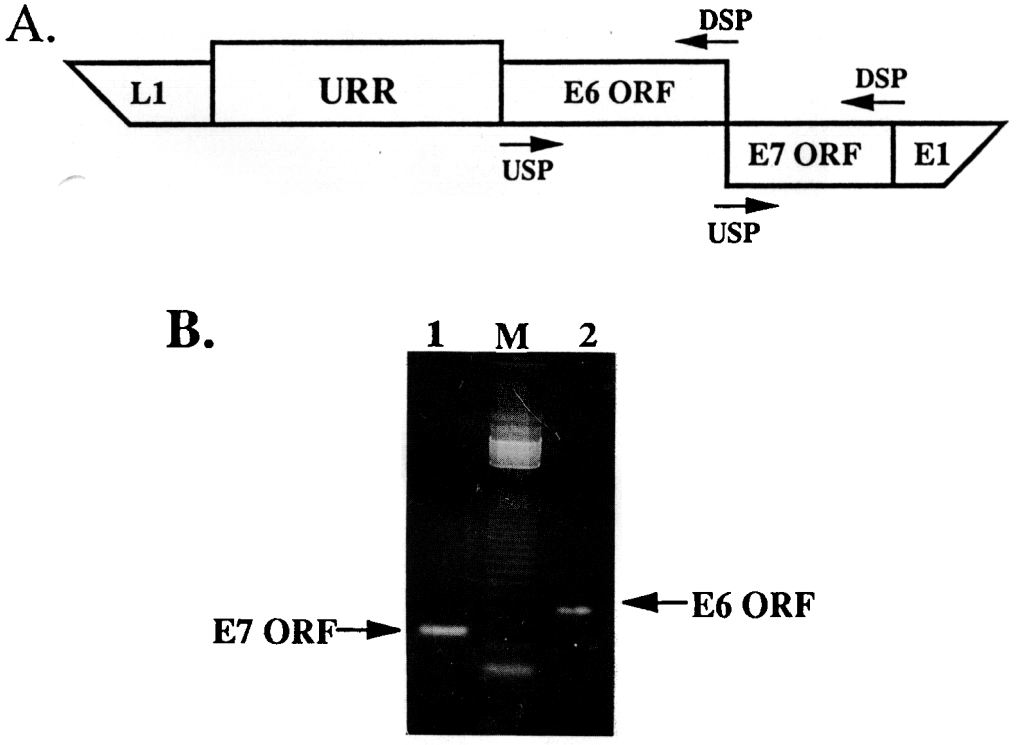

Fig. 1. *PCR amplification of HPV type 16 E6 and E7 ORF DNA fragments.*
A. Schematic diagram of PCR amplification for E6 and E7 ORF URR denotes upstream regulatory region of HPV type 16.
B. Agarose gel(1%) electrophoresis of PCR products. 505 bp size amplified E6 ORF DNA(lane 1) and 337 bp size amplified E7 ORF DNA (lane 2) DNA fragments are indicated. Lane M represents 123 bp ladder DNA size marker.

minutes in paraformaldehyde fixative, washed 3 times in PBS and incubated in 50 μL of 1:100 dilution of mouse anti-E6 ORF protein monoclonal Ab or mouse anti-E7 ORF protein monoclonal Ab for 1 hour at 25℃. Culture slides were then washed three times in PBS and incubated in FITC-conjugated goat anti-mouse IgG Ab for 30 minutes in a darkroom and washed another three times in PBS before viewing.

RESULTS

PCR amplification of HPV type 16 E6 and E7 ORF DNA

After PCR, agarose gel (1%) electrophoresis was performed to identify amplified E6 or E7 ORF DNA. E6 ORF DNA had about 500bp and E7 DNA had about 330 bp that were nearly the same size as calculated from sequence data(Seedorf *et al.* 1985) (Fig. 1).

Construction of recombinant plasmid

Blunt end ligated DNA was used for transformation of DH5 *E.coli* competent cells and plasmid was isolated from each transformant using the miniprep plasmid isolation method (Sambrook *et al.* 1989). Isolated plasmid DNA was digested with a HindIII restriction enzyme for analysis. HindIII digestion analysis revealed that E6 or E7 ORF DNA was inserted in pSC11 vector with correct orientation (Fig. 2) and named as pSC11-E6 and pSC11-E7, respectively.

Selection of recombinant vaccinia virus

CV-1 cells infected with the wild type vaccinia virus were transfected with calcium phosphate precipitated pSC11-E6 or pSC11-E7. Blue plaques made by homologous recombination between vaccinia viral DNA and transfected recombinant plasmid were identified (Fig. 3) and then through two successive

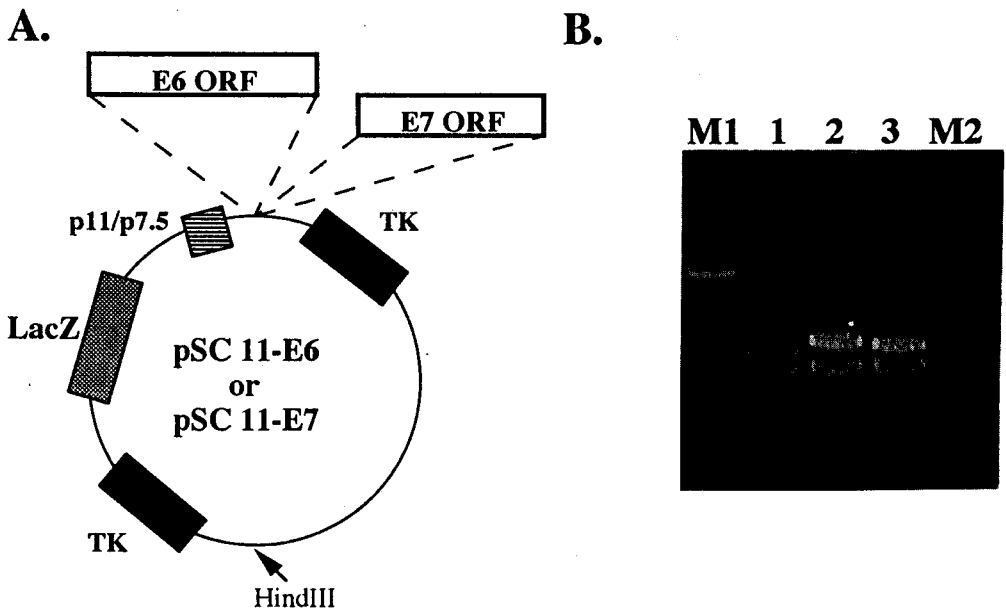

Fig. 2. *Subcloning of amplified E6 and E7 ORF DNA into pSCII vaccinia virus transfer vector.*
A. Construction and restriction map of pSC11-E6 and pSC11-E7.
B. Agarose gel(1%) electrophoresis of HindIII digested pSC11-E6 and pSC11-E7. Lane M1 and M2 represent HindIII digested DNA size marker and 1kb ladder DNA size marker, respectively. Lanes 1, 2, and 3 contain HindIII digested pSC11, pSC11 E6, and pSC11-E7, respectively.

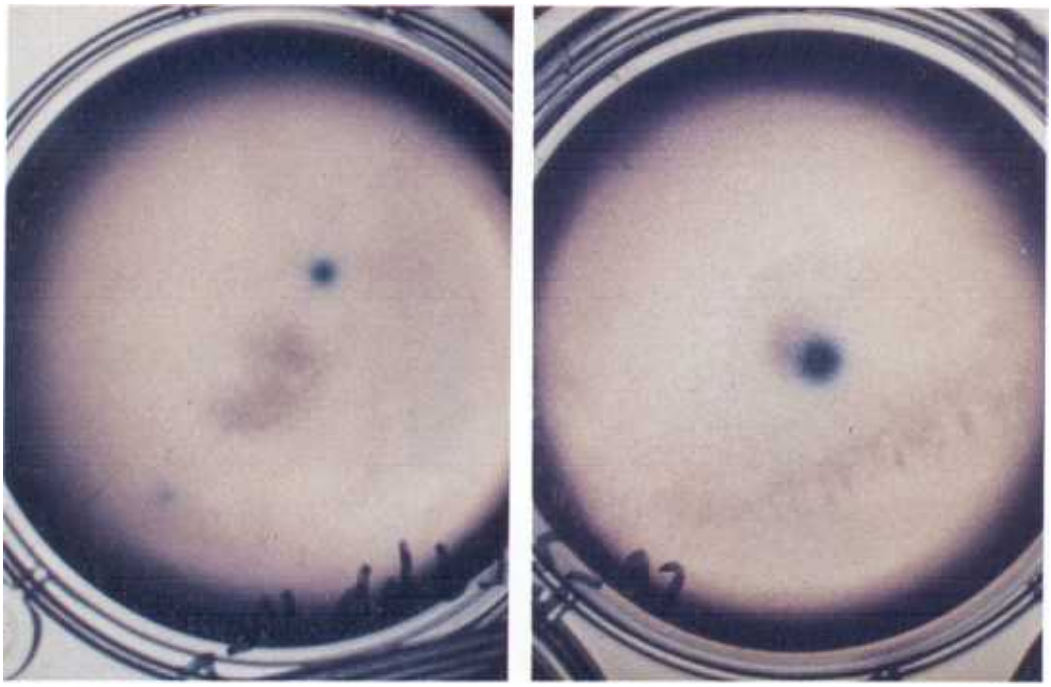

Fig. 3. *Recombinant vaccinia virus plaque isolation.*
CV-1 cells infected with wild type vaccima virus were transfected with calcium phosphate precipitated pSC11-E6 or pSC11-E7. VV-E6(A), VV-E7 (B) recombinants were identified by a plaque assay on Hu. TK⁻ 143 B cells in the presence of BUdR and X-Gal.

plaque purifications, purified plaques were obtained. The recombinant vaccinia virus isolated from purified plaques were designated as VV-E6 and VV-E7, respectively. The titer of each recombinant vaccinia virus was determined as 3.6×10^9 pfu/mL for VV-E6, and 1.8×10^9 pfu/mL for VV-E7.

Indirect immunofluorescence staining

VV-E6 or VV-E7 infected RHEK-1 cells were immunostained with mouse anti-E6 or anti-E7 ORF monoclonal Ab followed by FITC-conjugated goat anti-mouse IgG Ab. Immunostained cells were observed with an indirect immunofluorescence microscope. Indirect immunofluorescence microscopy revealed that fluorescence signals by E6 and E7 ORF proteins of HPV type 16 were observed only in the cytoplasm of the RHEK-1 cells and all nuclei were spared(Fig. 4).

DISCUSSION

The major conclusion from the above results is that E6 or E7 proteins of HPV type 16 were located in the cytoplasm of RHEK-1 cells. Smotkin and Wettstein (1986) have shown that the E7 protein is a cytoplasmic phosphoprotein and has a half life ranging between 55 and 70 minutes (Barbosa *et al.* 1989). Sato *et al.* (1989) also have shown that E7 protein expressed from a pSV2-E7, a SV40 based expression plasmid containing the E7 gene of HPV 16 (nt 509-875), was localized at the cytoplasm in monkey COS-1 cells by immunoprecipitation, but immunofluorescence staining showed that the E7 protein was also detected in the nuclei of the transfected COS-1 cells. They suggested that the release of the E7 protein from nuclei

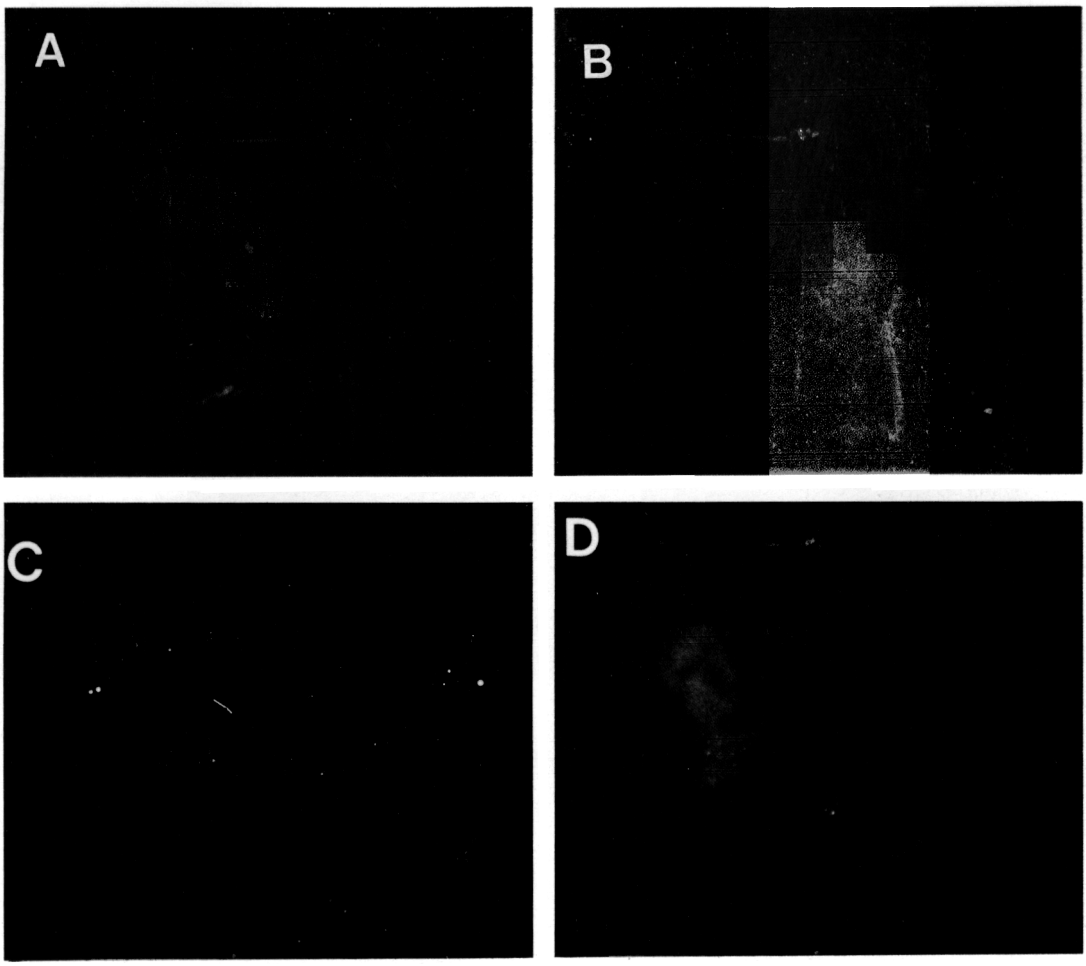

Fig. 4. *Indirect immunofluorescence of VV-E6 or VV-E7 infected RHEK-1 cells.*
RHEK-1 cells were cultured in 8 well culture slides. Cells were infected at a multiplicity of 4 pfu/cell with VV-E6 or VV-E7 recombinant vaccinia viruses fixed 12 hours post infection. The subcellular location of E6 or E7 protein was then detected by indirect immunofluorescence using mouse anti E6 ORF protein monoclonal antibody(Ab) or mouse anti E7 ORF protein monoclonal Ab and FITC conjugated goat anti-mouse IgG Ab as secondary Ab. (A) represents immunostained RHEK-1 cells infected with wild type vaccinia virus, and (B) represents immunostained RHEK-1 cells infected with pSC-11 recombinant vaccinia virus, (C) and (D) represent immunostained RHEK-1 cells infected with VV-E6 or VV-E7, respectively, (All cases are stained with respective Abs for control, but here we showed representative figures.)

might occur when the cell structure is broken.

In the case of the E6 protein, immunoprecipitation with monoclonal Ab raised against C-terminal polypeptide (23 amino acids (Aas)) of HPV 18 E6 ORF protein revealed that the E6 protein was located in the nucleus of the cell (Scheffner *et al.* 1990). When the E6 protein was synthesized in insect cells infected with E6 expressing baculovirus, the protein was localized in both the nuclear and the membrane fractions, with half lives of four and two hours, respectively, and changing the first five Aas of E6 did not alter the pattern of cellular localization of the protein (Grossman *et al.* 1989). Kanda *et al.* (1988)

constructed eukaryotic expression plasmid pSR α-E6 containing some sequences of the non-coding region and E6 ORF (HPV 16 nt 25 to 657). The E6 protein expressed in monkey COS-1 cells was detected primarily in the nucleus by immunofluorescence staining. However, the subcellular localization of E6 proteins was distinguished in the membrane and in nuclear fractions but not in the cytoplasmic fraction by immunoprecipitation.

Monoclonal Abs used in this experiment had the following characteristics: Ab to E6 ORF protein recognized epitope in the E6 protein but its precise location has not been identified, and Ab for the E7 ORF protein recognized the epitope composed of the N-terminal 20 Aa. Tindle *et al.* (1990) reported that there were three linear epitopes in the E7 protein composed of Aa 10-14, Aa 38-41, and Aa 39-54. Kanda *et al.* (1991) also produced monoclonal antibodies that recognized Aa 8-22 (region I) and Aa 39-54 (region II) epitopes of the HPV E7 protein and used them for immunofluorescence staining to detect the E7 protein in COS-1 cells. In this experiment, they could not observe the fluorescence signal when the cells were fixed with acetone. However, the E7 protein was observed in the nuclear and cytoplasmic fractions of the cells when treated with formaldehyde. These results suggested that E7 proteins present in the nucleus of COS-1 cells might not have been detected because the epitopes recognized by these monoclonal Abs were not exposed. This may have occured because E7 proteins formed complexes with other cellular proteins, polymerized with each other, or folded into themselves.

Other DNA tumor viruses such as Adenovirus (Ad) and Simianvirus (SV) 40 encode similar transforming proteins, Ad E1A and SV 40 large T Ag, respectively. These proteins are located in the nucleus and phosphorylated (Graham 1984; Livingston and Bradly 1987). By analogy, E6 or E7 proteins encoded by HPV were expected to be located in the nucleus, but our results revealed that E6 and E7 proteins of HPV 16 were present only in the cytoplasm. These observations might result from the masking of epitopes present in the E6 or E7 proteins following their interaction with other proteins. Thus, it is not possible to conclude the true subcellular location of the E6 or E7 proteins based on our results alone. To support these conclusions, it could be necessary to rule out the possible nuclear location of E6 or E7 protein by using pooled monoclonal Abs or polyclonal Ab against the E6 or E7 protein. Taken together with the fact that E6 or E7 proteins bind to p53 or p105 RB tumor suppressor proteins *in vitro*, respectively, the following hypothesis can be considered. Namely, E6 and E7 proteins are present in the cytoplasm and bind to the tumor suppressor counterparts, thereby preventing transport of these proteins into the nucleus. These antioncogene products that fail to be rapidly transported out of the cytosol may be degraded by certain proteases such as the ubiquitin dependent system. In this way, the precise function of antioncogene products in the regulation of cell growth could be destroyed, and abnormal cell growth could occur. To support this hypothesis, further study is required.

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