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Expression of Human Papillomavirus Type 16, Prototype and Natural Variant E7 Proteins using Baculovirus Expression System

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=Abstract=

Human papillomavirus (HPV) 16, E7 proteins derived from the prototype (Bac73) and natural variant (Bac101) E7 open reading frame were produced in Sf9 insect cells. The variant E7 gene occurred naturally by substitution mutation at the position of 88 nucleotide, resulting serine instead of asparagine. Using E7 specific monoclonal antibody (VD6), both E7 proteins were identified in recombinant baculovirus infected SF9 cells. Radiolabelling and immunoprecipitation analysis revealed that both E7 proteins were phosphoproteins. Immunostaining result showed that E7 proteins were mainly localized in the cytoplasm. Nuclear form of E7 proteins was also detected after a sequential fractionation procedure for removing chromatin structure. Considering that the VD6 recognition site in E7 protein is located within 10 amino acid at the N-terminus, this region appears to be blocked by the nuclear component. Western blot analysis revealed that nuclear form was more abundant than cytoplasmic E7 proteins. Time course immunostaining showed that the primary location of E7 protein was the nucleus and exported to the cytoplasm as proteins were accumulated. These events occurred similarly in both Bac73 and Bac101 infected Sf9 cells, suggesting that these two proteins may have similar biological functions.

Key Words: Human papillomavirus type 16 (HPV-16), Prototype and natural variant E7, Expression, Subcellular localization

INTRODUCTION

More than 70 types of human papillomavirus (HPV) have been identified to date and are closely associated with benign and malignant lesions of mucocutaneous epithelia [1]. Among them, over the 20 genotypes of HPV have been found to infect genitalia and are classified as one of two groups (high or low risk) depending upon oncogenic potential [2, 3]. Especially, human papillomavirus type 16 (HPV 16) is the most frequently detected type in cervical cancer specimens [3]. Among HPV derived proteins, the E7 protein has been known as the major oncogenic protein with strong transforming potential [4]. Recently, it was reported that the E7 natural variant which had the substitution mutation at 29th amino acid residue (asparagine to serine) was more

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frequently found in HPV 16 infected cervical cancer than prototype E7 gene [5]. Considering that detection rate of variant was correlated with disease progression and the mutated serine could be phosphorylated, natural occurrence of this mutation could have some biological significance.

To study biological features of E7 natural variant, we produced baculovirus-derived HPV 16, prototype and variant E7 proteins. Many viral proteins produced in insect cells using baculovirus vectors exhibit authentic post-translational modification, such as glycosylation, phosphorylation and acylation and are targeted to the correct cellular location [6]. In this report, the phosphorylation status and subcellular localization of prototype and variant baculoviral expressed E7 proteins were studied.

MATERIALS AND METHODS

Construction of E7 expression plasmids, transfection and isolation of recombinant baculovirus

DNAs extraction from cervix cancer specimens and the nucleotide sequence analysis after the double nested PCR were previously described [5]. Among the analyzed E7 gene sequences, the prototype and variant gene which had the mutated amino acid sequence (29th Asp to Ser) were re-amplified using oligonucleotide primer E7-USP (5'-CTC GGATCC ATGCA TGGAG ATACA-3') and E73end (5'-TTATG GTTTC TGAGA CAGA-3'). The nucleotide sequence of E7-USP had the attached restriction enzyme recognition sequence (*Bam*H1) for the directional cloning. This PCR product was cloned using T-vector modified from pBluescript SK(-) [7]. After the direction and nucleotide sequence of cloned gene was confirmed, the insert DNA was isolated using *Bam*H1 and *Eco*RI digestion, and this DNA was inserted into pVL1393 baculovirus transfer vector. The final clones, p73 had a prototype amino acids sequence and p101 had a substitution mutation in Asn-29 to Ser-29 in E7 ORF.

The co-transfection into *Spodoptera frugiperda* (Sf9) cell with *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA and recombinant transfer plasmid DNA and selection of recombinant baculovirus were performed as previously described [8]. The Sf9 insect cells were grown in Grace's insect tissue culture medium (GIBCO BRL, USA) supplemented with 10% fetal bovine sera at 27℃. Infection and assay in Sf9 cells were performed according to standard methods [8, 9].

Western blot analysis

Cell lysates of Sf9 cells or CaSki cells (ATCC No. CRL 1550) were separated by 15% SDS-PAGE, transferred to nitrocellulose membranes, and stained with VD6 antibody as previously described [10]. The antibody-reactive bands were visualized by using chemiluminescence substrates (ECL, Amersham, UK) with appropriate secondary antibodies. For the preparation of crude cytosolic and nuclear extracts, Sf9 cells were harvested using hypotonic buffer (10 mM Tris [pH7.5], 1.5 mM $MgCl_2$, 10 mM NaCl, 300 mM sucrose) and cell membranes were disrupted by Dounce homogenization. The supernatants obtained by low speed centrifugation (750 g) were subjected to high speed centrifugation (10,000 g) to separate soluble crude cytosolic fraction and insoluble fraction. The pellets obtained by low speed centrifugation were resuspended using high salt buffer (1% Nonidet P-40, 10 mM Tris [pH7.5], 1.5 mM $MgCl_2$, 500 mM NaCl), sonicated and fractionated by centrifugation into nuclear soluble and insoluble fractions.

Phospho-labelling and Immunoprecipitation assay

The phosphate labelling and immunoprecipitation was performed as previously described [11] with some modifications. The Sf9 cells in 25 cm^2 flasks were incubated in serum free

Table 1. Oligonucleotide primers used and plasmid construction

Primers	Sequences of primers (5' to 3')	Use
	1* 12	
E7-USP	TC GGA TCC ATG CAT GGA ACA	forward primer
	297 278	
E73end	TTA TGG TTT CTG AGA ACA GA	reverse primer
	1 3 31 46	
E7d2-10	CA GGA TCC ATG TAT ATG TTA GAT TTT A	forward primer
	1 3 61 74	
E7d2-20	AG GGA TCC ATG GAT CTC TAC TGT TA	forward primer
	19 30 61 72	
E7d11-20	ACA TTG CAT GAA GAT CTC TAC TGT	forward primer
	1 30	
E7-N30	ATG CAT GGA GAT ACA CCT ACA TTG CAT GAA	forward primer
	13 21 46 57	
E7d8-15	ACA CCT ACA CAA CCA GAG ACA	forward primer
	1 21	
E7-N21	ATG CAT GGA GAT ACA CCT ACA	forward primer

Plasmid construction

Plasmid	Mutant gene production**	Characteristics
pE7d2-10	PCR using E7d2-10 and E73end	deletion from 2nd His to 10th Glu
pE7d2-20	PCR using E7d2-20 and E73end	deletion from 2nd His to 20th Thr
pE7d11-20	1st PCR using E7d11-20 and E73end 2nd PCR using E7-N30 and E73end 3rd PCR usng E7-USP and E73end	deletion from 11th Tyr to 20th Thr
pE7d8-15	1st PCR using E7d8-15 and E73end 2nd PCR using E7-N21 and E73end 3rd PCR using E7-USP and E73end	deletion from 8th Leu to 15th Leu

*: Numericals mean the nucleotide number of E7 open reading frame
**: The final PCR products were cloned into pMalc-2 plasmid as described in Materials and Methods section
The underlined sequences were the *Bam*HI recognition sequence

media containing 200 μCi orthophosphate (NEN, USA) for one hour without phosphate starvation step. The labelled cells were washed with phosphate buffered saline (PBS) and harvested in RIPA buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS), and centrifuged. The supernatant was precleared with normal mouse sera and then mixed with VD6 (5 μg). Immune complexes were precipitated with protein A-agarose (Bio-Rad, USA). After washing five times with RIPA buffer, these immunoprecipitates were subjected to SDS-PAGE and then to autoradiography or Western blot analysis.

Immunostaining

Infected Sf9 cells on cover slip were washed with PBS twice, fixed with 4% paraformaldehyde, permeabilized with 0.5% triton X-100 in PBS, and then subjected to reaction with primary antibodies, VD6. After subsequent washing, the Sf9 cells were reacted with FITC or peroxidase-conjugated anti mouse secondary antibody and observed with fluorescence microscope (Zeiss, Germany) or light microscope after color development using DAB substrate. In some cases, for removal of chromatin structure in nucleus, a sequential fractionation pro-

cedures [12] were performed prior to immunostaining. Briefly the cultured Sf9 cells were sequentially treated with 0.5% Triton X-100, 0.5% Tween 20 / 1% deoxycholate, DNase I, 0.25 M ammonium sulfate and 2 M NaCl for 10 min. except Triton X-100 treatment (for 2 min.) at room temperature. All chemicals were dissolved in PBS. Then the Sf9 cells were fixed with 4% paraformaldehyde and immunostained.

Epitope mapping

For the precise mapping of antigenic site in E7 protein for monoclonal antibody VD6, four deletion mutant plasmids (p5d2-10, p5d2-20, p5d11-20, and p5d8-15) were constructed using pMalc2 bacterial expression vector and expressed as maltose bining protein (MBP) fusion form. These deleted E7 genes were obtained from p73 plasmid by PCR amplification using deletion primers (Table 1). The cloning of these deleted E7 gene were performed using the same method for construction of p73 except that the *PstI* site instead of *Eco*RI in pBluescript vector was used for 3' cohesive end generation. The p5d2-10, p5d2-20, p5d11-20 and p5d8-15 plasmids had E7 ORF deleted from 2nd His to 10th Glu, from 2nd His to 20th Thr, from 11th Tyr to 20th Thr, and from 8th Leu to 15th Leu respectively. The expression and purification of these E7 mutant proteins were performed as described previously [13]. The reactivities of these mutants proteins with VD6 antibody were analyzed by Western blot.

RESULTS

Expression of E7 proteins in Sf9 cells

Among the previously analyzed E7 gene from cervical cancer specimens [5], the prototype and 29th Asp to Ser mutated variant E7 open reading frame (ORF) were cloned into pVL1393 baculovirus transfer vector (Fig. 1A) and recombinant baculoviruses expressing E7 proteins were generated by co-transfection of E7 recombinant plasmid DNA and baculovirus genomic DNA (AcNPV) into Sf9 cells. The cell lysates of Sf9 cells infected with pro-

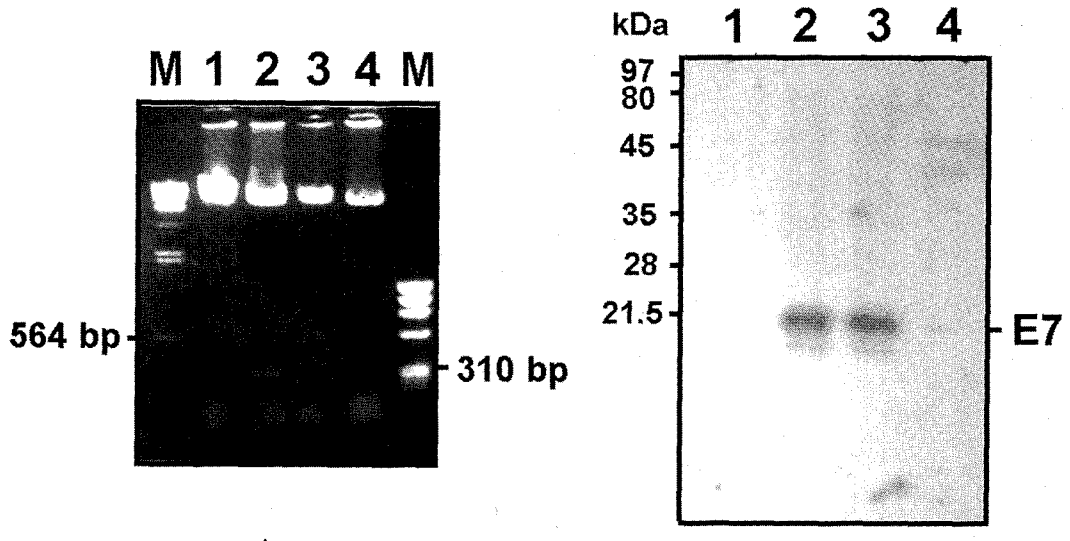

Fig. 1. Agarose gel electrophosresis of E7 baculovirus expression plasmids, p73 and p101, **(A)** and Western blot analysis of Sf9 cell lysates infected by Bac73 and Bac101 **(B)**. A, lane 1: p73 digested with *Bam*HI, lane 2: digested with *Bam*HI and *Eco*RI, lane 3: p101 digested with *Bam*HI, lane 4: digested with *Bam*HI and *Eco*RI. M lanes were molecular standards. Marker at left side was λ/*Hin*dIII digests (BRL, USA) and marker at right side was ϕX174 RF/*Hae* III digests (promega, USA).

totype and variant E7-recombinant baculovirus (Bac73 and Bac101) were analyzed by Western blot using HPV16-E7 specific monoclonal antibody, VD6 [13]. The specific recognition of both E7 proteins in lysates could be observed (Fig. 1B). Comparing with the reactivity to E7 protein expressed in CaSki cells which had the more than 500 copies of HPV genomes in its chromosome, infected Sf9 cells abundantly expressed E7 proteins.

Post-translational modification of E7 proteins

To assess whether baculoviral expressed E7 proteins had the similar features to native state E7 protein, the phosphorylation status of E7 protein was analyzed. E7 protein has been known to be phosphorylated at 31, 32th serine residues [14]. The Sf9 cells were metabolically labelled using radio active [^{32}P] orthophosphate at day 1, 2, 3, 4, 5 post-infection and the cell lysates were immunoprecipitated using VD6 antibody and protein A-agarose. The specific VD6 reactive phosphoprotein could be found in both Bac73 and Bac101 infected cells but not in mock or wild type virus infected cells (Fig. 2). This results showed that both E7 proteins were authentically modified similar to native state E7 protein.

Subcellular localization of E7 proteins

Since E7 protein interacts with nuclear pro-

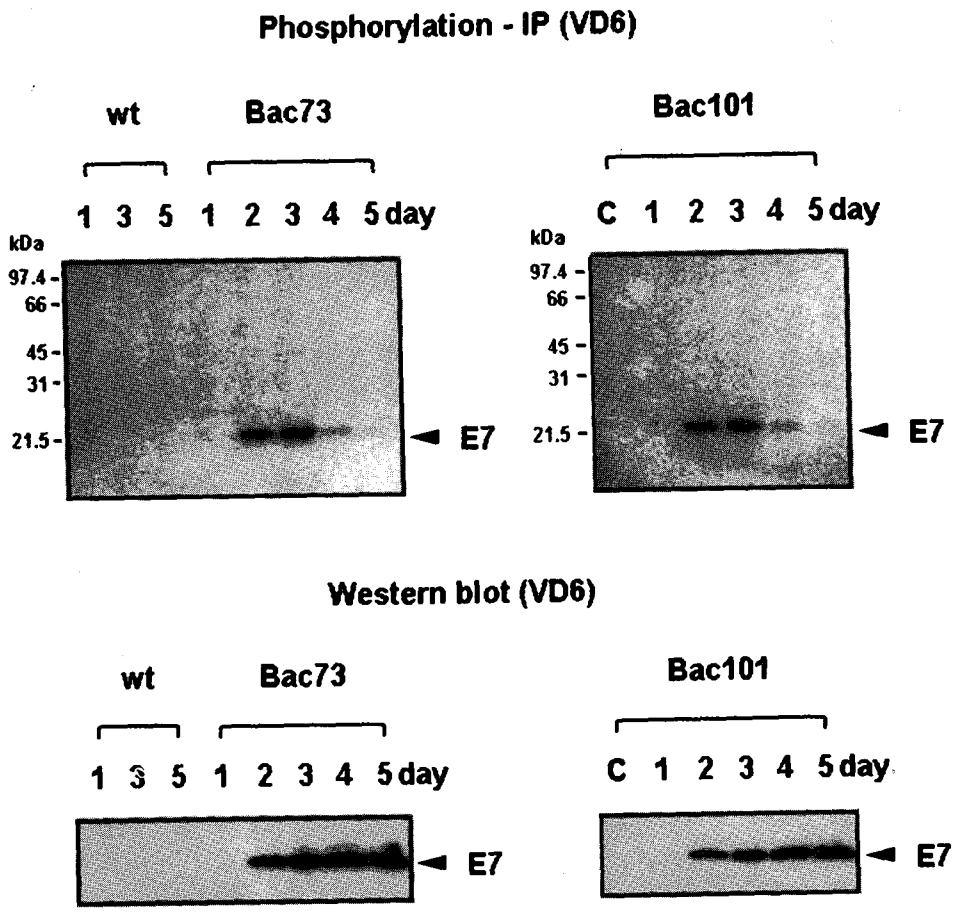

Fig. 2. Phosphate labelling and immunoprecipitation assay (**A**) and Western blot analysis of immunoprecipitated E7 proteins (**B**). Sf9 cell infected by wild type AcNPV (wt), by Bac73, and by Bac101. Control lane in Bac101 means that Sf9 cell lysate did not infected any virus (C; control).

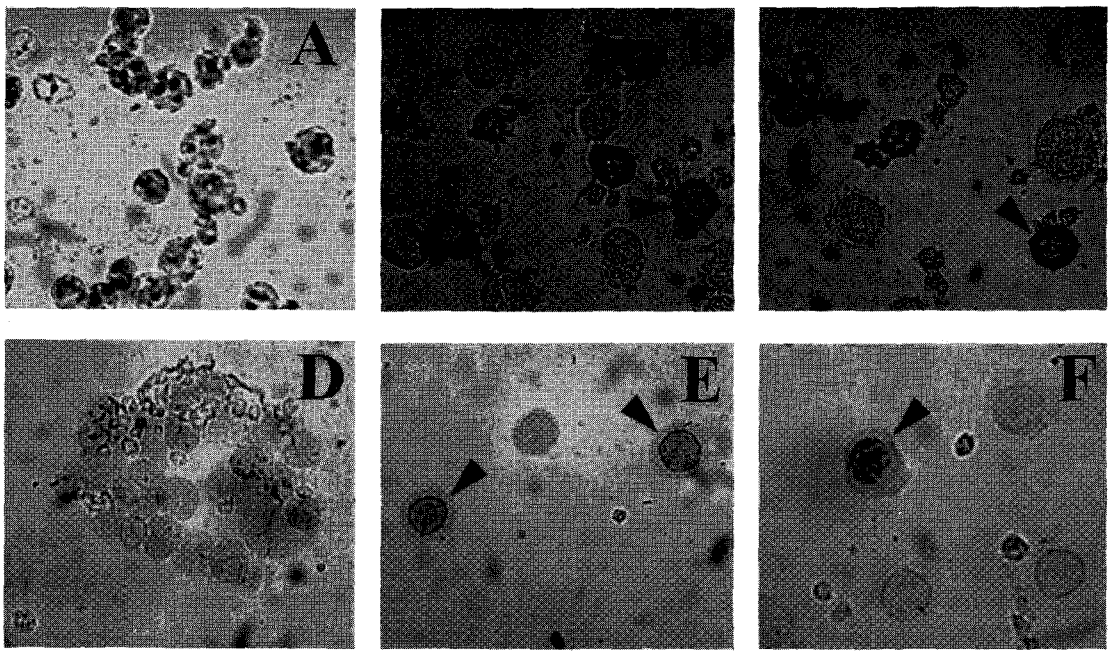

Fig. 3. Immunoperoxidase (IP) staining of Sf9 cells infected by wild type virus (A, D), Bac73 (B, E), and Bac101 (C, F). **A**, **B**, and **C** were the IP stained Sf9 cells after convential paraformaldehyde fixation. **D**, **E**, and **F** were the IP stained Sf9 cells after sequential fractionation procedure.

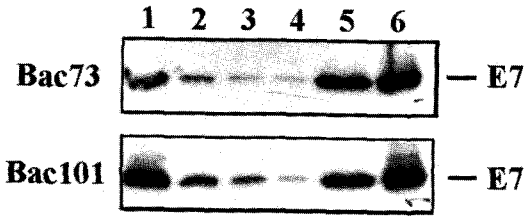

Fig. 4. Western blot analysis of crude cytosolic and nuclear extracts of Sf9 cells at day 3 post-infection. Lane 1, were the whole cell extracts. Suspension of Sf9 cells in hypotonic solution was subjected to Dounce homogenization and low speed centrifugation. The supernatant (lane 2-4) and pellet (lane 5-6) were collected. This supernatant was further centrifuged at high speed and lane 2 was supernatant (crude cytosolic extracts), lane 3 was washout solution of insoluble pellet, and lane 4 was insoluble pellet. lane 5 was crude nuclear soluble extracts in hypertonic solution and lane 6 was insoluble nuclear extracts.

tein p105 Rb [15, 16] and has transactivating activity [17], it could be assumed that E7 protein may be localized in nucleus. But results from the immunofluorescence (IF) and immunoperoxidase (IP) staining of Bac73 or Bac101 infected Sf9 cells at day 3 post-infection showed that the majority of E7 protein was expressed in the cytoplasm (Fig. 3 B, C). To investigate whether the nuclear form of E7 protein exist or not, the cytosolic and nuclear chromatin structure were removed by sequential fractionation procedures [12] prior to IP staining. This result revealed that abundant E7 proteins were localized in nucleus (Fig. 3 E, F). Subsequently, the amount of E7 protein in cytoplasm and nucleus was quantitated by Western blot analysis using crude cytosolic and nuclear extracts and we found that about five times more E7 protein was observed in nucleus than in cytoplasm (Fig. 4). We found no difference in subcellular localization of prototype and variant E7 proteins.

Time course of Immunostaining of E7 proteins

To analyze the primary localization of E7 proteins, the recombinant baculovirus infected Sf9 cells were immunostained at days 1, 2, 3

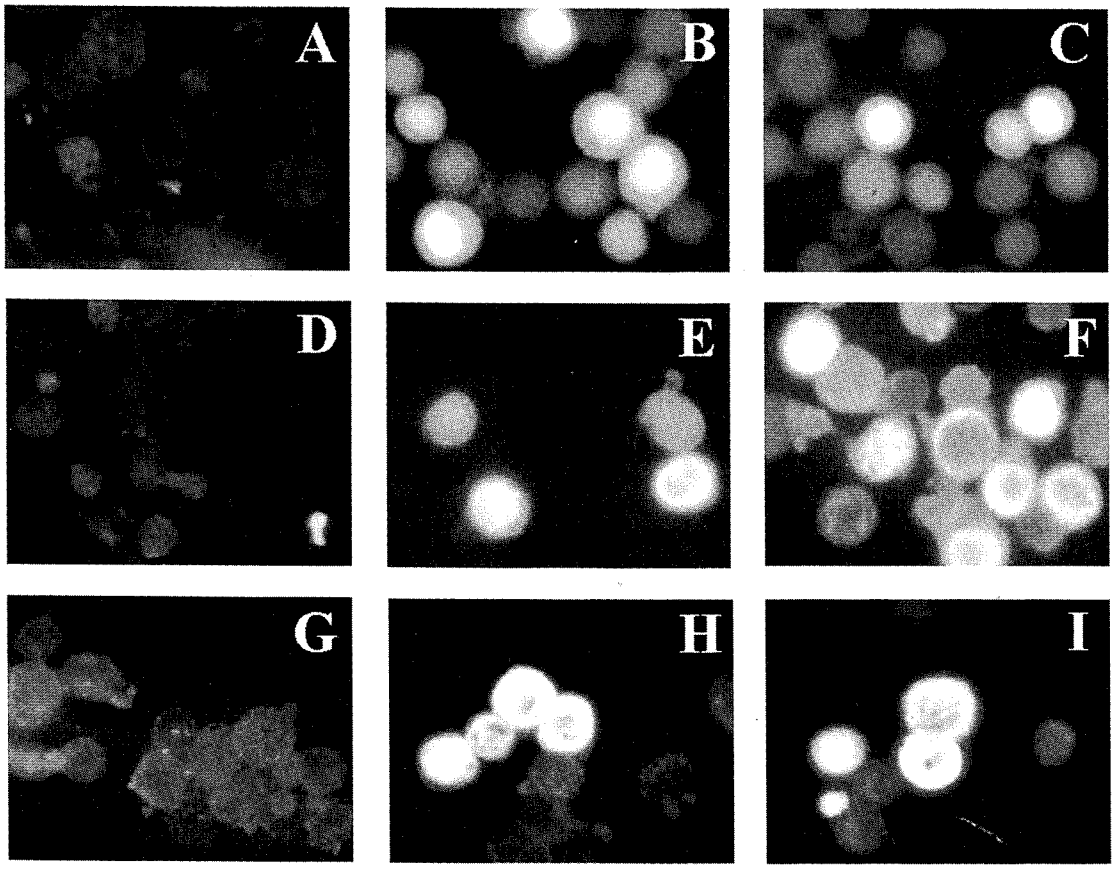

Fig. 5. Immunofluorescence staining of Sf9 cells infected by wild type virus (A, D, G), Bac73 (B, E, H), and Bac101 (C, F, I). IF staining was performed after convential paraformaldehyde fixation at day 1 post-infection (**A, B, C**), day 2 post-infection (**D, E, F**) and day 3 post-infection (**G, H, I**).

post-infection. After day 4 post-infection, most of cells were under lytic phase. The IF staining at day 1 post-infection showed the discrete nuclear E7 protein (Fig. 5 B, C) and at day 2, the amount of cytosolic E7 protein increased as fluorescence in nucleus diminished (Fig. 5 E, F). At day 3 post-infection, the cytosolic E7 protein was dominantly detected (Fig. 5 H, I). Similar result was observed in both Bac73 and Bac101 infected Sf9 cells, although this phenomenon was more prominent in Bac101 infected cells. From the day 2 post-infection, the nuclear E7 proteins were masked by extractable nuclear components and this event made some limitation in convential immunohistochemical detection of E7 proteins.

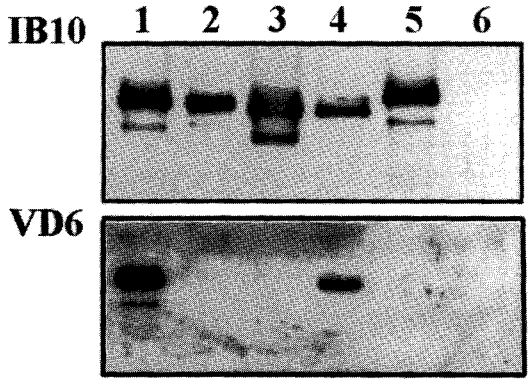

Fig. 6. Antigenic site estimation of monoclonal antibodies. E7 protein derived from pE7-MBP (lane 1), pE7d2-10 (lane 2), pE7d2-20 (lane 3), pE7d11-20 (lane 4), pE7d8-15 (lane 5), and pMalc-2 (lane 6). The monoclonal antibody, IB10, was reactive to middle domain of E7.

Epitope mapping of monoclonal antibody VD6

The precise antigenic site of VD6 antibody was analyzed to assess the region in E7 masked by nuclear component. The VD6 antibody recognize the E7 protein expressed by p5d11-20 plasmid but not by p5d2-10, p5d2-20 and p5d8-15 (Fig. 6). Based on this result, epitope of VD6 was mapped within a region of amino acid number 5 and 10 at the N-terminus. And this region in E7 protein was masked by nuclear structure as baculoviral E7 expression began to stabilize.

DISCUSSION

The major difficulty to study the biological properties of HPV is the inability to propagate HPVs *in vitro* and HPV derived proteins must be obtained through the recombinant technology. In this study, we used recombinant baculovirus expression system to obtain high level of functional proteins. The E7 protein had been known to be phophorylated by casein kinase II and phosphorylated E7 protein has been reported to have different biological features such as transforming potential and antigenicity [13, 14]. In this regards, E7 proteins expressed in eukaryotes were preferred to these expressed in prokaryotic expression system. Previously, yeast [18, 19] and baculovirus expression systems [20, 21] were used to study the host immune reaction to E7 proteins [21].

It has been reported that E7 gene as well as other HPV derived protein coding gene was naturally mutated [5, 22, 23]. The most frequently found variant which was studied in this report had the substitution mutation of serine at the 29th asparagine. This mutated serine was located near the potential CKII mediated phosphorylation sites (31 & 32th serine), thus this newly mutated serine could be phosphorylated. To investigate if there is any difference in biological prototype and variant E7 proteins, these two proteins were produced as native forms using a baculovirus expression system.

In Western blot analysis using the monoclonal antibody which recognized the N-terminal portion of E7 protein (Fig. 6), the baculovirus derived prototype and variant E7 proteins displayed molecular weight of approximately 20 kDa similar to the protein previously described [20, 21] (Fig. 1B). The size of E7 proteins determined by SDS-PAGE was larger than predicted size from E7 ORF. Similar observations have been reported in both prokaryotic and eukaryotic systems [11, 24]. Both E7 proteins expressed in insect cells were phosphorylated (Fig. 2).

Next, we analyzed the correct subcellular localization of these E7 proteins because the biological function of E7, such as binding with Rb tumor suppressor protein and adenoviral transactivation potential [15, 16, 17], strongly indicated the nuclear localization of E7 protein. Moreover, one of the known function of the CKII, which phosphorylated the E7 protein, was the enhancement of nuclear translocation of protein. Some nuclear proteins phosphorylated by CKII enhanced the binding affinity to nuclear localization sequence (NLS) binding protein [25] with its NLS region. This led us assume that CKII phosphorylation of E7 might be related to the E7 nuclear localization although there was no homologous sequence to NLS in E7 ORF. In this regard, we performed the immunostaining of E7 protein in recombinant baculovirus infected Sf9 cells using conventional paraformaldehyde fixation and found that the majority of E7 was in cytoplasm (Fig. 3). In fact, there were numerous reports about cytoplasmic E7 expression [11, 26, 28] although the E7 nuclear localization was also reported [18, 20, 28]. Moreover, some suggested that the interaction of E7 with Rb protein and resulting proteosomal degradation of Rb occurred in cytoplasm [27]. To clarify these contradictory phenomena, we performed the Western blot analysis and im-

munostaining after removal of nuclear chromatin structure using sequential fractionation procedures and found more E7 proteins in nucleus. Subsequent time course immunostaining revealed that the primary expression site was nucleus in both E7 proteins. So it could be assumed that E7 was primarily expressed in nucleus and part of it might be exported to the cytoplasm later on. Also it could be suggested that the interaction of E7 with nuclear structure resulted in the limitation of the nuclear E7 immunohistochemical detection as E7 expression stabilized. This interaction of E7 with nuclear structure was previously reported [12]. These findings were largely similar in both prototype and variant E7 proteins and it could be thought that the variant form might be have similar to prototype E7 protein within cells.

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