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Synthesis and Assembly of Virus-like Particles of Human Papillomaviruses Type 6 and Type 16 in Fission Yeast *Schizosaccharomyces pombe*

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We have synthesized capsid proteins of human papillomavirus types 6 (HPV 6) and 16 (HPV 16) in fission yeast *Schizosaccharomyces pombe* and produced virus-like particles (VLP). The capsid proteins were localized in the nucleus by indirect immunofluorescence and cell fractionation analyses. The VLP were produced in both yeast clones synthesizing L1 alone and L1/L2 and purified by sulfato-cellulofine chromatography. Electron microscopic examination showed that these VLP were similar in structure to native HPV particles. Two HPV 16 L1 variants (16 B27L1 and 16 T3L1), isolated from benign cervical samples, produced many more (68- and 14-fold) VLP than the prototype L1 (16 PL1) derived from cervical carcinoma. Coexpression of the HPV 6 L2 protein with 6 L1 and 16 B27L1 proteins increased the production level of the VLP four- and twofold, respectively. The L2 was not detected in the VLP purified with sulfato-cellulofine column, although the L2 was purified in the same fraction containing HPV 6 and 16 B27-VLP by size-fractionation using Sepharose column. Interaction between 6 L2 and 6/16 L1 proteins was not detected by the coimmunoprecipitation assays with either L1 or L2 antibodies. These results suggest that the L2 is not incorporated into the VLP synthesized in yeast. © 1995 Academic Press, Inc.

INTRODUCTION

Papillomaviruses infect the epithelia of a wide variety of animals and generally induce benign proliferative lesions at the site of infection. Some types of papillomavirus can induce malignant tumors. More than 70 types of human papillomavirus (HPV) have been isolated and some of these HPVs are etiological factors for various warts in the skin or the mucosal epithelium. Each HPV type has a strong host cell specificity for infection. Among the mucosal types, HPV 6 and HPV 11 are associated with benign warts, while the HPV 16 and the HPV 18 are associated with anogenital cancer and its precursor lesions (zur Hausen, 1991). Although HPV 6 or HPV 11 are generally benign in adults, they can also induce more severe lesions in children such as juvenile laryngeal papillomatosis (Strong *et al.,* 1979). This disease is extremely resistant to any clinical treatment. Therefore, it is important to establish human prophylactic vaccines against HPV 6 and 11, as well as the malignant types of HPV such as HPV 16 and 18, to prevent these viral infections. The study of these viral structural proteins has progressed less than that of the oncoproteins of these viruses, because of the lack of a suitable propagation system for HPV virions *in vitro.*

Immunization with BPV or HPV 11 virions has elicited antibodies in animals which inhibit the infectivity of these viruses for cultured cells (Dvoretzky *et al.,* 1980) or for xenograft tissue (Ghim *et al.,* 1991). Some of these neutralizing antibodies arising in these animals recognized conformational epitopes of the viral capsid proteins (Christensen *et al.,* 1990). Neutralizing antibodies could also be generated by immunization with bacterially derived BPV L1 and L2 proteins, but titers of these antibodies were very low (Pilacinski *et al.,* 1984; Jin *et al.,* 1989). Therefore, immunization with the native capsid structure of HPV seems to be essential to produce effective vaccination against human papillomaviruses.

Native virions of papillomaviruses are nonenveloped 50- to 60-nm spherical structures composed of 72 pentameric capsomeres in icosahedral symmetry. Each capsomere is composed of five L1 molecules and form a virion shell with a $T = 7$ symmetrical lattice (Baker *et al.,* 1991). Several groups have established systems for making virus-like particles (VLP) which resemble native HPV particles but lack viral DNA. HPV 16 and HPV 1 particles have been produced in a vaccinia virus system (Zhou *et al.,* 1991a; Hagensee *et al.,* 1993) and HPV 11, HPV 16, and HPV 33 particles in a baculovirus system (Kirnbauer *et al.,* 1992; Rose *et al.,* 1993; Volpers *et al.,* 1994). Conformational epitopes present in native HPV 11 infectious virions were also present on the baculovirus-produced HPV 11 VLP and reacted with sera obtained from individuals with condyloma acuminata (Rose *et al.,* 1993). These VLP may

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substitute for native virions of human papillomaviruses as prophylactic vaccines for humans. However, using these *in vitro* production systems, the production level of the VLP from HPV 16 was still very low (Kirnbauer *et al.,* 1992).

Kirnbauer *et al.* (1993) recently reported that two isolates of HPV 16, which shared a nonconservative change of histidine 202 to aspartic acid in the L1 sequence, were able to produce VLP much more efficiently than prototype HPV 16 in a baculovirus system. We have recently isolated 10 HPV 16 L1 variants from clinically obtained cervical samples in UK and Trinidad containing high copy numbers of HPV 16 genomes (Pushko *et al.,* 1994). All these variants had aspartic acid at amino acid 202 of L1.

Both vaccinia virus and baculovirus systems are expensive and require laborious manipulations to increase the yield of VLP. An expression system using yeast would be an attractive alternative to these higher eukaryotic systems if correctly assembled capsid proteins could be obtained. We expressed variant HPV 16 L1 capsid genes as well as the prototype L1 genes of HPV 16 and HPV 6 in fission yeast *Schizosaccharomyces pombe* and characterized the structural function of each capsid protein. Two HPV 16 L1 variants and the HPV 6 L1 assembled to form VLP resembling native capsids in contrast to the prototype L1 of HPV 16. The HPV particles produced in yeast would be good candidates as prophylactic vaccines for humans.

MATERIALS AND METHODS

Preparation and isolation of recombinant yeast

Fragment DNA coding L1 protein (nt 5789 to 7291) and L2 protein (nt 4554 to 5802) of HPV 6 were generated by polymerase chain reaction (PCR) with PFU polymerase (Stratagene). Both genes were cloned into *Bam*HI–*Xho*I sites of the pBSK (Stratagene) and in *Nco*I–*Eco*RI sites of pET vector (Novagen), respectively. The PCR primers for HPV 6 L1 and L2 were designed to contain *Bam*HI–*Xho*I and *Nco*I–*Eco*RI restriction sites for the L1 and L2 genes of the HPV 6, respectively. The sequence of these primers are as follows: HPV 6 L1 primers, 5′-primer, 5′-GGGGATCCATGTGGCGGCCTAGCCGACAG-3′ and 3′-primer, 5′-CCCTCGAGTTACCTTTTAGTTTTGGCGCG-3′, HPV 6 L2 primers, 5′-CCCATGGCACATAGTAGGGCCCGACGACG-3′ and 5′-GGGAATTCCTAGGCCGCCACATCTGAAAA-3′. The cutting sites of each restriction enzyme on these primers are underlined. A prototype and two mutant types of L1 capsid genes of HPV 16 (B27L1 and T3L1) had been generated by PCR were cloned into pBSK vector (Pushko *et al.,* 1994). All these capsid genes were sequenced with a T7 sequence kit (Pharmacia) and were confirmed to have full coding sequences. All capsid genes were cut out from all the recombinant pBSK and pET and introduced into yeast expression vectors pREP 3 and pREP 4 containing thiamine-repressible promoter (Maundrell, 1993). HPV 16 L1 was cloned at *Bal*I and *Sal*I sites of pREP 3 and the HPV 16 L2 was cloned at the same site of pREP 4. The HPV 6 L1 was cloned in *Bam*HI–*Xho*I sites of pREP 4-20 and a fragment of HPV 6 L2 gene was cloned at *Bal*I site of pREP 3-20 by blunt end ligation. All the constructs of the recombinant genes used in this study are shown in Fig. 1.

The *leu-* and *ura*-auxotrophic mutant of *S. pombe* was transformed with the recombinant plasmids. We introduced both L1 and L2 genes via pREP 3 (leu) and pREP 4 (ura) vectors into a mutant yeast (*leu-*, *ura-*) by allowing the recombinant clone to grow selectively in minimal essential medium without leucine and uracil.

Immunoblot analysis

Yeast cells were suspended in yeast lysis buffer (50 m*M* Tris–Cl, pH 8.0, 0.1% Triton X-100, 0.5% SDS), boiled for 2 min, and broken with glass beads (0.45–0.60 mm, Sigma) using a strong vortex for four periods of 1 min intermittently cooling on ice. Total cell extracts were obtained after centrifugation at 10,000 *g* for 10 min. Equal amounts of denatured protein were loaded onto 10% SDS–polyacrylamide mini gels and run at 100 V for 1 hr. The proteins were transferred onto nitrocellulose membrane (Hybond-C, Amersham) using electroblot apparatus (Bio-Rad) and incubated in PBS-M buffer (PBS containing 5% dried milk and 1% fetal bovine serum) at 37° for 30 min. After washing once with PBS, the membrane was incubated with PBS-M containing the appropriate antibody diluted 1:250 and incubated at 4° overnight. After three 20-min washes with PBST (PBS containing 0.1% Tween 20), the membrane was incubated in an anti-mouse–peroxidase conjugate diluted 1:1000 in PBST at room temperature for 40 min, followed by three further washes of the membrane with PBST. Protein signals were detected by chemoluminescence using ECL Western blotting detection reagents (Amersham) according to the manufacturer's instructions.

Indirect immunofluorescence

Thirty milliliters of cultured yeast induced to express the introduced genes was fixed with 3% formaldehyde and 0.2% glutaraldehyde in PEM buffer (100 m*M* PIPES, pH 7.4, 1 m*M* EDTA, 1 m*M* $MgSO_4$) at 30° for 60 min. The cells were digested with 0.25% mutanase and 0.0625% of zymolase (20000 U Seikagaku Kogyo). Protoplasts were prepared by the method of Hagan and Hyams (1988). Permeabilized protoplasts were pelleted and resuspended in 5–7 vol of PEMBAL solution (1% bovine serum albumin, 0.1% sodium azide, 100 m*M* lysine hydrochloride in PEM buffer) containing the appropriately diluted antibodies. The optimum dilution of each antibody was determined by preliminary experiments. Following 16 hr incubation, the cells were washed three times in PEMBAL and resuspended in 5–7 vol of fluorescent labeled anti-mouse antibody at 2% in PEMBAL. Incubation of the secondary antibody was for 4 hr and followed by three washes in PEMBAL. For observation, cells were air dried

onto poly-L-lysine-coated coverslips and inverted onto glass slides. The stained samples were examined on a Con-focal Fluorescence Imaging System MRC-500 Bio-Rad microscope.

Cell fractionation analysis of *S. pombe*

Yeast cells induced for expression of the introduced genes were cultured in 1000 ml of minimal low glucose medium for 16 hr at 30°, centrifuged at 800 *g* for 5 min, and resuspended in 50 ml of 50 m*M* potassium phosphate buffer (pH 6.5) containing 25 m*M* EDTA. After re-centrifugation, the pellet was resuspended with 10 ml of KKC buffer (20 m*M* potassium phosphate buffer, pH 6.5, 800 m*M* KCl, 0.1 m*M* $CaCl_2$) containing 5 mg/ml of Novo-Zym 234 (Novo Nordisk Biolabs) and then incubated at 32° for 30 min to digest the cell walls. The sample was then layered onto a 35-ml cushion of cold KKC/G (KKC buffer containing 10% glycerol) and centrifuged at 1000 *g* for 10 min at 4°. The supernatant containing small particles of cell wall debris was removed and later analyzed as a membrane fraction of yeast. The pellet was resuspended in 1 ml of cold KKC/G and diluted with 7 ml of hypotonic KC buffer (20 m*M* potassium phosphate buffer, pH 6.5, 0.1 m*M* $CaCl_2$) supplemented with protease inhibitors, 0.5 m*M* PMSF, 1 mg/ml leupeptin, 1 mg/ml aprotinin, and 1 mg/ml pepstatin (Sigma). Separation of the cytoplasm from the nucleus was achieved by adding Nonidet P-40 to the sample to a final concentration of 0.1%. Five milliliters of the sample was then layered onto a 25-ml cushion of KM/F (20 m*M* potassium phosphate buffer, pH 6.5, 2 m*M* $MgCl_2$, 10% Ficoll) and centrifuged at 26,900 *g*, 4° for 60 min, separating the cytoplasmic and nuclear fractions in the supernatant and pellet, respectively. Ten micrograms of protein from each fraction was subjected to immunoblot analysis.

Purification of virus-like particles by sulfato-cellulofine chromatography and preparation of the sample for electron microscopy

Yeast cells induced for synthesis of the capsid proteins were harvested from 1600 ml of cultures by centrifugation and resuspended in 20 ml of PBS. The yeast cells were broken mechanically with glass beads in a cell-breaking machine (Melsungen AG, Braun) for 2 min with repeated cooling. The supernatant was obtained after centrifugation at 10,000 rpm, 4° for 10 min using a Sorvall SS 34. After filtration with an 0.45-μm filter, this cell extract was applied onto a sulfato-cellulofine (Seikagaku Kogyo) column (2.6 × 13 cm) equilibrated with 0.15 *M* PBS (10 m*M* phosphate buffer, 0.15 *M* NaCl). After washing with 5× bed volumes of 0.15 *M* PBS followed by the same volume of 0.5 *M* PBS (10 m*M* phosphate buffer, 0.5 *M* NaCl), the fraction containing capsid proteins was eluted with 2 *M* PBS (10 m*M* phosphate buffer, 2 *M* NaCl). The eluate was then applied to a Sepharose CL 4B column (2.6 × 100 cm) equilibrated with 2 *M* PBS and a first peak fraction of this column was collected.

To examine the yield of VLP in different yeast clones, each 0.5 ml of the VLP sample purified with a sulfato-cellulofine column was concentrated with a Ultra CL-filter 30,000 NMWL (Millipore) and then negatively stained for electron microscopic examination.

Transmission electron microscopy

Two microliters of the sample was placed onto a glow-discharged carbon-coated grid and allowed to absorb for about 1 min in a moist chamber; it was then washed with 10 drops of 1% aqueous potassium phosphotungstic acid (pH 7.0) or 1% aqueous uranyl acetate. The high resolution electron micrographs were recorded using an (Philip EM 301 A and Zeiss 10 C) electron microscope at ×34,000 magnification.

Purification of virus-like particles with equilibrium gradient centrifugation

Cell extract was obtained from 10 liters of yeast culture by the same procedure described above. After adding cesium chloride (CsCl) to the cell extract to a density of 1.30 g/ml, the sample was centrifuged at 27,000 rpm for 40 hr at 10° using a Beckman SW 28 rotor. Thirty fractions were collected and checked for presence of L1 protein by immunoblot analysis. The fractions containing L1 protein were diluted in PBS and centrifuged at 35,000 rpm for 4 hr at 4° using Beckman SW 41 rotor. The pellet was resuspended in buffer A (Tris–HCl, pH 7.5, 2 *M* NaCl, 2 m*M* $MgCl_2$, 15 m*M* mercaptoethanol) by rotation overnight at 4°. To obtain purer sample, the fractions containing L1 protein were repurified by CsCl gradient ultracentrifugation for 24 hr.

RESULTS

Synthesis of HPV 6 and HPV 16 capsid genes in *S. pombe*

To test the ability for self-assembly *in vivo,* we constructed yeast recombinant REP vectors and synthesized different capsid proteins from HPV 6 and HPV 16 in fission yeast *S. pombe* under the control of a thiamine-repressible promoter (Tommasino and Maundrell, 1991; Maundrell, 1993). Three different HPV 16 L1 (PL1, B27L1, and T3L1 and HPV 6 L1 (6 L1) genes were tested (Fig. 1). The PL1 was derived from the prototype HPV 16 DNA isolated from a cervical carcinoma (Durst *et al.,* 1983) and the B27L1 and T3L1 were isolated from two benign cervical cell samples containing high copy number of viral genomes (Pushko *et al.,* 1994). The B27L1 and T3L1 contained several point mutations compared with the prototype L1 sequence (Seedorf *et al.,* 1985), including the aspartic acid at position 202, as reported by Kirnbauer *et al.* (1993).

HPV 6 L1 (lane 3 in Fig. 2C) and PL1, B27L1, and T3L1 of HPV 16 (lanes 1–6 in Fig. 2A) proteins were detected as a 57-kDa band in immunoblot analysis of induced cultures. The size of the band was the same as that of HPV16 L1 synthesized in insect cells (lane 7 in Fig. 2A). No bands

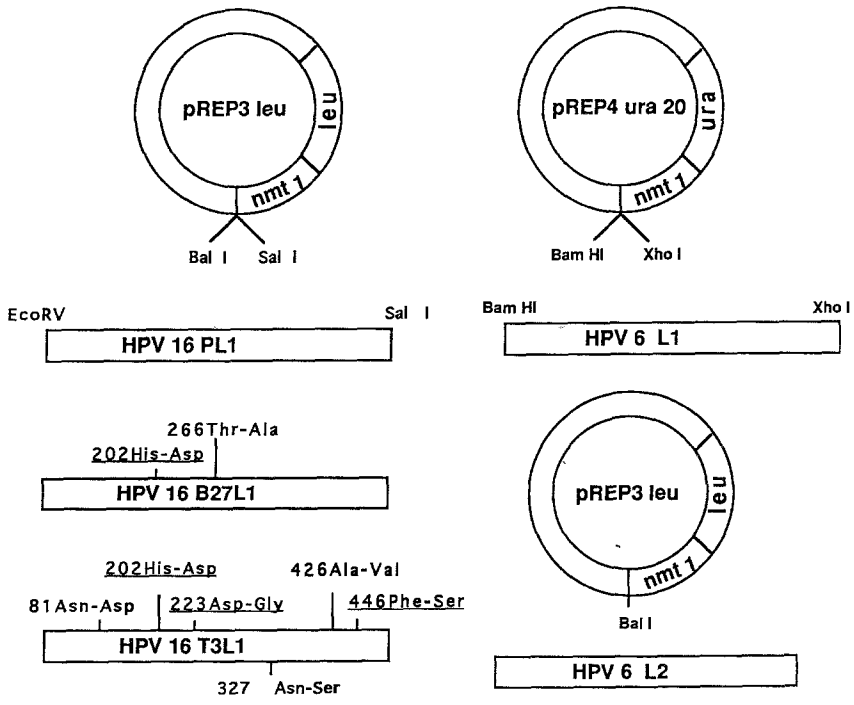

FIG. 1. Constructs of the yeast recombinant vectors used in this study. The L1 capsid genes of HPV 16 and L1/L2 genes of HPV 6 were introduced in yeast vectors pREP 3 and pREP 4. The amino acid changes in the variant L1 genes of HPV 16, B27L1, and T3L1 are indicated. Nonconservative changes are underlined.

were detected in the samples which were not induced for expression of L1 proteins (lane 2 in Fig. 2C) nor in normal yeast (lane 8 in Fig. 2A and lane 1 in Fig. 2C). Several smaller bands (less than 49 kDa) were often observed in three different L1 proteins of HPV 16 and are probably degradation products of L1. No differences were observed in size or level of expression between variant (B27L1 and T3L1) and prototype (PL1) L1 proteins of HPV 16 (lanes 1, 3, and 5 in Fig. 2A). The HPV 6 L2 protein was detected as a 75-kDa band in every sample expressing the 6 L2 gene (lanes 4, 5, and 6 in Fig. 2B). None of these bands were detected in the other clones containing no 6 L2 (lanes 1, 2, and 3 in Fig. 2B) or in the same clones not induced for expression of the L2 gene (data not shown).

Localization of the capsid proteins

Native virions of human papillomaviruses are known to be propagated in the nucleus of infected cells. It was reported that both L1 and L2 proteins of HPV 16 and HPV 1 were localized in the nucleus (Zhou *et al.*, 1991b; Hagensee *et al.*, 1993). All the capsid proteins are presumed to be directed from the cytoplasm to the nucleus immediately after translation (Zhou *et al.*, 1991b). The nu-

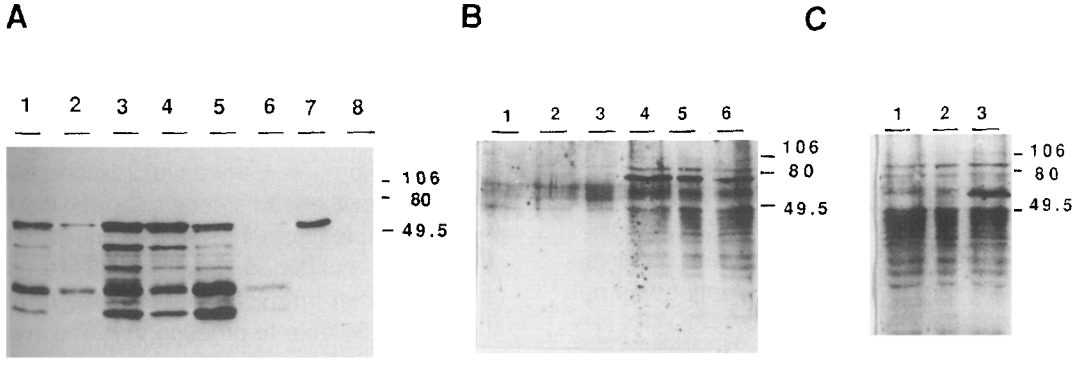

FIG. 2. Western blot analyses for expression of HPV 6/16 capsid genes in yeast. (A) HPV 16 L1 was detected as a 57-kDa band in lanes 1–7 with anti-16 L1 antibody (Camvir-1). Lane 1, 16 PL1; lane 2, 16 PL1/6L2; lane 3, 16 B27L1; lane 4, 16 B27L1/6 L2; lane 5, 16 T3L1; lane 6, 16 T3L1/6 L2; lane 7, HPV 16 PL1 expressed in the insect cells infected with the recombinant baculovirus. Lane 8, normal yeast. (B) HPV 6 L2 was detected as a 75-kDa band in lanes 4–6 with anti-HPV 6 L2 antibody (3F7). Lane 1, HPV 6L1; lane 2, HPV 16 PL1; lane 3, HPV 16 B27L1; lane 4, HPV 6L1/6L2; lane 5, HPV16 PL1/6L2; lane 6, HPV16 B27L1/6L2. (C) HPV 6 L1 was detected as a 57-kDa band in lane 3 with anti-HPV 33 L1 antibody. Lane 1, normal yeast; lane 2, HPV 6L1/6L2, noninduced; lane 3, HPV 6 L1/6 L2, induced.

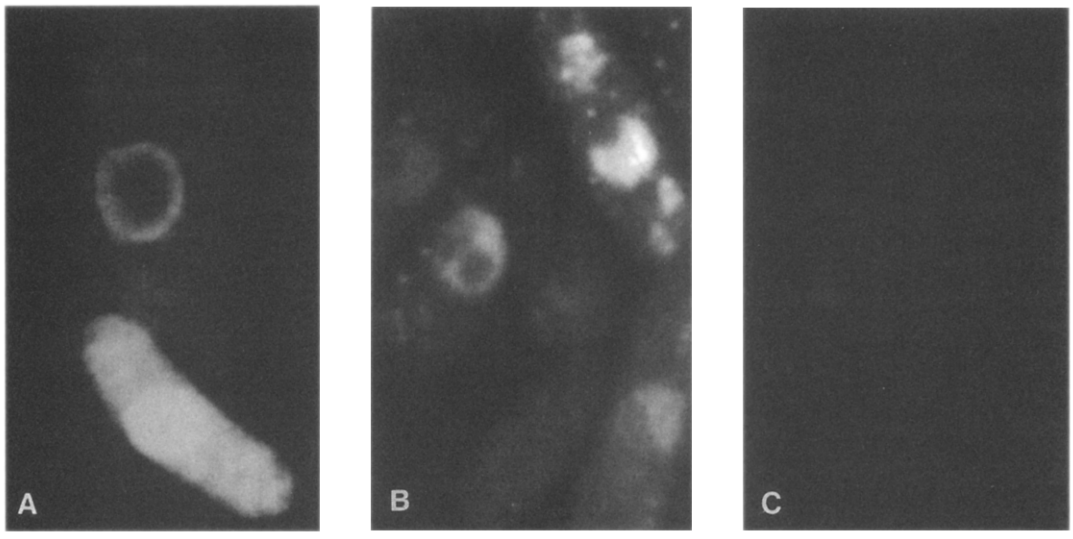

FIG. 3. Localization of L1 and L2 capsid proteins by indirect immunofluorescence. Yeast cells induced for expression of HPV 6L1/L2 genes were stained with a neutralizing antibody against HPV 11 (B2) (A), with anti HPV6 L2 (3F7) (B), and with a control antibody (anti-HPV 16 E2 protein) (C). Strong L1 protein signals are observed in the nucleus of the yeast (A). L2 is predominantly stained in the nucleus but spotty staining is also seen in the cytoplasm (B). No signals are observed with a control antibody (C).

clear localization of the L1 protein may be important for viral capsid assembly or for maturation of viral particles. To investigate this in a yeast system, we carried out an indirect-immunofluorescence analysis on each clone using the L1 or L2 monoclonal antibodies. After induction for expression of the introduced capsid genes, cells were stained with appropriate antibodies. In the HPV 6 L1 clone, 30–50% of the cells were positively stained with the neutralizing monoclonal antibody (B2) against HPV 11 which can prevent infection with HPV 11 (Christensen *et al.*, 1990). Only nuclear or perinuclear staining was observed in 10–30% of these positively stained cells. The other cells were stained in both the nucleus and the cytoplasm, with nuclear staining still more predominant than cytoplasmic (Fig. 3A). However, nuclear staining was not observed with the other anti-HPV 11 antibody (H3) or with an anti-HPV 33 L1 antibody which could detect denatured HPV 6 L1 protein by Western blot analysis (Fig. 3C).

HPV 6 L2 protein was also detected in the yeast coexpressing HPV 6 L1 and 6 L2. The frequency and intensity of the L2 staining on these cells was similar to that of 6 L1. The L2 protein was detected in both nucleus and cytoplasm, although the nuclear staining was dominant in more than half of the cells (Fig. 3B). The staining pattern of L1 was diffuse (Fig. 3A) while that of L2 looked spotty and irregular (Fig. 3B). No staining was observed in control yeast cells with any L1 and L2 antibodies nor a control antibody (Fig. 3C).

For HPV 16 the L1 signals were localized in either the nucleus or the perinucleus of all recombinant clones expressing PL1, B27L1, or 3T L1 proteins, and the staining pattern was similar in all these clones (data not shown). HPV 6 L2 expression was also observed in all chimeric clones containing 16 L1 and 6 L2. The staining pattern of the L2 protein in these chimeras was identical to that of the yeast containing HPV 6 L1/6 L2 (data not shown).

To confirm the nuclear localization of these capsid proteins, we fractionated cell components using a Ficoll separation method and compared the expression level of capsid proteins in each fraction by immunoblot analysis. The HPV16 L1 and HPV 6 L2 were detected in the nuclear fractions (lanes 7 and 8 in Fig. 4A and lanes 7 and 9 in Fig. 4B), whereas no apparent bands of L1 and L2 were observed in the membrane and cytoplasmic fractions (lanes 1–6 in Figs. 4A and 4B).

Purification and electron microscopic analysis of VLP from the yeast

We purified VLP using sulfato-cellulofine and size-fractionation chromatography as described under Materials and Methods. The 57-kDa band for HPV 16 L1 capsid protein was detected in the elution fraction in high salt buffer (2 *M* PBS) (lane 4 in Fig. 5A), whereas it was not detected in the flow through fraction (lane 1) and the elution fraction in low salt buffer (0.5 *M* PBS) (lanes 2 and 3 in Fig. 5A). The same results were observed in HPV 6 L1 capsid protein. Electron microscopic examination of the negatively stained samples showed that the 2 *M* PBS elution samples from the yeast synthesizing HPV 6 L1 or HPV 16 L1 contained several virus-like particles (Fig. 6A) as did that from HPV 6L1/6L2. The L1 protein alone seemed to be able to produce VLP. The average size of the VLP was 50 nm (40–55 nm) in diameter and pentagonal capsomere structure was apparent. The morphologies of these particles were similar to HPV 6 VLP produced in a baculovirus system (provided by Martha Ladner and Robert Ralston, Chiron Corp., U.S.A.) (Fig. 6E) and native viral particles of HPV 1 (Fig. 6F). Many smaller particles

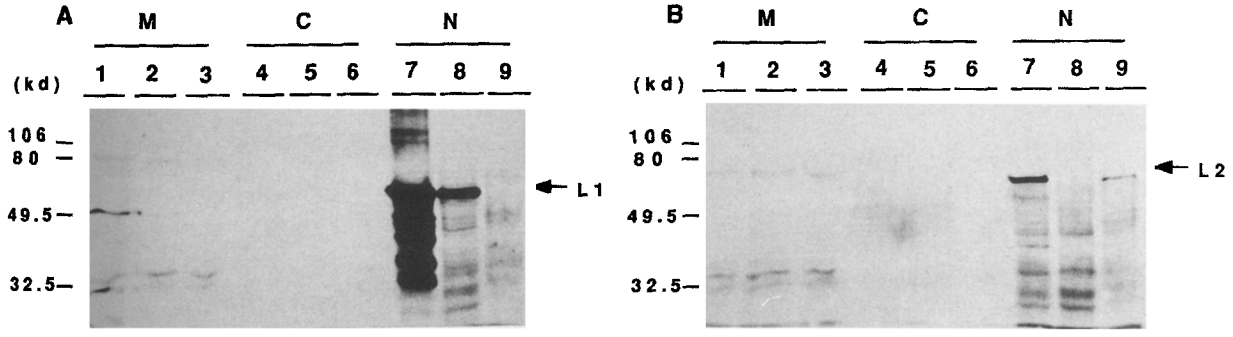

FIG. 4. Detection of the capsid proteins in different cell components of *Schizosaccharomyces pombe.* Ten micrograms of total protein from each cell fraction from the different yeast clones was subjected to SDS–PAGE and analyzed by immunoblot with anti-HPV 16 L1 antibody (Camvir-1) (A) and anti HPV 6L2 antibody (3F7) (B). M, membrane fraction; C, cytoplasmic fraction; N, nuclear fraction. Lanes 1, 4, and 7, yeast containing HPV 16 B27L1 and 6L2 genes. Lanes 2, 5, and 8, yeast containing HPV 6 L1 gene. Lanes 3, 6, and 9, yeast containing HPV 6L2 gene. HPV 16 and 6 L1 proteins (lanes 7 and 8 in Fig. 4A) and HPV 6L2 protein (lanes 7 and 9 in Fig. 4B) are detected in the nuclear fractions as indicated by arrows.

with diameter about 17 × 23 nm (Fig. 6A) were also observed. These smaller particles showing barrel-like structures were always copurified with the VLP in our procedure. The morphology of this particle is very similar to a subunit of yeast fatty acid synthase observed in *Saccharomyces cerevisiae* (Stoops *et al.,* 1992). We used these particles as an internal control when we compared the production level of VLP between individual recombinant yeast. Some tubular structures were occasionally observed (Fig. 6B). The similar tubular structures have been reported to be copurified with the viral particles from rabbit tissue infected with Shope papilloma virus (Finch *et al.,* 1965) and in human papillomaviruses (Kiselev *et al.,* 1969). The surface appearance of the tubular structures is very similar to that of the virus particles and they are probably composed of similar morphological units in a pentagonal array but arranged over the surface of a cylinder instead of a sphere. Many smaller particles less than 10 nm were also identified in the sample, some of which are aggregated (Fig. 6C), seem to be unassembled capsomeres of HPV. All of these particles except for the tubular structures were observed in all the samples from the other yeast clones, although numbers of each particle were different in individual clones. Purer samples of VLP were obtained by further purification with a gel-filtration column (Figs. 5B and 6D).

Comparison of the efficiency for capsid self-assembly *in vivo*

To compare the yield of VLP from different capsid proteins, we counted total numbers of both VLP and fatty acid synthase particles in 10 fields at ×34,000 randomly on the samples which were only purified with a sulfato-cellulofine column (Fig. 6A). These results are shown in Table 1. Efficiency of each VLP formation *in vivo* was roughly estimated by the VLP: fatty acid synthase ratio. HPV 6 L1, HPV 16 B27L1, and T3L1 alone could produce 24, 68, and 14 times more VLP, respectively, than HPV 16 PL1. When we compared the yield of VLP between L1 alone and L1/L2, approximately four times as many VLP were produced in HPV 6 L1/6 L2 than in 6 L1 alone. A twofold increase in the yield of VLP was also observed in the chimeric clones HPV 16 B27 L1 and HPV 6 L2, but

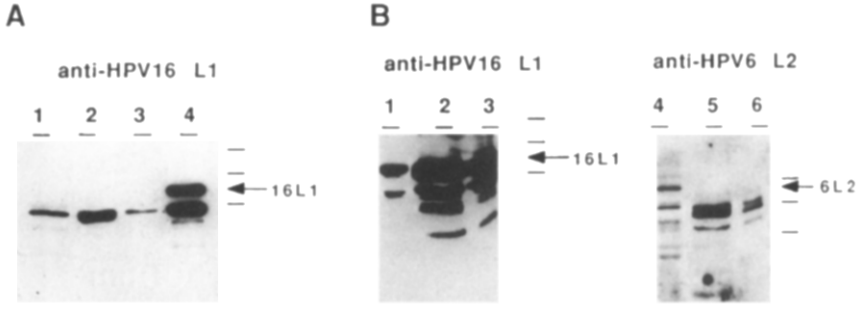

FIG. 5. Immunoblot analyses in the purified samples containing virus-like particles of HPV 16. One milliliter of each sample was tested in these experiments. Cell extract from the yeast induced for expression of HPV 16 B27L1 capsid genes was applied into a sulfato-cellulofine column and several fractions were collected and tested by Western blot analysis with anti HPV 16 L1 antibody (A). Lane 1, flow through fraction; lanes 2 and 3, two elution fractions in PBS (0.5 *M* NaCl); lane 4, an elution fraction in PBS (2 *M* NaCl). The samples purified by sulfato-cellulofine or sulfato-cellulofine/gel filtration chromatography were analyzed by Western blot with anti-HPV 16 antibody (left) and with anti-HPV 6 L2 antibody (right) (B). The protein markers indicate sizes of 106, 80, and 49.5 kDa (A, the left figure in B), and 80, 49.5, and 32.5 kDa (the right figure in B). Lanes 1 and 4, total cell extract; lanes 2 and 5, the sample purified with sulfato-cellulofine column; lanes 3 and 6, sample purified with both sulfato-cellulofine and gel-filtration columns.

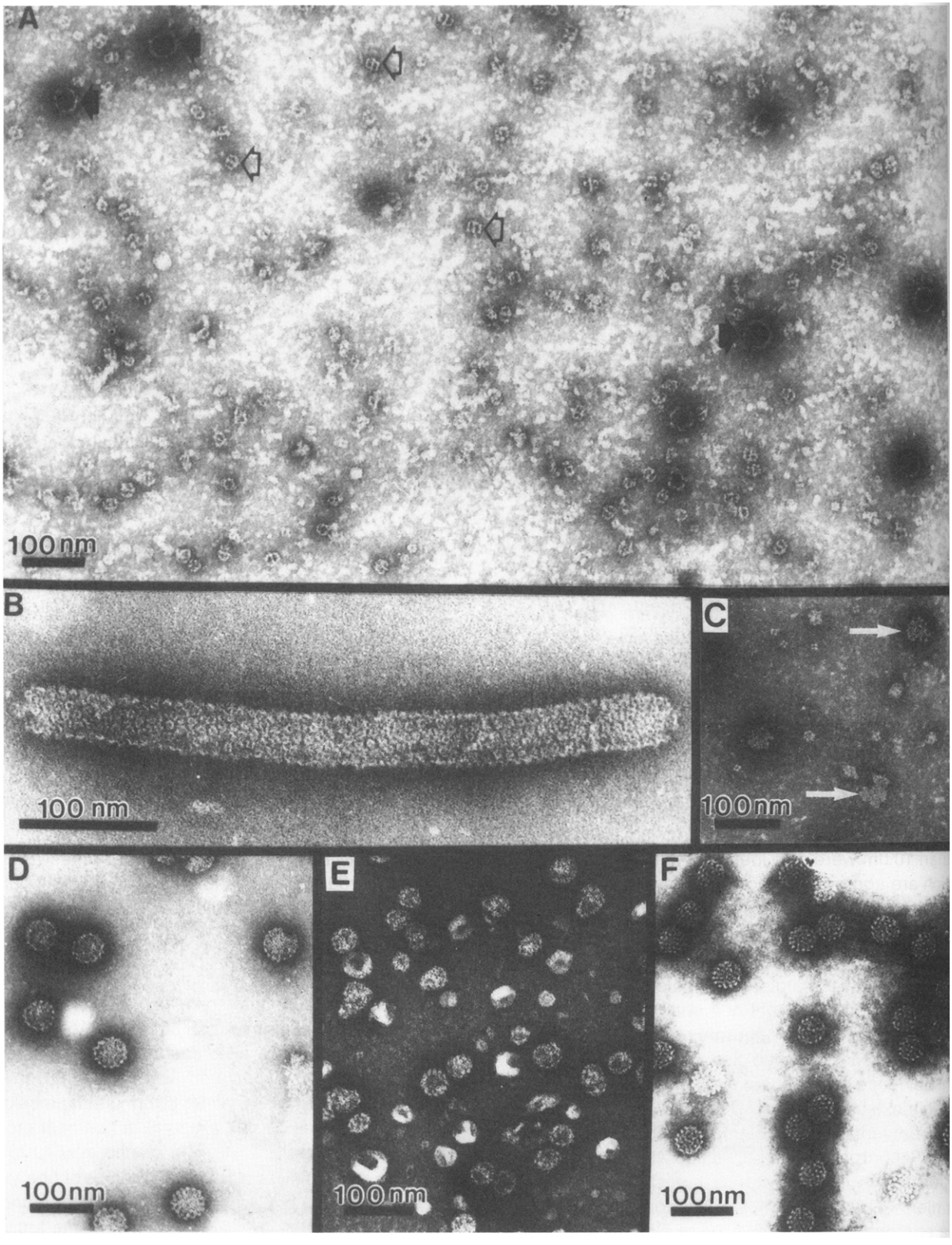

FIG. 6. Electron micrographs of purified HPV particles. HPV particles were stained with either uranyl acetate or potassium phosphotungstate and examined by TEM. (A) A sample of HPV 6 L1/6 L2 purified with sulfato-cellulofine column. Several virus-like particles (VLP, indicated with black closed arrows) and fatty acid synthase molecules (indicated with open arrows). The VLP are 40–55 nm in diameter (scale bar is 100 nm). (B) Tubular structures are occasionally seen in the samples and have the same capsomere structure as VLP. (C) Clumps of unassembled capsomeres. (D) A sample of HPV 16 B27 VLP obtained by purification with both sulfato-cellulofine and gel-filtration columns. (E) HPV 6 VLP derived from insect cells infected with recombinant baculovirus. (F) Native HPV 1 virions.

TABLE 1

YIELD OF THE VIRUS-LIKE PARTICLES IN THE RECOMBINANT YEAST EXPRESSING DIFFERENT CAPSID PROTEINS

Recombinant yeast expressing capsid proteins	Number of VLP/ number of YFASP (10 fields[a] × 34,000)	Efficiency of VLP formation (No. of VLP × 100/ No. of YFASP[b])
H6 L1	68/970	7.01 (24)[c]
H6 L1/L2	225/757	29.7 (100)
H16 PL1	1/335	0.29 (1.0)
H16 PL1/6L2	5/1290	0.38 (1.3)
H16 27L1	326/1672	19.56 (68)
H16 27L1/6L2	230/650	35.4 (122)
H16 T3L1	35/849	4.1 (41)
H16 T3L1/6L2	34/1344	2.7 (9.3)

[a] The mean number of particles in 10 fields which was calculated from three different preparations is shown.

[b] Yeast fatty acid synthase particles.

[c] Comparative efficiency of production of the virus-like particles (prototype L1 of HPV 16 is set at 1.0).

no stimulatory effect of L2 was observed in HPV 16 PL1/6L2. The yield of the VLP was slightly decreased in the T3L1/6L2 compared with that of the T3L1 alone. When we examined the expression levels in cell extracts, both 16 PL1 and 3T L1 proteins were repressed for their expression in the chimeric clones PL1/6 L2 and T3L1/6 L2 (lanes 2 and 6 in Fig. 2A) compared with the PL1 and the T3L1 alone (lanes 1 and 5 in Fig. 2A). The stimulatory effect of L2 protein to capsid assembly seems to be less apparent in yeast than in the other systems (Zhou *et al.*, 1991a; Hagensee *et al.*, 1993; Kirnbauer *et al.*, 1993).

Interaction of L1 and L2 proteins in capsid assembly

The function of L2 protein is not known and there is no clear evidence as to whether it is incorporated into virus-like particles or not. In our study, the yields of VLP of HPV 6 L1 and 16 B27L1 were slightly increased by coexpression of HPV 6 L2. The 6 L2 protein comigrated with 6 and 16 L1 proteins, when we loaded crude yeast lysate onto a gel-filtration column (fractions 5 to 9 in Fig. 7). However, no 6 L2 band (75 kDa) was detected in the VLP samples 6L1/6 L2 and B27L1/6 L2, which were purified with an sulfato-cellulofine column or both sulfato-cellulofine and gel-filtration columns (lanes 2 and 3 in Fig. 5B). No interaction of the L1 and L2 proteins in the cell lysate was detected by the coimmunoprecipitation assay with either L1 or L2 antibodies (data not shown). These findings suggest that the L2 protein was neither incorporated into the virus-like particles nor associated with the L1 protein in the yeast system.

DISCUSSION

We demonstrate that HPV capsid proteins derived from fission yeast *S. pombe* can assemble to form VLP and tubular structures as seen in native HPV infection. Although some size variation was observed (40–55 nm) in these VLP, the size was relatively uniform and the average size (50 nm) was similar to the native virion (Finch and Klug, 1965; Finch and Crawford, 1975) and the HPV 16 particles produced in baculovirus systems (Kirnbauer *et al.*, 1993). The VLP were also isolated from yeast by cesium equilibrium gradient ultracentrifugation (data not shown). The buoyant density of the VLP in the gradient was 1.29 g/ml, which was the same as that of the HPV 16 VLP (Kirnbauer *et al.*, 1993) and HPV 33 VLP (Volpers *et al.*, 1994) synthesized in baculovirus systems. All these results suggest that the capsid proteins synthesized in yeast are similar to that of native HPV and synthetic capsid proteins by the other systems.

In our results, smaller irregularly assembled particles were observed in the sample from the yeast expressing prototype L1 of HPV 16 (data not shown). Zhou *et al.* (1991a) also first reported that the HPV 16 particles produced in a vaccinia virus system were 40 nm in diameter. Since their particles were also derived from prototype HPV 16, this type seems to form smaller irregularly assembled particles and this may be correlated with inefficient production of the VLP of this type. We introduced two L1 variants of HPV 16 (B27L1 and T3L1) into yeast and compared them with the prototype L1. The B27 L1 could produce 68 times more VLP than the prototype 16 L1 and the T3 L1 produced 14 times more VLP than the prototype. These L1 variants share the same mutation at residue 202 as the two isolates (114K and 114B) reported by Kirnbauer *et al.* (1993). They reported that the HPV 16 114KL1 produced about much more VLP than the cells expressing the HPV 16 prototype L1 in a baculovirus system. The B27 L1 has two amino acid changes at histidine 202 to aspartate and threonine 266 to alanine (Fig. 1), whereas the 114K has one amino acid change at histidine 202 to aspartate and the 114B has three amino acid changes at valine 194 to isoleucine, histidine 202 to aspartate, and threonine 266 to alanine. From these sequences, the B27 L1 seems to be closely related to these 114K L1 and 114B L1. Our results therefore support the idea proposed by Kirnbauer *et al.* (1993) that an amino acid change at histidine 202 to aspartate is crucial for self-assembly of L1 and the sequence with aspartate at position 202 may constitute the wild type of HPV 16.

HPV 16 T3L1 was similar to prototype L1 in some respects and no stimulatory effect of HPV 6 L2 on capsid assembly was observed in 16 PL1/6 L2 or 16 T3L1/6 L2. This is probably because the expression levels of PL1 and T3 L1 were greatly reduced by coexpression of the L2 protein (Fig. 2B). Both PL1 and T3L1 proteins become insoluble in PBS suspension when kept at 4° for several days, while the HPV 6L1 and HPV 16 B27 L1 remained soluble (data not shown). A similar finding with the HPV 16 PL1 was reported by Kirnbauer *et al.* (1993). The T3 L1 protein shared the same mutation at amino acid position 202 as the other new isolates of HPV 16, but it has in addition five other changes, asparagine 81 to aspartate, aspartate 223

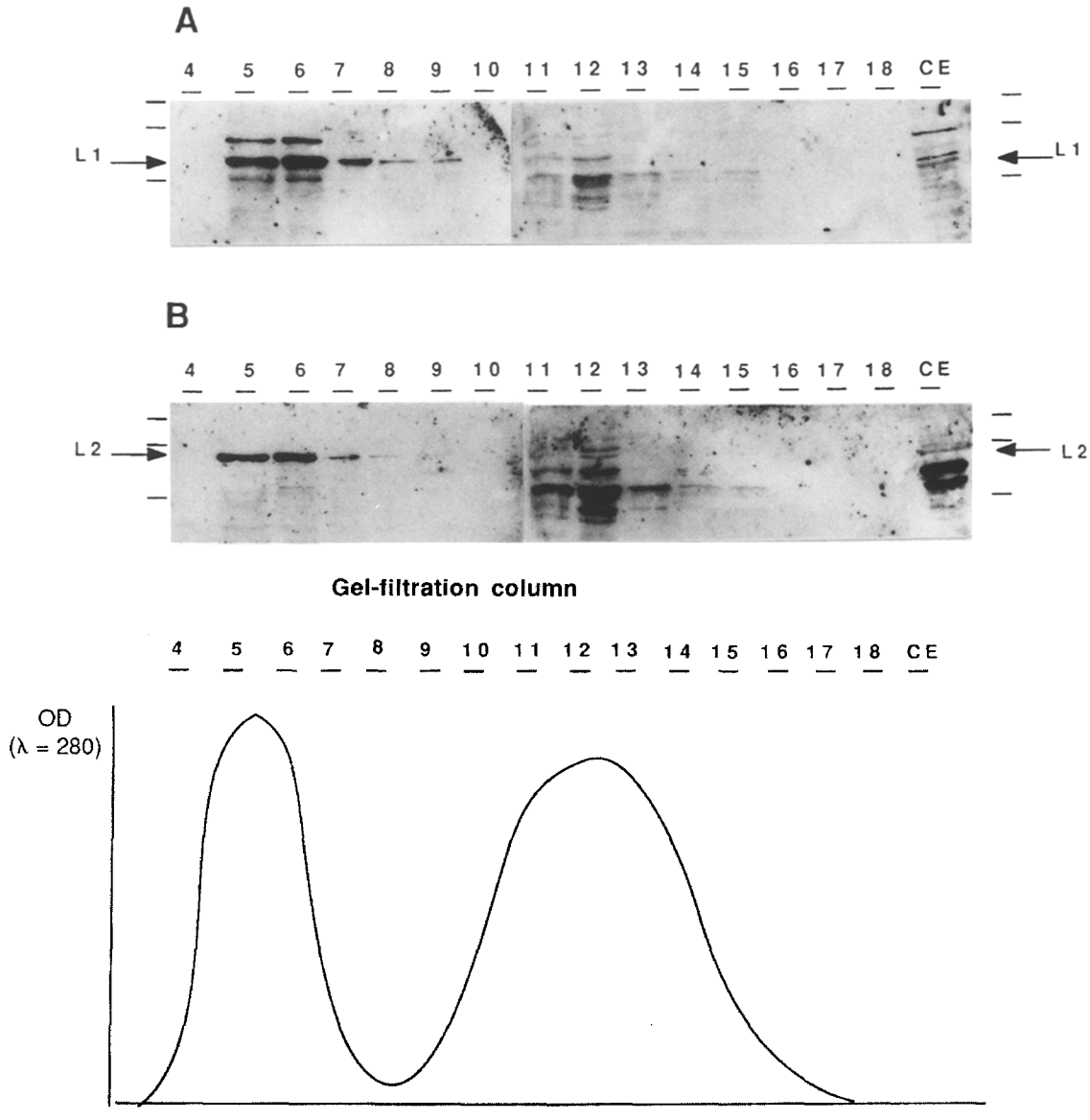

FIG. 7. Migration of L1 and L2 proteins by gel-filtration chromatography. One milliliter of the crude cell extract from HPV 6 L1/L2 yeast was applied to a Sepharose CL 4B column (1 × 30 cm) equilibrated with PBS. Half-milliliter fractions were collected and run on a SDS–PAGE gel. The proteins were transferred to a membrane which was used for both reactions with anti-L2 and anti-L1 antibodies. Strong signals of L1 (A) and L2 (B) proteins indicated by arrows are observed in the same fractions from the first peak which contained VLP (fractions 5–6) and unassembled capsomeres (fractions 7–9). Total cell extract was loaded as control in the last lane of both experiments (CE).

to glycine, asparagine 327 to serine, alanine 426 to valine, and phenyl alanine 446 to serine (Fig. 1). Two nonconservative mutations aspartate 223 to glycine and phenyl alanine 446 to serine may be responsible for an adverse effect on self-assembly of this capsid protein.

Native papillomavirus particles are known to propagate in the nucleus of the cells infected with the virus. However, there is no clear evidence whether nuclear localization is essential for self-assembly of the capsid proteins generated in *in vitro* expression systems, although nuclear localization of the L1 and L2 proteins was reported in vaccinia virus systems using HPV 16 (Zhou *et al.*, 1991b) and HPV 1 (Hagensee *et al.*, 1993). In the present study, we also showed that L1 proteins of both HPV 6 and HPV 16 were predominantly localized in nucleus by immunofluorescent staining (Fig. 3) and cell-fractionation analysis (Fig. 4). The nuclear staining of HPV 6 L1 may represent the nuclear localization of the assembled capsid proteins, because it was detected only with the neutralizing antibody against HPV 11 which cross-reacted with HPV 6 particles by immunogold electron microscopy (data not shown).

At present, the function of L2 protein of papillomaviruses is not clear. When we expressed both L1 and L2 proteins of HPV 6 in yeast, slightly more VLP was produced than with L1 alone, even in a heterologous system (16 B27L1 with HPV 6 L2). Previous reports (Zhou *et al.*, 1991a; Hagensee *et al.*, 1993; Kirnbauer *et al.*, 1993) indicated that coexpression of L2 with homologous L1 promoted the production of VLP, although the stimulation

was different in each case. In some reports (Hagensee *et al.*, 1993; Kirnbauer *et al.*, 1993; Volpers *et al.*, 1994), incorporation of L2 proteins into the VLP of HPV 1, HPV 16, and HPV 33 was suggested, and complex formation of L1 and L2 of HPV 16 was also reported by Xi *et al.* (1991), although VLP formation was not observed in that report. In contrary, Rose *et al.* (1993) reported that there was no evidence of L1 and L2 complex formation in HPV 11 capsid proteins expressed in baculovirus system, although the L1- and L2-labeled proteins were immunoprecipitated with cognate antisera. In the present study, the L2 protein seems to be neither incorporated in the virus-like particles nor associated with L1 protein, because no L2 protein (75 kDa) was detected in purified VLP and no interaction between the L1 and L2 proteins was observed in the coimmunoprecipitation assay. This may be the reason why production level of VLP was not greatly increased by coexpression of L2 protein in our yeast system as compared with the other reports (Hagensee *et al.*, 1993; Kirnbauer *et al.*, 1993).

We have shown that preparative amount of VLP could be purified by chromatography. The yeast system provides an alternative method to produce virus-like particles for use as vaccines against human papillomaviruses. Problems of toxicity, purity, and high cost in other systems can be avoided in yeast. The yeast system presented here also may be applicable to other viruses for particle production and for structural protein analysis.

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