JOURNAL OF VIROLOGY, Jan. 1993, p. 315–322
0022-538X/93/010315-08$02.00/0

Vol. 67, No. 1

Self-Assembly of Human Papillomavirus Type 1 Capsids by Expression of the L1 Protein Alone or by Coexpression of the L1 and L2 Capsid Proteins

MICHAEL E. HAGENSEE,[1,2] NOBUO YAEGASHI,[1]† AND DENISE A. GALLOWAY[1,3]*

Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, Washington 98104-2092,[1] and Departments of Medicine[2] and Pathology,[3] University of Washington, Seattle, Washington 98195*

Received 3 August 1992/Accepted 29 September 1992

Vaccinia virus vectors were used to express the major (L1) and minor (L2) capsid proteins of human papillomavirus type 1 (HPV-1) with the vaccinia virus early (p7.5K) or late (pSynth, p11K) promoters. All constructs expressed the appropriate-sized HPV proteins, and both L1 and L2, singly or in combination, localized to the nucleus. Capsids were purified by cesium chloride density gradient centrifugation from nuclei of cells infected with a vaccinia virus-L1 (vac-L1) recombinant or a vac-L1-L2 recombinant but not from vac-L2-infected cells. Electron microscopy showed that the particles were 55 nm in diameter and had icosahedral symmetry. Immunogold-labeled antibodies confirmed the presence of the L1 and L2 proteins in the HPV-1 capsids. Capsids containing L1 alone were fewer and more variable in size and shape than capsids containing the L1 and L2 proteins. The L1-plus-L2 capsids were indistinguishable in appearance from HPV-1 virions obtained from plantar warts. The ability to produce HPV capsids in vitro will be useful in many studies of HPV pathogenicity.

Human papillomaviruses (HPVs) have been frequently associated with benign and malignant lesions of the anogenital tract (22, 40) as well as being responsible for common hand and foot warts (28). DNA from HPV types 6 and 11 (HPV-6 and HPV-11) is commonly found in condylomata acuminata; HPV-16 and HPV-18 are seen in anogenital malignancies (40); and HPV-1 and HPV-2 are found in common warts.

Studies on the life cycle of HPVs have been hampered by the inability to propagate the virus in culture, the lack of an animal model system, and the paucity of virions in clinical lesions. The lack of a tissue culture system for propagating HPVs is presumably due to the tight coupling of viral transcription and DNA replication with differentiation of the host cell. Despite advances in epithelial cell culture (35) and differentiation in organotypic cultures (1, 21), adequate culture techniques to propagate HPVs have not been accomplished. A nude mouse xenograft system has been developed to propagate HPV-11 (23) as well as HPV-1 (24). This method is quite laborious, is restricted to a few HPV types, and does not allow large-scale preparation of virions. Recently, it was shown that explants of the HPV-11-containing cysts can be grown in organotypic cultures (7). Clinical lesions do not provide a ready source of viral particles. The amount of HPV-1 virions obtained from plantar warts is several orders of magnitude greater than the number of virions found in genital tract lesions; however, the abundance of HPV-1 particles in a given lesion is quite variable.

Papillomaviruses contain a single circular molecule of double-stranded DNA of approximately 7,900 bp associated with histones inside a nonenveloped icosahedral capsid (for a review, see reference 13). Virion capsids consist of 72 capsomeres arranged on a T=7 icosahedral lattice with a right-hand (dextro) skew lattice (10). Like simian virus 40 and polyomavirus (2, 3, 14, 31), the capsomeres of bovine papillomavirus type 1 and HPV-1 have been shown to possess fivefold symmetry (4) and therefore are likely to be pentamers of the major capsid protein. The L1 open reading frame encodes the major capsid protein, and studies with virions and other systems expressing L1 have identified a protein with an electrophoretic mobility consistent with its predicted size of approximately 55 kDa (8, 12, 25, 30, 33, 37). A minor capsid antigen is encoded by the L2 ORF that also specifies a protein of 55 kDa. However, studies on isolated proteins from warts as well as L2 expression in a variety of systems, including in vitro transcription and translation, have shown that the L2 protein migrates with a molecular mass of approximately 72 kDa (8, 12, 20, 34, 37). The location of the minor capsid protein (L2 for HPV and VP2 and VP3 for simian virus 40 and polyomavirus) within virions has not been determined unambiguously. The appearance of electron density in the hollow of each pentamer has suggested a plausible location for these proteins (26). Negatively stained HPV virions examined by electron microscopy have a diameter of 55 nm (10) and sediment at a density of 1.30 to 1.33 g/ml in cesium chloride gradients (9).

Vaccinia virus expression systems have been used to express large quantities of foreign genes in mammalian cells with proper processing and posttranslational modification (29). The structural proteins of human immunodeficiency virus type 1 (15, 16) and hepatitis A virus (36) have been expressed from recombinant vaccinia viruses, and those proteins have been self-assembled into viral particles. Recently, Zhou et al. (38) have reported the production of 40-nm "virion-like" particles from vaccinia virus vectors expressing HPV-16 L1 and L2.

In this report, we demonstrate the production of HPV-1 capsids using a vaccinia virus system. These capsids migrate appropriately by cesium chloride density centrifugation, are of the correct size and shape, and possess the L1 and L2 capsid proteins as demonstrated by immunoelectron micros-

* Corresponding author.
† Present address: Tohoku University School of Medicine, Sendai, Japan.

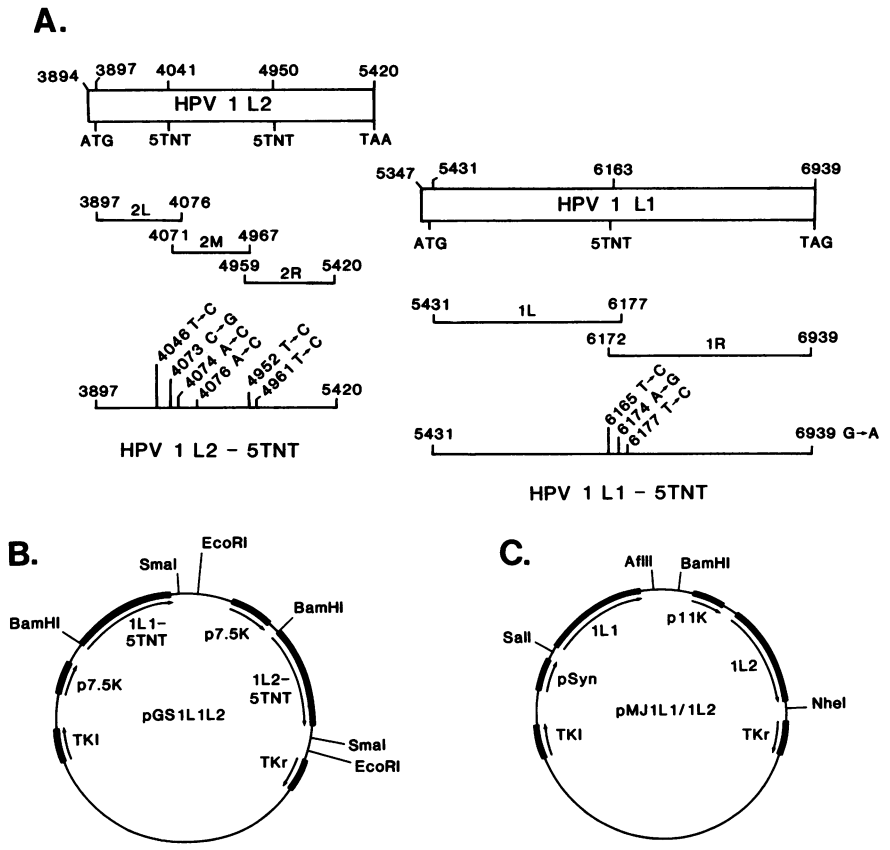

FIG. 1. Construction of pGS and pMJ series of plasmids. (A) Changes in the HPV-1 L1 and L2 sequences that were made to alter the 5TNT sequences. (B) Mutagenized fragments were cloned into pGS1L1/1L2. (C) Original 1L1 and 1L2 sequences were cloned as described in Materials and Methods to give pMJ1L1/1L2.

copy. We also report capsid formation using vaccinia virus constructs expressing only the major structural protein, L1.

MATERIALS AND METHODS

Construction of recombinant vaccinia virus vectors. Vaccinia virus expression vectors were constructed containing the HPV-1 L1 (1L1) and L2 (1L2) genes separately or in tandem. Each of the HPV genes was driven by either the constitutive promoter (p7.5K) or the late promoters (p11K, pSynth) of vaccinia virus. The HPV-1a DNA sequence was described previously (5), and the L1 and L2 genes that had been cloned into pATH vectors (37) were the starting material for the constructions described here.

The pGS series of plasmids containing p7.5K were constructed by using the plasmids pGS20 and pGS62 obtained from Shiu-lok Hu, Bristol Myers/Squibb Pharmaceutical Research Institute, Seattle, Wash. (16). Early vaccinia virus promoters are inhibited by the transcriptional termination signal 5TNT (TTTTTNT); thus, it was necessary to remove these sequences from the coding region of HPV-1 L1 and L2. Utilizing the redundancy of the genetic code, L1 and L2 were modified by site-directed mutagenesis with specific polymerase chain reaction (PCR) primers to remove the 5TNTs without altering the amino acid sequence. Figure 1 outlines the constructions.

HPV-1 L1 was constructed in two segments to alter the 5TNT signal beginning at nucleotide (nt) 6163 (5). Segment 1L contains the start of translation at nt 5431 through 6177 with a T→C change at nt 6165 to alter the 5TNT and A-to-G and T-to-C changes at nt 6174 and 6177, respectively, to create a *Bss*HII site. Upstream of the ATG linkers for *Bam*HI and *Eco*RI, restriction sites were added, and downstream of the *Bss*HII site, a *Sal*I recognition sequence was added. Primers used to construct 1L were CCCGAATTCG GATCCATGGCTGTCTGGTTACCAGCGCAGAATAAG (L1 nt 5431 to 5460) and CAGGTCGACGCGCGCAAAAA AGAACATAGAGTTGCCATAGGCTTC (L1 nt 6177 to 6142). The other segment, 1R, was constructed to alter nt 6174 and 6177 to create a *Bss*HII site for rejoining the 1L and 1R segments and a G→A change at nt 6939 to create a more efficient translation termination codon. To prevent readthrough in vaccinia viruses, a 5TNT signal was added after the stop codon. Recognition sequences for *Eco*RI were added upstream of 1R, and for *Hin*dIII and *Sma*I, they were added downstream. Primers used to construct 1R were GACGAATTCGCGCGCCGCGAGCAAATGTATACCAG GCACTTTTTT (L1 nt 6172 to 6207) and GATAAGCTTCC CGGGATAAAAATTAAGCCTTACGCCTGCGCTTGGCTG ACGTAGA (L1 nt 6939 to 6907).

The fragments were inserted into the *trpE* fusion protein expression vector pATH 11 (11, 19) by digestion with *Eco*RI and *Sal*I for 1L and by digestion with *Eco*RI and *Hin*dIII for 1R. Expression of the appropriate-sized fusion protein was confirmed in *Escherichia coli*. HPV-1 L1 was regenerated by digestion of both plasmids with *Bss*HII and *Hin*dIII, isola-

tion of fragments, and religation to produce p1L1pcr.m1. Protein expression was again examined, and the mutagenized nucleotides were confirmed by DNA sequencing. p1L1pcr.m1 was digested with *Bam*HI and *Sma*I and inserted into the similarly cleaved vaccinia virus expression vector pGS62 to produce pGS1L1.

In a similar but more complicated fashion, the two 5TNT sequences in HPV-1 L2 located at nt 4041 and 4950 were altered by constructing three segments encoding L2. Segment 2L contains the start of translation at nt 3897 through 4076 with a T-to-C change at nt 4046 to disrupt the 5TNT signal and at nt 4073, 4074, and 4076 (C to G, A to C, and A to C, respectively) to create a *Bss*HII site. Recognition sequences for *Bam*HI and *Eco*RI were added upstream of the ATG, and a *Xba*I site was added downstream of 2L. Primers used in the construction were CCCGAATTCGG ATCCATGTATCGCCTACGTAGAAAACGCGCTGCC (L2 nt 3897 to 3926) and GACTCTAGAGCGCGCTGTTCCA ATGCCCAAACCTCCCAAGAAAAC (L2 nt 4076 to 4041). Segment 2M was constructed to alter nt 4952 from T to C to disrupt 5TNT, at nt 4073, 4074, and 4076 to create a *Bss*HII site as described above, and at nt 4961 (T to C) to create an *Afl*II site. A recognition sequence for *Eco*RI was added upstream of the *Bss*HII site, and a *Sal*I site was added downstream of the *Afl*II sequence. The primers used were CCCGAATTCGCGCGCGGCTCTGGAGGAAGAATTGGT TATACTCCC (L2 nt 4071 to 4106) and CAGGTCGACACT TAAGTCATAGAAGAAGTGGGTTCTGGCGCCAATTGC (L2 nt 4967 to 4929). Segment 2R was altered at nt 4961 to create an *Afl*II site, and an *Eco*RI site was added upstream. Downstream of the translation codon at nt 5420, a 5TNT signal was added along with recognition sequences for cleavage by *Sma*I and *Hin*dIII. The primers used were CCCGAATTCGACTTAAGTTCTATTGCTCCAGAAGAC TCAATTGAA (L2 nt 4959 to 4994) and GATAAGCTTCC CGGGATAAAAATTATACATAAGCTCTTTTACGACGC TTTCTAAG (L2 nt 5420 to 5388).

Each of the PCR-generated segments of L2 was cloned into pATH 11. Expression of appropriate-sized fusion proteins was confirmed by Western immunoblot. 2L and 2M were combined by digestion with *Bss*HII and *Sal*I and ligation to form 2LM. 2R was then added by digestion with *Afl*II and *Hin*dIII and ligation. Mutations were confirmed by sequencing, and expression was confirmed in pATH 11. The resultant construct, p1L2pcr.m1, was cleaved with *Bam*HI and *Sma*I and inserted into the vaccinia virus expression vector pGS20 to produce pGS1L2. The single expression vectors pGS1L1 and pGS1L2 were combined to produce one vector containing both 1L1 and 1L2, with each HPV gene driven by the p7.5K promoter. pGS1L1 and pGS1L2 were both cleaved with *Eco*RI. The 1.5-kb fragment containing 1L2 was isolated and ligated into pGS1L1, producing pGS1L1/1L2 (Fig. 1B).

The pMJ series of constructs were derived from the vaccinia virus expression vector pMJ601 that contains a strong vaccinia virus promoter derived from consensus sequences of late promoters, designated pSynth, kindly provided by B. Moss, National Institutes of Health (6). *Sal*I and *Afl*II sites were added to HPV-1 L1 by PCR utilizing the primers AGAGTCGACATGGCTGTCTGGTTACCAGCGC AGAAT (L1 nt 5431 to 5457) and GGGAAGCTTAAG CTAAGCCTTACGCCTGCGCTTGGCTGA (L1 nt 6939 to 6911). The fragment was inserted into pMJ601 cleaved with *Sal*I-*Afl*II, producing pMJ1L1. The 1L1 gene was thus under control of pSynth.

The vaccinia virus vector pMJ1L2 contains the 1L2 gene driven by the p11K vaccinia virus late promoter. This was produced by designing a PCR primer that included the entire p11K promoter sequence upstream of the translation initiation codon for L2 and a *Bam*HI recognition sequence. An *Nhe*I site was added downstream of the L2 termination codon. The primers used were CGGGGATCCAATTTCA TTTTGTTTTTTTCTATGCTATAAATGTATCGCCTCCG GAGAAAACGCGCTGCCCCC (L2 nt 3897 to 3929) and ATCATCGATGCTAGCTTATACATAAGCTCTTTTACGA CGCTT (L2 nt 5420 to 5384). The PCR product was inserted into pMJ601 to produce pMJ1L2. The combined plasmid expressing HPV-1 L1 and L2 was constructed by digesting pMJ1L1 and pMJ1L2 with *Bam*HI and *Nhe*I, isolating the 1.5-kb L2 fragment, and ligating the fragment into pMJ1L1. The resultant construct, pMJ1L1/1L2, contains the 1L1 gene driven by the pSynth promoter and the 1L2 gene driven by the p11k promoter (Fig. 1C).

Production of recombinant vaccinia virus stocks. Vaccinia virus recombinants were generated essentially as described by Mackett et al. (27), utilizing the thymidine kinase gene of vaccinia virus. BSC-1 monkey kidney cells were infected with wild-type vaccinia virus, vNY, at a multiplicity of infection of 0.03. Plasmid DNA (20 μg) was added by calcium phosphate precipitation 3 h later. The resulting virus pool was selected for thymidine kinase-negative viral recombinants by growth in bromodeoxyuridine on thymidine kinase-negative cells. Conformation of plasmid DNA recombination into the viral genome was performed by dot-blot hybridization with HPV 1L1 and 1L2 sequences as a probe. Appropriate isolates were plaque purified twice and amplified to a high-titer virus stock. Expression of HPV proteins was confirmed by Western blot. Viral isolates are designated by vac- followed by the plasmid name.

Purification of HPV-1 capsids. BSC-1 cells (20- to 150-mm plates) were infected with recombinant vaccinia virus at a multiplicity of infection of 30. Cells were incubated at 37°C for 72 h in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and antibiotics. Cells were then collected by aspiration. Cells were pelleted by centrifugation (1,000 × *g*, 10 min) and washed once with phosphate-buffered saline (PBS). A nuclear fraction was prepared by resuspending the cell pellets in 10 ml of HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid] buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$) and incubating on ice for 10 min. Cells were lysed by homogenization, 50 strokes with a tight-fitting pestle. Nuclei were collected by centrifugation at 2,000 × *g* for 10 min. The nuclei were resuspended in 2 ml of PBS and sonicated for 2 min, and 2.1 g of cesium chloride was added. The nuclear suspension was placed into ultracentrifuge tubes, and the density was adjusted to 1.340 g/ml. Samples were spun at 45,000 rpm for 36 h at 4°C in an SW55 rotor. Fractions were collected by puncturing the bottom of the tube or by needle aspiration of the capsid band. Density of the resultant fractions was determined with a Bausch & Lomb refractometer. Fractions were dialyzed against three changes of PBS, and HPV proteins were detected by Western blot. Fractions containing 1L1 and 1L2 proteins were analyzed for capsid formation by electron microscopy. The yield was approximately 10^{10} particles.

SDS-PAGE and Western immunoblot assay. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot were performed as described by Jenison et al. (17, 18). Murine monoclonal antibodies specific for 1L1 and 1L2 were obtained from David Baker at SUNY Stony Brook and have been characterized by Yaegashi et al. (37).

Immunofluorescent staining of HPV-1 L1 and L2. Recombinant cells were grown on microscope slides (Nunc, Naperville, Ill.) to confluency and then infected with recombinant vaccinia vectors at a multiplicity of infection of 10 to 30 for 16 h. The cells were then fixed in methanol-acetone, air dried, and washed in PBS–0.05% Tween. A 1/25 dilution of primary antibody (mouse monoclonal antibody to L1 or L2) was layered on the slides and incubated at 37°C for 60 min. This was followed by washing in PBS and then adding a 1/50 dilution of fluorescein-conjugated goat anti-mouse antibody (Sigma Chemical Co., St. Louis, Mo.) for 30 min at 37°C. Slides were washed, mounted in Dapco [α-1,4-diazabicyclo(2,2,2)-octane (25 mg/ml), spectrograde glycerol (90%), PBS (0.1×)], sealed with fingernail polish, and viewed on a Zeiss microscope equipped with epiluminescence.

Electron microscopy. Samples to be assayed for the presence of HPV-1 capsids were spun onto carbon-coated 300-mesh copper grids in a Beckman Airfuge at approximately 100,000 × *g* for 10 min. Negative staining was done with 3% uranyl acetate. Samples were observed in a JEOL 1005X electron microscope. Pictures were taken at 50,000× at 80 kV.

For immunogold antibody labeling, after being spun onto grids, the samples were blocked in a drop of BLOTTO (nonfat powdered milk [5%], NaCl [0.9%], antifoam A [0.1%], sodium azide [0.1%]) for 10 min. The samples were incubated with a 1/25 dilution of first antibody for 30 min or overnight. Mouse monoclonal antibodies to HPV-1 L1, HPV-1 L2, or HPV-16 E7 (Triton Diagnostics, Alameda, Calif.) were used. Samples were then washed with BLOTTO and incubated for 60 min in a 1/25 dilution of gold-conjugated (5-nm particles) goat anti-mouse antibodies (Ted Pella, Inc., Redding, Calif.). The samples were then washed twice with water and negatively stained as above.

The number of HPV particles produced was estimated by filtering through a 0.45-μm-pore-size filter. A known amount of 0.31-μm latex spheres were added, and samples were spun onto grids as above. Grids were dropped onto cacodylate buffer briefly and then fixed for 15 min in one-half strength Karnovsky's fixative. Grids were washed twice with water and negatively stained as above.

RESULTS

Expression of the HPV-1 L1 and L2 proteins from recombinant vaccinia virus vectors was initially confirmed in cell lysates. BSC-1 cells were infected overnight with both the GS and MJ series of vaccinia virus vectors. Cells were collected, lysed by sonication, and analyzed by Western blot utilizing monoclonal antibodies to 1L1 and 1L2. As can be seen in Fig. 2A, lanes 4 and 6, vac-GS1L1- and vac-GS1L1/1L2-infected cells showed a band at 55 kDa that reacted with the 1L1 monoclonal antibody α-L1. Similarly, infection with vac-GS1L2 and vac-GS1L1/1L2 (Fig. 2B, lanes 5 and 6) showed a band at 72 kDa that reacted with the 1L2 monoclonal antibody α-L2. No bands were seen in uninfected cells (lane 1) or in cells infected with vNY (lane 2). The molecular weights for the 1L1 and 1L2 proteins are in accordance with sizes reported previously with authentic virus (8). Thus, the recombinant vaccinia virus vectors appear to be making correctly sized HPV-1 capsid proteins.

Vectors utilizing the vaccinia virus late promoters were similarly analyzed (Fig. 2). These vectors also produced capsid proteins of the correct size. There appeared to be approximately 10 to 100 times more protein produced with the late promoters (lanes 8 to 10). Also, minor additional

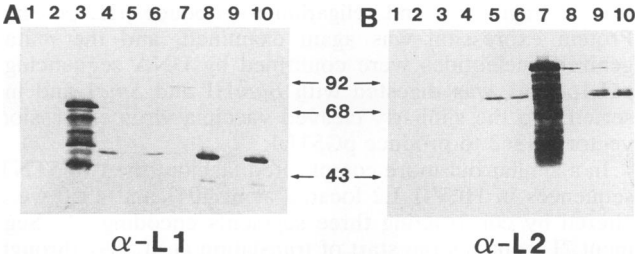

FIG. 2. Expression of recombinant vaccinia virus vectors in cell extracts. Western blot with monoclonal antibody to 1L1 (A) and monoclonal antibody to 1L2 (B). Lanes contain similar concentrations of cell extracts from vaccinia virus-infected cells or bacterial fusion proteins. Lanes: 1, mock infected; 2, vNY (wild-type vaccinia virus); 3, trp1L1; 4, vac-GS1L1; 5, vac-GS1L2; 6, vac-GS1L1/1L2; 7, vac-trp1L2; 8, vac-MJ1L1; 9, vac-MJ1L2; 10, vac-MJ1L1/1L2. Molecular weight markers are shown (×10^3).

bands of 48 and 50 kDa could be seen (Fig. 2A, lanes 8 and 10), which are likely to be proteolytic degradation products of L1.

The location of the HPV-1 L1 and L2 proteins produced in the vaccinia virus-infected cells was analyzed by indirect immunofluorescence. BSC-1 cells infected with vac-MJ1L1/1L2 demonstrated nuclear fluorescence when utilizing monoclonal antibodies to L1 or L2 (Fig. 3A and B). Omitting the first antibody revealed no fluorescence (data not shown). Infection with vac-MJ1L1 or vac-MJ1L2 also demonstrated nuclear fluorescence, indicating that both the L1 and L2 proteins are able to transport independently into the nucleus (Fig. 3C and D).

To determine whether the L1 and L2 proteins could self-assemble to form capsids, cells infected with vac-GS1L1/1L2 or vac-MJ1L1/1L2 were collected and nuclei were isolated, lysed, and layered onto cesium chloride gradients. After centrifugation for 48 h, fractions were collected and analyzed for 1L1 and 1L2 protein by Western blot. A typical gradient is illustrated in Fig. 4. The majority of the L1 and L2 protein was located in fraction 5, which had a density of 1.30 g/ml. This corresponded to the density of virus isolated from clinical specimens (9). Fraction 5 was also analyzed by electron microscopy. Particles that were 55 nm and icosahedral were found (Fig. 5B).

Approximately 100 to 1,000 more particles were produced from vac-MJ1L1/1L2-infected cells than from vac-GS1L1/1L2-infected cells. Comparison of these particles with particles purified from authentic HPV-1 warts showed them to be identical in appearance (Fig. 5). The HPV-1 virions from authentic warts appear to contain two sorts of particles: those that appear to be more electron dense and presumably contain HPV DNA in a nucleoprotein core, and those that are less dense and are likely to be empty capsids lacking DNA. The HPV-1 capsids produced in vitro resemble the empty capsids in the virion preparation. Particle counts of the capsids produced in vitro demonstrated approximately 10^{10} particles per ml.

Cells infected with vaccinia virus vectors expressing only the major capsid protein L1 were also analyzed by cesium chloride centrifugation. vac-MJ1L1 produced a large amount of L1 protein migrating at a density of approximately 1.30 g/ml. When analyzed by electron microscopy, 55-nm icosahedral particles were found (Fig. 6, bottom panels). The quantity was approximately 100-fold less than the vac-MJ1L1/1L2-produced capsids. No particles could be found

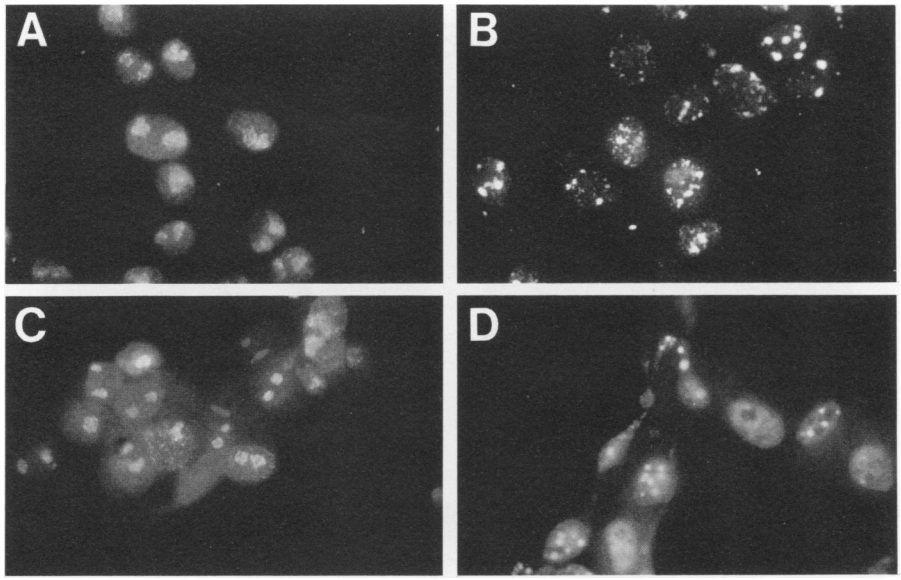

FIG. 3. Localization of vaccinia virus-produced HPV proteins. BSC cells were infected with the noted recombinant vaccinia virus, and immunofluorescence was performed with the monoclonal antibody noted. (A) vac-MJ1L1/1L2, anti-L1; (B) vac-MJ1L1/1L2, anti-L2; (C) vac-MJ1L1, anti-L1; (D) vac-MJ1L2, anti-L2.

by using recombinants expressing only L2 (vac-MJ1L2) or by using wild-type vaccinia virus (vNY) (data not shown).

The HPV capsids were further characterized by immunoelectron microscopy (Fig. 6). Capsids purified by cesium chloride centrifugation from vac-MJ1L1/1L2- and vac-MJ1L1-infected cells were compared with authentic HPV-1 virions isolated from plantar warts. As can be seen in Fig. 6 (left-hand panels), gold particles were associated with L1 in the vac-MJ1L1/1L2 and vac-MJ1L1 panels as well as the authentic warts. This confirmed the presence of L1 in the capsid particles. Similarly, gold particles associated with L2 (Fig. 6, middle panels) were seen in vac-MJ1L/1L2 capsids and authentic warts, but no L2 was found in the capsids produced by vac-MJ1L1. As a control, no gold labeling was seen with a monoclonal antibody against a nonstructural protein, HPV-16 E7 (right-hand panels). These experiments confirmed the presence of L1 and L2 in the vaccinia virus-produced HPV capsids.

DISCUSSION

This study demonstrated the successful production of HPV-1 capsids utilizing a vaccinia virus expression system. The genes coding for the two structural proteins L1 and L2 were inserted into vaccinia virus recombinants and used to infect monkey kidney cells. L1 and L2 proteins were produced in high quantity, and their molecular weights were as previously described from naturally occurring HPV infections. Examination with the electron microscope demonstrated 55-nm particles that were icosahedral. The in vitro-synthesized capsids were indistinguishable from the empty capsids that were plentiful in similarly purified specimens of HPV-1 obtained from plantar warts. Finally, immunoelectron microscopy demonstrated that the capsids contained both L1 and L2. For these reasons, it is concluded that the vaccinia virus expression system produced authentic HPV-1 capsids in vitro.

Vaccinia virus vectors expressing only the major capsid protein L1 also demonstrated capsid formation. The particles were also 55 nm, icosahedral, and shown to possess only L1 by immunoelectron microscopy. This finding is similar to results with other papovaviruses. It has been shown that polyomavirus can assemble into particles with only the major structural protein, VP1 (32). The roles of the other structural proteins, L2 or VP2 and VP3, are less well defined. There appeared to be greater size and shape heter-

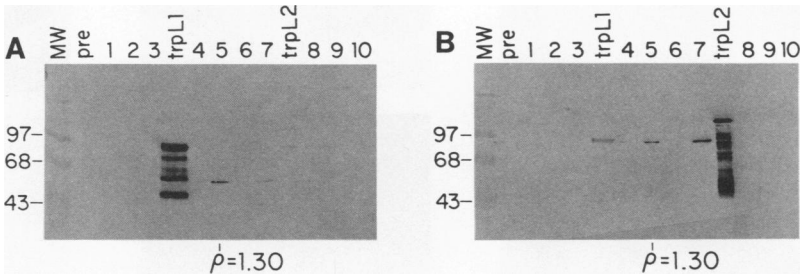

FIG. 4. Western blot of fractions from cesium chloride centrifugation of vac-GS1L1/1L2-infected cells. Numbers 1 to 10 represent various gradient fractions. pre designates sample before centrifugation, and trpL1 and trpL2 represent bacterial fusion proteins producing 1L1 and 1L2, respectively. Molecular weight markers are shown ($\times 10^3$). (A) anti-1L1; (B) anti-L2.

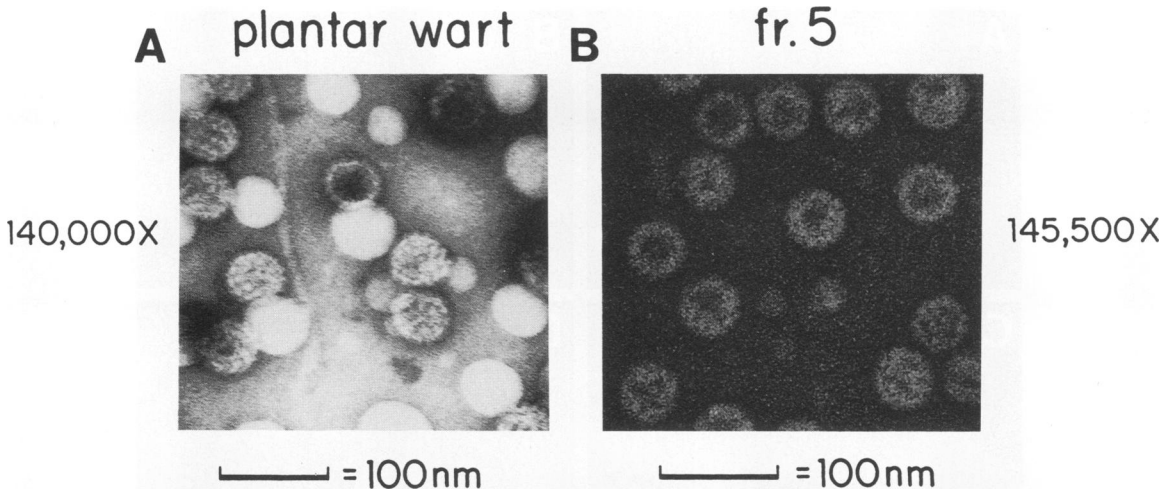

FIG. 5. Comparison of isolated warts and vaccinia virus-produced HPV-1 capsids. (A) purified warts; (B) vac-GS1L1/1L2.

ogeneity among the capsids containing only L1, as well as a quantitative difference, with the L1 capsids producing 10- to 100-fold fewer particles. This implies that L2 may be important in stabilization of the capsid structure. The immunoelectron microscopy data suggest that at least an epitope of L2 is exposed in the intact capsid, although some denaturation of the capsid during purification or preparation for microscopy cannot be ruled out. The exact location of L2 in the capsid is still unknown.

The data described here differ somewhat from those obtained by Zhou et al. (38), using a vaccinia virus system expressing HPV-16 L1 and L2. They demonstrated 40-nm particles migrating at the proper density for capsids by cesium chloride density gradient centrifugation but could not demonstrate any particle formation with vectors expressing L1 alone. No clinical specimens of HPV-16 with abundant virions are available, so it is impossible to compare the in vitro-synthesized HPV-16 capsids with authentic HPV-16 virions. In addition, no immunoelectron microscopy was performed to indicate which viral proteins were actually in the capsids. It is possible that HPV-16 capsids are inherently less stable, leading to the smaller particle and the inability to

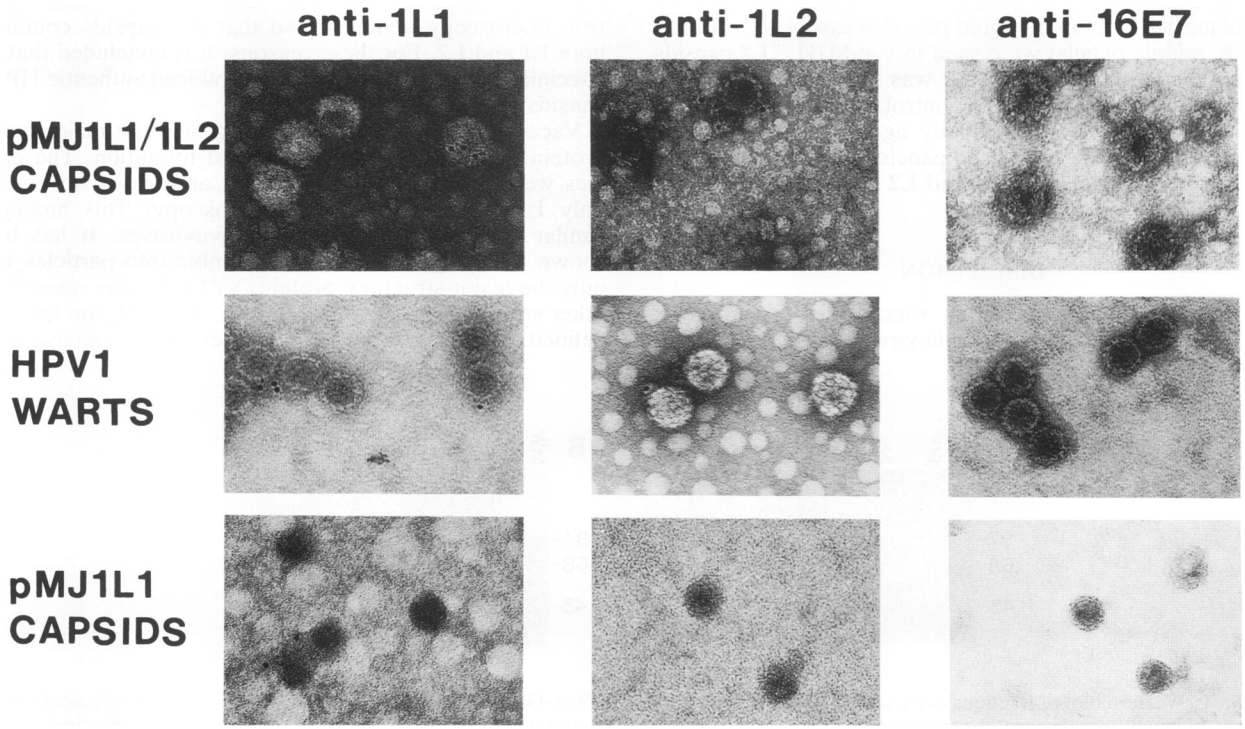

FIG. 6. Immunoelectron microscopy of warts and vaccinia virus-produced HPV-1 capsids.

produce capsids containing L1 only. Chimeric capsids of HPV-1 and HPV-16 vaccinia virus-produced structural proteins could help answer this question.

The immunofluorescent staining presented here demonstrated nuclear localization of both the L1 and L2 proteins. In addition, constructs containing L1 alone or L2 alone also localized to the nucleus. This implies that both the L1 and L2 proteins contain sequences that will localize them to the nucleus. L1 demonstrated a generalized nuclear staining with increased uptake in the nucleolus, whereas L2 demonstrated a more punctate pattern. In contrast, a more diffuse nuclear staining has been seen for both L1 and L2 in thin sections from clinical specimens (8, 20, 33, 34). This different pattern of fluorescence, especially for L2, may be due to the vaccinia virus expression system but may be due to the overexpression of the HPV proteins, differences in magnification, or differences in specimen preparation. Recent studies have identified a nuclear localization sequence in L1 of HPV-16 (39). The actual site of assembly has not been defined by these studies, but electron microscopy of thin sections of infected cells have not demonstrated HPV capsids in the cytoplasm (data not shown). This is consistent with previous studies showing HPV particles confined to the nucleus (28).

Finally, the number of particles produced was on the order of 10^{10} from fifty 100-mm plates. This can easily be scaled up for large-scale preparation. The uses for HPV capsids are numerous, and the capsids will likely be important in dissecting many aspects of the pathogenesis of HPVs, including identification of the cellular receptor for HPVs, the requirements for capsid assembly and perhaps packaging of viral DNA, and characterization of the host immune response with more-authentic antigen targets.

ACKNOWLEDGMENTS

We received considerable assistance in constructing the recombinant vaccinia virus vectors as well as plasmids and cells from Shiu-lok Hu and Bruce Travis, plasmids from Bernard Moss, and antibodies and a generous gift of HPV-1 virions from David Baker. The electron microscopy was performed by Liz Caldwell, artwork by Paul Su, and manuscript preparation by Marci Wright.

This work was supported by grants from the National Cancer Institute (P01 CA42792) and from the Fred Hutchinson Cancer Research Center. M.H. is supported by an NIAID training grant (T32 AI07044).

REFERENCES

1. **Asselineau, D., and M. Prunieras.** 1984. Reconstruction of 'simplified' skin: control of fabrication. Br. J. Dermatol. **111**(Suppl. 27):219–222.
2. **Baker, T. S., J. Drak, and M. Bina.** 1988. Reconstruction of the three-dimensional structure of simian virus 40 and visualization of the chromatin core. Proc. Natl. Acad. Sci. USA **85**:422–426.
3. **Baker, T. S., J. Drak, and M. Bina.** 1989. The capsid of small papovaviruses contains 72 pentameric capsomeres: direct evidence from cryo-electron microscopy of simian virus 40. Biophys. J. **55**:243–253.
4. **Baker, T. S., W. W. Newcomb, N. H. Olson, L. M. Cowsert, C. Olson, and J. C. Brown.** 1991. Structures of bovine and human papillomaviruses. Analysis by cryoelectron microscopy and three-dimensional image reconstruction. Biophys. J. **60**:1445–1456.
5. **Danos, O., L. W. Engel, E. Y. Chen, M. Yaniv, and P. M. Howley.** 1983. Comparative analysis of the human type 1a and bovine type 1 papillomavirus genomes. J. Virol. **46**:557–566.
6. **Davison, A. J., and B. Moss.** 1990. New vaccinia virus recombination plasmids incorporating a synthetic late promoter for high level expression of foreign proteins. Nucleic Acids Res. **18**:4285–4286.
7. **Dollard, S. C., J. L. Wilson, L. M. Demeter, W. Bonnez, R. C. Reichman, T. R. Broker, and L. T. Chow.** 1992. Production of human papillomavirus and modulation of the infectious program in epithelial raft cultures. Genes Dev. **6**:1131–1142.
8. **Doorbar, J., and P. H. Gallimore.** 1987. Identification of proteins encoded by the L1 and L2 open reading frames of human papillomavirus 1a. J. Virol. **61**:2793–2799.
9. **Favre, M., F. Breitburd, O. Croissant, and G. Orth.** 1975. Structural polypeptides of rabbit, bovine, and human papillomaviruses. J. Virol. **15**:1239–1247.
10. **Finch, J. T., and A. Klug.** 1965. The structure of viruses of the papilloma-polyoma type. III. Structure of rabbit papilloma virus, with an appendix on the topography of contrast in negative-staining for electron-microscopy. J. Mol. Biol. **13**:1–12.
11. **Firzlaff, J. M., C.-N. L. Hsia, C. L. Halbert, S. A. Jenison, and D. A. Galloway.** 1987. Polyclonal antibodies of HPV type 6b and type 16 bacterially derived fusion proteins, p. 105–113. *In* B. M. Steinberg, J. L. Brandsma, and L. B. Taichman (ed.), Cancer cells. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **Firzlaff, J. M., N. B. Kiviat, A. M. Beckmann, S. A. Jenison, and D. A. Galloway.** 1988. Detection of human papillomavirus capsid antigens in various squamous epithelial lesions using antibodies directed against the L1 and L2 open reading frames. Virology **164**:467–477.
13. **Galloway, D. A., and J. K. McDougall.** 1989. Human papillomaviruses and carcinomas. Adv. Virus Res. **37**:125–171.
14. **Griffith, J. P., D. L. Griffith, I. Rayment, W. T. Murakami, and D. L. D. Caspar.** 1992. Inside polyomavirus at 25 Å resolution. Nature (London) **355**:652–654.
15. **Haffar, O., J. Garrigues, B. Travis, P. Moran, J. Zarling, and S.-L. Hu.** 1990. Human immunodeficiency virus-like, nonreplicating, *gag-env* particles assemble in a recombinant vaccinia virus expression system. J. Virol. **64**:2653–2659.
16. **Hu, S.-L., B. M. Travis, J. Garrigues, J. M. Zarling, P. Sridhar, T. Dykers, J. W. Eichberg, and C. Alpers.** 1990. Processing, assembly, and immunogenicity of human immunodeficiency virus core antigens expressed by recombinant vaccinia virus. Virology **179**:321–329.
17. **Jenison, S. A., J. M. Firzlaff, A. Langenberg, and D. A. Galloway.** 1988. Identification of immunoreactive antigens of human papillomavirus type 6b by using *Escherichia coli*-expressed fusion proteins. J. Virol. **62**:2115–2123.
18. **Jenison, S. A., X. P. Yu, J. M. Valentine, and D. A. Galloway.** 1989. Human antibodies react with an epitope of the human papillomavirus type 6b L1 open reading frame which is distinct from the type-common epitope. J. Virol. **63**:809–818.
19. **Koerner, T. J., J. E. Hill, A. M. Myers, and A. Tzagoloff.** 1990. High-expression vectors with multiple cloning sites for construction of *trp*E fusion genes: pATH vectors. Methods Enzymol. **194**:477–490.
20. **Komly, C. A., F. Breitburd, O. Croissant, and R. E. Streeck.** 1986. The L2 open reading frame of human papillomavirus type 1a encodes a minor structural protein carrying type-specific antigens. J. Virol. **60**:813–816.
21. **Kopan, R., G. Traska, and E. Fuchs.** 1987. Retinoids as important regulators of terminal differentiation: examining keratin expression in individual epidermal cells at various stages of keratinization. J. Cell Biol. **105**:427–440.
22. **Koutsky, L. A., D. A. Galloway, and K. K. Holmes.** 1988. Epidemiology of genital human papillomavirus infection. Epidemiol. Rev. **10**:122–163.
23. **Kreider, J. W., M. K. Howett, A. E. Leure-Dupree, R. J. Zaino, and J. A. Weber.** 1987. Laboratory production in vivo of infectious human papillomavirus type 11. J. Virol. **61**:590–593.
24. **Kreider, J. W., S. D. Patrick, N. M. Cladel, and P. A. Welsh.** 1990. Experimental infection with human papillomavirus type 1 of human hand and foot skin. Virology **177**:415–417.
25. **Li, C. C., K. V. Shah, A. Seth, and R. V. Gilden.** 1987. Identification of the human papillomavirus type 6b L1 open reading frame protein in condylomas and corresponding anti-

bodies in human sera. J. Virol. **61**:2684–2690.
26. **Liddington, R. C., Y. Yan, J. Moulai, R. Sahli, T. L. Benjamin, and S. C. Harrison.** 1991. Structure of simian virus 40 at 3.8-Å resolution. Nature (London) **354**:278–284.
27. **Mackett, M., G. L. Smith, and B. Moss.** 1984. General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes. J. Virol. **49**:857–864.
28. **Orth, G., M. Favre, and O. Croissant.** 1977. Characterization of a new type of human papillomavirus that causes skin warts. J. Virol. **24**:108–120.
29. **Piccini, A., and E. Paoletti.** 1988. Vaccinia: virus, vector, and vaccine. Adv. Virus Res. **34**:43–64.
30. **Pilacinski, W. P., D. L. Glassman, R. A. Krzyzek, P. L. Sadowski, and A. K. Robbins.** 1984. Cloning and expression in Escherichia-coli of the bovine papilloma-virus-L1 and papilloma-virus-L2 open reading frames. Bio/Technology **2**:356–360.
31. **Rayment, I., T. S. Baker, D. L. D. Caspar, and W. T. Murakami.** 1982. Polyoma virus capsid structure at 22.5 Å resolution. Nature (London) **295**:110–115.
32. **Salunke, D. M., D. L. D. Caspar, and R. L. Garcea.** 1986. Self-assembly of purified polyomavirus capsid protein VP1. Cell **46**:895–904.
33. **Tomita, Y., H. Shirasawa, H. Sekine, and B. Simizu.** 1987. Expression of the human papillomavirus type 6b L2 open reading frame in Escherichia coli: L2-beta-galactosidase fusion proteins and their antigenic properties. Virology **158**:8–14.
34. **Tomita, Y., H. Shirasawa, and B. Simizu.** 1987. Expression of human papillomavirus types 6b and 16 L1 open reading frames in *Escherichia coli*: detection of a 56,000-dalton polypeptide containing genus-specific (common) antigens. J. Virol. **61**:2389–2394.
35. **Watt, F. M., and H. Green.** 1982. Stratification and terminal differentiation of cultured epidermal cells. Nature (London) **295**:434–436.
36. **Winokur, P. L., J. H. McLinden, and J. T. Stapleton.** 1991. The hepatitis A virus polyprotein expressed by a recombinant vaccinia virus undergoes proteolytic processing and assembly into viruslike particles. J. Virol. **65**:5029–5036.
37. **Yaegashi, N., S. A. Jenison, J. M. Valentine, M. Dunn, L. B. Taichman, D. A. Baker, and D. A. Galloway.** 1991. Characterization of murine polyclonal antisera and monoclonal antibodies generated against intact and denatured human papillomavirus type 1 virions. J. Virol. **65**:1578–1583.
38. **Zhou, J., J. Doorbar, X. Y. Sun, L. V. Crawford, C. S. McLean, and I. H. Frazer.** 1991. Identification of the nuclear localization signal of human papillomavirus type 16 L1 protein. Virology **185**:625–632.
39. **Zhou, J., X. Y. Sun, D. J. Stenzel, and I. H. Frazer.** 1991. Expression of vaccinia recombinant HPV 16 L1 and L2 ORF proteins in epithelial cells is sufficient for assembly of HPV virion-like particles. Virology **185**:251–257.
40. **zur Hausen, H.** 1991. Human papillomaviruses in the pathogenesis of anogenital cancer. Virology **184**:9–13.